

AMERICAN UNIVERSITY OF BEIRUT

EVALUATING *E. COLI* CONTAMINATION EFFECTS ON
LETTUCE GROWTH AND ASSESSING THE IMPACT OF
HEAVY METALS ON BACTERIAL VIABILITY IN WATER,
PLANT, AND SOIL

by
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ABSTRACT OF THE THESIS OF

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Despite the continuous awareness of food safety and improvements in sanitary infrastructure, foodborne illnesses continue to burden public health significantly. Numerous research studies have shown the occurrence of foodborne outbreaks linked to *Escherichia coli* contamination in fresh produce. In Lebanon, specifically within the Litani River Basin (LRB), investigations have reported high levels of both *E. coli* and Heavy Metals (HM) in the water. This is particularly concerning given that the agricultural sector in Lebanon relies on this water source for irrigation.

This study aimed to shed light on food safety issues concerning romaine lettuce within the basin. We explored the occurrence of *E. coli* in water soil and lettuce and evaluated the influence of different irrigation systems on the level of *E. coli* contamination. We also focused on examining the impact of four HM (Manganese (Mn), Copper (Cu), Zinc (Zn), and Nickel (Ni)) on the behavior of three *E. coli* strains isolated from water, soil, and lettuce by evaluating the growth of the bacteria under varying concentrations of HM after 10 hours and 20 hours of treatment. Additionally, we assessed their antibiotic resistance. We found that LRB was highly contaminated with *E. coli*. To cope with that contamination, the hydroponic system was the best system to mitigate the lettuce contamination. In addition, distinct responses to HM exposure were revealed. By highlighting the significance of agriculture in this area, especially in economically challenged regions, the study raises awareness of contamination occurrences and transmission pathways. It seeks to propose a better understanding of the situation, with suggestions for optimizing agricultural practices in association with environmental sustainability and food safety standards within the LRB.

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ABBREVIATIONS

HM: Heavy Metals

Escherichia coli: *E. coli*

Litani River Basin: LRB

World Health Organization: WHO

Nickel: Ni

Copper: Cu

Zinc: Zn

Manganese: Mn

Lead: Pb

Mercury: Hg

Barium: Ba

Arsenic: As

Chromium: Cr

Molybdenum: Mo

Cobalt: Co

Cadmium: Cd

Iron: Fe

Phosphorous: P

Center for Disease Control and Prevention: CDC

Minimum Inhibitory Concentration: MIC

Minimum Bactericidal Concentration: MBC

CHAPTER I

LITERATURE REVIEW AND INTRODUCTION

A. Introduction

According to the WHO and FAO food safety is the assurance that food will not cause harm to the consumer when it is prepared or eaten according to its intended use (Kopper et al., 2023). To sustain life and promote good health, people should have good access to sufficient amounts of safe and nutritious food. Food contamination is a global safety concern, where each year 1 in 10 people develop illness due to the consumption of food that has been contaminated (WHO, 2022). As for the *E. coli* contaminated vegetables, leafy vegetables especially lettuce has been proven to be highly vulnerable to *E. coli* contamination (Luna-Guevara et al., 2019). The Center for Disease Control and Prevention (CDC), along with public health and regulatory authorities in various states, Canada, and the U.S. Food and Drug Administration, investigated a widespread occurrence of Shiga toxin-producing *E. coli* infections. These cases were traced back to romaine lettuce originating from the Central Coastal growing areas in northern and central California (CDC, 2019, 2021). After a scientific study revealed substantial levels of *E. coli* contamination in Akkawi cheese sold in Beirut, a team of researchers and students conducted a proof-of-work examination to uncover the extent of bacterial contamination in salads containing fruits and vegetables (shkair, 2021, 2021). Moreover, romaine lettuce serves as a crucial ingredient in various authentic Lebanese dishes like Fattoush and Tabbouleh, raising significant and concerning food safety issues. Owing to its geographical location, spanning from the interior to the coastal region and traversing diverse topographic surfaces with a mix of human settlements, the Litani River has

consistently been viewed as a vital component in Lebanon's socioeconomic development. Covering approximately 9% of the Lebanese population, the Litani River plays a pivotal role in the country's overall progress. Specifically, the agricultural sector in the Litani River Basin (LRB) is predominantly influenced by the availability of Litani water. As a result, 31% of the income generated within the basin is derived from agriculture, employing 6% of the 370,000 inhabitants in the agricultural sector. This substantial contribution underscores the significant role of agriculture in the economic landscape of the region (Shaban & Hamzé, 2018). The deterioration in the water quality of the Litani River poses a potential health risk, particularly concerning agricultural production in the Bekaa region. Findings from a study reveal that irrigation with Litani River water lead to the accumulation of various microbiological (*E. coli*, *E. cloacae*, *E. aerogenes*, *K. pneumonia*, *K. oxytoca*, *S. marcescens*, *C. freundii*, *Sh. sonnei*, *C. diversus*, *Listeria spp*, and *P. aeruginosa*) and chemical contaminants (barium (Ba), arsenic (As), lead (Pb), cadmium (Cd), chromium (Cr), zinc (Zn), iron (Fe), nickel (Ni), copper (Cu), manganese (Mn), and molybdenum (Mo)) in vegetables such as lettuce, parsley, and potatoes, as well as in the surrounding soils. Notably, levels of these contaminants in leafy vegetables were higher than in tubular crops, particularly potatoes. Furthermore, the study highlights that exposure to polluted irrigation water, especially through sprinkling irrigation, emerged as a significant factor influencing the safety of the cultivated crops. This underscored the importance of addressing water quality concerns to safeguard both agricultural productivity and public health in the region (Madi, 2012). Our investigation was concentrated on evaluating the presence of *E. coli* contamination in the vicinity of the LRB and exploring measures to reduce this contamination. Additionally, our aim was to raise awareness about this food safety and environmental concern.

1. Objectives

- Assessing microbiological quality of lettuce, soil, and water
- Define the source of contamination.
- Study the effect of irrigation systems on lettuce contamination.
- Study the effect of HM on bacterial contamination.

2. Significance of the study

Through this study we will be able to evaluate the level of contamination in the agricultural areas used for lettuce planting around the LRB. Lettuce being an essential ingredient in our daily diet, its contamination would be a public health threat for that finding a solution to mitigate this contamination would be essential to reduce foodborne illnesses caused by leafy green vegetables.

B. Literature review

1. Biological contamination

Biological contamination of food is the most common cause of food poisoning. It is the presence of dangerous pathogens in food, like microorganisms, bacteria, viruses, mold, fungi, and toxins (Wilkinson, 2020). This type of contamination can happen due to either direct or cross-contamination. Direct contamination is due to the presence of pathogens previously produced in the food reaching unsafe levels. An example of this would include the bacteria and toxins found in spoiled meat. Whereas cross-contamination is considered when pathogens reach food from other sources and divide to risky levels (Lee, 2020).

Bacteria in short can be considered as simple forms of life. These organisms are ubiquitous and are present in every single aspect of life whether its biotic or abiotic. They

could be present in either free living or parasitic forms based on different conditions. The origin of most diseases has been proven to have a bacteriological etiology hereby relating most of the diseases to bacterial infections (Baron, 1996). Although bacteria are famous of being pathogenic, they are part of the human's normal flora of skin and mucous membranes from birth till death where the human body is made of almost 10^{13} cells occupied with 10^{14} bacteria (Davis, 1996). Since the very beginning of biotechnology, these tiny microbes have been used as vectors to produce a broad range of therapeutic drugs such as sirolimus which is a bacterium-derived macrolide- used in the treatment of some cancers (Amedei & D'Elios, 2012). Though it's a major part of the human's body and life bacterial contamination and its subsequent infections impose a serious threat to human's health (Alexandre et al., 2018). 91% of all foodborne illnesses are related to bacterial contamination with *E. coli*, *Salmonella*, *Staphylococcus*, *Listeria*, and *Campylobacter* (Jha, 2016).

a. *E. coli* characteristics

In 1855, Theodor Escherich (1857–1911), a German-Austrian pediatrician, discovered and first described a bacterium called “*Escherichia coli* (*E. coli*)” by isolating it from babies' feces (Basavaraju et al., 2022). As a definition, *E. coli* is a rod-shaped, non-spore forming, gram-negative, facultative anaerobic coliform bacterium (Lim et al., 2010). It belongs to the genus *Escherichia* that is mostly found in the environment, foods, and warm-blooded animals' lower gut. It is non-acid fast bacilli that could exist as single cells or in pairs. These rod-shaped cells range between $1\text{-}3\mu\text{m} \times 0.4\text{-}0.7\mu\text{m}$ in size, $1\mu\text{m}$ in length, $0.35\mu\text{m}$ in width, and $0.6\text{-}0.7\mu\text{m}$ in volume (Basavaraju et al., 2022). Its motility is due to the presence of peritrichous flagella and only a few discovered strains are

nonmotile. Strains surrounded by fimbria can be either motile or nonmotile. It has a thin cell wall with only one or two layers of peptidoglycan. *E. coli* can grow at temperatures ranging from 10°C to 40°C but its optimum growth temperature for *E. coli* is the same as that of the body which is 37°C (98°F) and some strains can even multiply at temperatures up to 49°C (120.2°F). It can survive at 4.5–9.5 pH but the maximum growth is observed at 7.0 that is neutral pH (Percival & Williams, 2014). This bacterium is characterized by its ability to divide in a very short period where its life cycle takes as little as 20mins in favorable conditions. That's why it was called the “workhorse” of molecular biology (Samie, 2017). Some *E. coli* strains isolated from extraintestinal infections are characterized by the presence of a polysaccharide capsule. These capsules can be seen as bright halos over a dark backdrop using negative staining procedures (Basavaraju et al., 2022). *E. coli* is classified into 150–200 serotypes or serogroups based on 3 antigens, somatic (O) or cell wall antigen, capsular (K) antigen, and flagellar (H) antigen. Seventy-five types of the H or flagellar antigen and 173 types of O or somatic antigens 103 types of the K or capsular antigens have been recognized (Mueller & Tainter, 2023).

b. *E. coli* in the environment

E. coli cycles between two major habitats warm-blooded animals' intestines and the environment (water, sediment, and soil), which is considerably different in terms of physical conditions, range, and quantity of nutrients availability. It can live on a wide variety of substrates where it can also be found in hotter conditions, such as on the edge of hot springs and on ground meats due to slaughterhouse processing. *E. coli* can live for long periods of time in feces, soil, and water. This bacterium can live and multiply rapidly in fresh feces for a period of 2-3 days under aerobic conditions but beyond that its growth and

numbers start to cease. Whereas in the intestine of mammals *E. coli* is considered as the inhabitants of the mucous layer of mammalian colon accounting for around 0.1% of the gut microbiota and its well known for its ability to compete with other types of bacteria in this organ thus being ranked as the most abundant facultative anaerobe in the human intestinal microflora. *E. coli* in the colon synthesizes K and B complex vitamins and protects the gastrointestinal tract against colonization with pathogenic microbes, while the host offers an ecological niche and nutrients. Thus, several strains of *E. coli* have been used as probiotics and have already been used in pharmaceuticals. It colonizes the intestines of newborns within few hours after birth and aids in keeping the digestive tract healthy (Martinson & Walk, 2020). Although this bacterium has been extensively studied, till now the literature couldn't find the reason behind the symbiosis between *E. coli* and the intestines (Leimbach et al., 2013). A hypothesis suggested that *E. coli* might have a great ability to metabolize gluconate in the colon more efficiently than other resident species, thereby occupying a highly specific metabolic niche. These commensal strains that already exist in the intestines of mammals rarely cause any disease unless its host is immunocompromised, or the normal gastrointestinal barriers are breached such as in the case of peritonitis.

c. Pathogenicity of *E. coli*

Until late 1950's, *E. coli* species was known to be nonpathogenic cohabitants since it's a part of the intestinal normal flora of animals and humans but due to its ability of certain strains to impose diseases it was considered as potential pathogenic bacteria (Olsvik et al., 1991). Some *E. coli* strains evolved into pathogenic *E. coli* by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity

islands. Infections by pathogenic *E. coli* might be limited to the mucosal surfaces or could disseminate throughout the body causing bacteremia. *E. coli* strains can cause enteric/diarrhogenic or extraintestinal (ExPEC) infections in humans. ExPEC infections are primarily urinary tract (caused by uropathogenic *E. coli*, UPEC) and sepsis/ meningitis (caused by neonatal meningitis *E. coli*, NMEC) (Basavaraju et al., 2022).

Based on their pathogenicity profiles (virulence factors, clinical disease, and phylogenetic profile) enteric *E. coli* infections are also subdivided into 6 pathotypes that are: Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC, including *Shigella* sp), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and Diffusely Adherent *E. coli* (DAEC) (Basavaraju et al., 2022). Two more pathotypes emerged recently that are Adherent Invasive *E. coli* (AIEC) that might be associated with Crohn disease but does not cause diarrhogenic infection and the Shiga Toxin (Stx) producing Enteroaggregative *E. coli* (STEAEC). Each pathotype contains many serotypes and some serotypes can belong to more than one pathotype, thus giving strains a serotype might not provide definitive identification of pathotypes (Clements et al., 2012; Kaper et al., 2004).

d. Food safety concerns and *E. coli* contamination

According to the CDC, 95,000 cases of *E. coli*-related food poisoning in the US each year, with symptoms ranging from mild to extremely severe (CDC, 2023). But the question is how the food gets contaminated and is washing enough? The use of manure as a fertilizer in agriculture is a direct route of fecal contamination. Although most manure is free of harmful *E. coli*, a small percentage of cattle carry pathogenic *E. coli* in their intestines. This harmful *E. coli* is then deposited in the cattle's manure and, and thus

reaches the crops after manure application without first being composted. The absence of efficient hygiene and processing of the produce, it might be contaminated with *E. coli* when it reaches the supermarket. Wildlife may also be a source of contamination for food. Deer, birds, and pests can drop pathogenic *E. coli* containing feces on plants. Moreover, manure can cause contamination problems in food even when not used as fertilizer. Raw milk can be cross contaminated during the milking process. During the slaughtering of the cattle, *E. coli* from their feces sometimes may reach the beef. In this case, only the surface of the beef becomes contaminated. Bacteria is likely to be killed by many cuts of beef, like steak, cooking. However, in ground meat, grinding of the meat and mixing it might lead to the distribution of the bacteria throughout the product, and that's the reason behind the necessity of well cooking the ground beef so that the heat reaches the core. Poor personal hygiene, like improper hand washing after using the toilet, can transmit harmful *E. coli* from infected food handlers to food products (Alegbeleye et al., 2018; American Society of Microbiology, 2011; Luna-Guevara et al., 2019). Water is also a source of *E. coli* contamination. Using contaminated water to irrigate, wash, or chill them can contaminate foods. If produce (especially leafy green vegetables) is grown in water that has been contaminated by manure, the *E. coli* can adhere to their surfaces and become extremely difficult to wash off. In some cases, *E. coli* can even find its way inside the vegetable cells where washing will have no impact since *E. coli* is an endophytic bacterium. Water mixed with *E. coli* is not only a problem on the farm since it's also used in food processing, or even to wash food at the supermarket, if it carries harmful *E. coli*, food can be contaminated (American Society of Microbiology, 2011; Machado-Moreira et al., 2021). Listing all the potential ways food might be contaminated with *E. coli* is challenging due to the several possibilities. This complexity points out the difficulty in guaranteeing food safety, as

safeguarding against *E. coli* and other pathogens is a tricky and complicated task (Ekici et al., 2019). *E. coli* isn't just a concern in food; it can also be ingested through recreational water. Pathogenic *E. coli* poses a risk in settings like swimming pools where inadequate cleaning and chlorination allow its survival. Additionally, ponds, rivers, and areas where humans intermingle directly with animals or their manure, like farms, petting zoos, and gardening with manure, are potential sources for harmful *E. coli* strains to enter our bodies (Mohamed & Habib, 2023).

e. Occurrence of *E. coli* in Lebanon and the Arab countries

Bacterial pathogens such as pathogenic *E. coli* are a common cause of foodborne infections in the Arab world, resulting in substantial economic losses and significant public health implications. The genetic material of these *E. coli* pathogens is often detected in a variety of food items throughout Arab countries (Mohamed & Habib, 2023). In a study that tested 145 samples of fresh produce from Lebanon for the occurrence of *E. coli*, *E. coli* was detected in 39 out of the samples (26.8%). The highest level of contamination was identified in leafy green produce, including items such as purslane, thyme, parsley, and lettuce, except for peppermint, which was free from fecal *E. coli* (Khatib et al., 2015). Leafy greens are specifically susceptible to *E. coli* for various reasons. Firstly, their fame has risen due to the distinguished health benefits, leading to boosted production to meet this mandate. However, the widespread cultivation over larger areas makes it difficult to trace contamination in the fields. The excessive consumption further raises the probability of consumption of contaminated leafy greens. Additionally, their growth near ground exposes the edible leafy parts to possibly contaminated water (Fontanazza, 2019). Contrasting with many other vegetables that are frequently cooked

before eating, leafy greens like romaine are commonly eaten raw, increasing the risk as bacteria aren't excluded through heating (Falcone, 2021).

2. *Chemical contamination*

Chemical food contaminants are substances that are not naturally found in the typical raw materials used for food production, nor are intentionally added during the standard production process (Schrenk, 2004). Chemical hazards are considered as a primary cause of food contamination and are frequently linked to outbreaks of foodborne illnesses (Faille et al., 2018). Chemical contaminants fall into one of two classes: natural and artificial (Canadian Institute of Food Safety, 2022). Chemical food contaminants are generally classified into four categories: natural toxins, environmental contaminants, agrochemical residues, food process toxicants, together with intentionally added chemicals (Oliver et al., 2015; Thakali & MacRae, 2021; Verma et al., 2022). So, the food production chain naturally carries both internal and external risks of contamination (Ng & von Goetz, 2017). Contamination can be introduced at various stages within the miscellaneous levels of food production, each phase presenting specific points of vulnerability (Lebelo et al., 2021). It's critical to distinguish the various types of food contaminants, which comprise environmental impurities, processing byproducts, unapproved adulterants or additives, and elements migrating from packaging materials (Mastovska, 2013). Environmental contaminants come from human introduction or natural incidents in water, air, or soil. Processing contaminants include undesired compounds formed during food preparation procedures like baking, roasting, canning, heating, fermentation, or hydrolysis (Schrenk, 2004). Direct contact between food and packaging materials can initiate chemical contamination through the migration of unsafe substances. Moreover, the use of

unapproved or incorrect additives possibly will lead to food contamination (Rather et al., 2017).

HM are naturally occurring elements that are found in the Earth's crust. Some of them, such as Cu, Zn, and Fe, are essential for several biological processes in living organisms in remnant amounts. Yet, at elevated levels, many HM can become toxic and pose critical health risks to both humans and other organisms (Jaishankar et al., 2014). HM are well known environmental chemical pollutants and they are characterized by being toxic and persistent in the environment, and with bioaccumulative nature. Their source could be natural or anthropogenic. Natural sources are weathering of metal-bearing rocks and volcanic eruptions, whereas anthropogenic sources (human activities), such as industrial processes, mining, and the use of certain products like pesticides and batteries, can contribute to the production of HM into the environment. After being released, these metals can accumulate in soil, water, and air, leading to escalated exposure for living organisms (Ali et al., 2019; Tchounwou et al., 2012). HM, coming from industrial areas, have the possibility to leach into the soil and afterward enter the food chain, contaminating the primary sources of food (Krishna & Govil, 2007). Elevated metal levels beyond specified thresholds can interrupt the natural microbial balance in soils and diminish their fertility thus affecting the quality of planted crops (Barbieri, 2016). Exposure to high concentrations of HM, such as Pb, Hg, Cd, and As, can have damaging effects on human health. These consequences may include damage to the nervous system, kidney and liver damage, developmental issues in children, and an increased risk of specific cancers (Balali-Mood et al., 2021). Efforts to mitigate the risks associated with HM exposure include employing guidelines and attempts to control industrial discharges, mastering waste disposal, and monitoring environmental quality. Furthermore, individuals can take steps to

reduce their exposure by being conscious of the products they use and implementing sustainable and environmentally friendly practices (Priya et al., 2023).

a. HM contamination in Lebanon

In recent times, there's been a global worry about the contamination of soil by HM and organic compounds. This concern is particularly pronounced in developing nations like Lebanon, where decades of mismanagement of solid waste have taken a toll (Borjac et al., 2020). In the latest updates, there have been reports of elevated levels of HM in various food items in Lebanon. This situation highlights the concern that a considerable number of Lebanese products may be tainted with HM and toxins. Given the absence of effective regulations, control measures, or corrective actions by the Lebanese government, there is a pressing need to evaluate the exposure of the Lebanese population to these contaminants through their consumption (Tahtouh, 2020). In the present times, the practices of soil contamination have significant environmental repercussions, making it crucial to prevent the integration of heavy metals into the food chain. The plant species chosen for a performed investigation constituted the components of the traditional Lebanese salad, Tabbouli. That included parsley and lemon selected from Damour, olive and onion from Ghazir, peppermint and tomato from Akkar, and bulgur. That selection aimed to address all the necessary criteria for assessing the predominant physicochemical properties and contamination levels in vegetables originating from soils with HM elements. The concentration ranges of As, Cu, Fe, Zn, Mn, Ni, Ba, Cd, Cobalt (Co), Cr, Pb, and Phosphorous (P) in the examined soil samples were assessed. The findings revealed that the concentrations of most of these elements in both soil and plant samples surpassed the permissible limits established by international standards for agricultural soils and

vegetables, as defined by FAO/WHO (FAO, 2023). Moreover, the transfer of these elements from soil to plants emerges as the primary pathway for human exposure to potential toxic contamination. It underscores the critical importance of adhering strictly to good manufacturing practices, agricultural practices, and safety measures (Assaad et al., 2020).

The evaluation of potential risks to human and ecosystem health arising from the detection of potentially harmful elements in Fattoush ingredients cultivated in Lebanon is of paramount importance. A recent study showed a significant contribution as it focuses, for the first time, on the plant species comprising the traditional Lebanese salad, Fattoush. The selected ingredients include lettuce, cucumber, tomato, onion, purslane, radish, lemon, and sumac, ensuring a comprehensive assessment of contamination levels in vegetables sourced from soils containing potentially harmful elements. Concentration ranges of As, Cu, Fe, Zn, Mn, Ni, V, Cd, Co, Cr, Pb, P, Sn, and Al in soils and the edible parts of plants collected from urban allotments in the South, Damour, Ghazir, and Akkar areas were determined. The findings revealed that concentrations of most studied elements in both soil and plant samples exceeded the permissible limits set by the WHO, particularly in purslane, lemon, and sumac. Remarkably, sumac, purslane, lemon, and lettuce were identified as "heavy metal hyperaccumulating plants," accentuating the importance of rigorous monitoring and adherence to safety measures in agricultural practices to mitigate potential health and environmental risks (Fadel et al., 2019).

CHAPTER II

IMPACT OF *E. COLI* CONTAMINATION ON LETTUCE GROWTH AND MITIGATION STRATEGIES THROUGH IRRIGATION SYSTEMS

A. Introduction

Nestled within the heart of Lebanon, the LRB is a geographical marvel, celebrated for its natural diversity and ecological significance. This region, renowned for its lush valleys and expansive plains, is not only a testament to nature's complexities but also a vital source of sustenance for the local communities. The agricultural sector, while contributing a modest 5% to Lebanon's Gross Domestic Product (GDP), plays a pivotal role in the basin's prosperity. In economically challenged regions such as the South, Northern Bekaa, Dinnyeh, and Akkar, agriculture's contribution soars, often constituting nearly 80% of the local GDP. Against this backdrop, the Litani River flows as the lifeblood of this region, nurturing not only crops but also a delicate balance of flora and fauna (ESCWA, 2013; FAO, n.d.).

In the midst of this bucolic landscape, the LRB confronts a pressing environmental conundrum characterized by *E. coli* contamination. Different research papers showed that *E. coli* is present in water (Dagher et al., 2021a), soil (Darwish et al., 2008), and vegetables (M. Halablab et al., 2011). Although there have been new initiatives to address pollution in the Litani River, significant challenges persist (Holtmeier, 2019). Despite the preventative measures The National Litani River Authority reports that the water quality in the Litani River's upper basin in the northern and central Bekaa does not meet international bacteriological standards for crop irrigation set by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations

(FAO). The analyses reveal that the percentage of total coliforms and heat-tolerant coliforms exceeds the maximum allowable limits according to these standards, indicating a significant deviation from accepted international irrigation water quality norms (National Authority for Litani River, 2021).

Romaine lettuce, like other leafy greens, can be contaminated by *E. coli* due to several factors. Environmental contamination can occur when fields are exposed to *E. coli* from animal feces, with irrigation water or manure-based fertilizers being potential sources. Interaction with wild animals and livestock in or around crop fields can also introduce *E. coli* to romaine lettuce. Inadequate processing, handling, and storage practices can lead to contamination during the post-harvest phase. Additionally, the use of contaminated water sources for irrigation and washing poses a risk. To mitigate *E. coli* contamination in romaine lettuce, it is crucial to implement strict agricultural and food safety practices and maintain high levels of hygiene and sanitation throughout the supply chain (Madi, 2012).

Our study started with the microbiological screening of water lettuce and soil around the LRB and extends to the evaluation of distinct irrigation systems, encompassing sprinklers, dripping, and hydroponics, aiming to provide guidance for optimizing agricultural practices that align with environmental stewardship.

In this comprehensive study, we aspired to not only deepen our understanding of the complex ecological challenges within the LRB but also to offer practical solutions. By addressing the impact of irrigation systems on *E. coli* contamination by comparing the levels of *E. coli* in soil water and lettuce across the three systems, our research aimed to guide ecologically responsible land and water resource management in this unique and vital region.

B. Materials and Methods

1. *Assessment of the samples' contamination*

a. Demo plot selection and sampling

In this study, we selected five plots located at different positions around the LRB. Each plot was divided into two sections acting as replicas. We collected water and soil samples before planting from each plot and subjected them to *E. coli* testing. During the final season, we collected lettuce, water, and soil samples for *E. coli* testing.

In sterilized one-liter bottles, we collected 2 L of irrigation water directly from the source. For lettuce samples, we gathered leaves from different lettuce plants, alternating between inner and outer leaves, and placed them in sterile bags. In the case of soil samples, each plot was divided into five sections (the four corners and the center), from which we collected five different soil samples that were then combined to create a representative 1 kg sample placed in sterile plastic bags. All collected samples were immediately placed on ice and transported to the Food Microbiology Laboratory at the Faculty of Agricultural and Food Sciences – American University of Beirut, where appropriate microbiological testing was conducted.

b. *E. coli* isolation

For the water sample testing: 1L of water was filtered using Whatman filter paper to collect the bacteria, and then the collected filter was placed on Rapid *E. coli* 2 agar (Bio-rad USA). It was subsequently incubated for 24 h at 37°C.

For the lettuce sample testing, leaves were cut using a sterile knife and a sterile cutting board. Four samples were then prepared by weighing 25 g of lettuce in a stomacher bag and adding 225 mL of Buffered Peptone Water (BPW) (Himedia, India). The samples were

blended using a stomacher. Serial dilution was performed, and the samples were plated on Rapid *E. coli* agar, followed by incubation for 24 h at 37°C.

For the soil samples, 25 g of soil were weighed in a stomacher bag, followed by the addition of 225 mL of BPW. The mixture was then blended using a stomacher. Serial dilution was performed, and the samples were plated on Rapid 2 agar, followed by incubation for 24 h at 37 °C.

For the confirmation of the suspected colonies, biochemical tests were conducted using Api20E strips (Biomérieux, France). MacConkey agar (Himedia, India) was utilized for the purification process, and the isolates were subsequently stored in 80% glycerol stocks at -80°C for further investigation.

c. Screening for *Salmonella*

For water samples, 1 L of water was filtered using Whatman filter paper to capture bacterial cells. The filter paper was then placed in a 9 mL falcon tube containing BPW and incubated for 24 h at 37 °C. For soil and lettuce samples, the pre-prepared mixtures (25g of the sample + 225 mL of BPW) were incubated for 24 h at 37 °C. The following day, 1 mL of the incubated BPW was transferred to a 9 mL Rappaport-Vassiliadis Salmonella (RVS) enrichment medium (Himedia, India) and further incubated for 24 h at 41.5 °C. After the second incubation, a full loop of the RVS culture was streaked on Xylose Lysine Deoxycholate agar (XLD agar) and Salmonella-Shigella agar (SS agar) (Himedia, India). Suspected colonies were confirmed using the Salmonella Latex Test (Bio-rad, USA) and Api20E tests.

2. *Effect of the irrigation systems on the bacterial contamination*

a. Lettuce plantlets

A total of 120 lettuce plantlets were purchased and planted in compost soil. These plantlets were irrigated three times a week with *E. coli*-free water for a period of three weeks to ensure their proper growth and root establishment.

b. Irrigation system establishment

After 2 weeks, the lettuces were divided into three groups: (1) a drip irrigation system; (2) a sprinkler irrigation system; (3) a hydroponic system. Each group consisted of 30 lettuces, with twenty-five being treated with *E. coli* and five serving as control plants.

c. Preparation of the treated water

From the *E. coli* stocks stored at -80°C, a full loop was taken and streaked on Rapid *E. coli* 2 agar, followed by incubation at 37°C overnight. Two to three colonies were then used to inoculate 1400 mL of BPW and incubated at 37°C with constant shaking overnight. The next day, Optical Density (OD) was measured at 600nm using a spectrophotometer to determine the concentration of the inoculum by comparing the results to a pre-prepared growth curve.

To prepare the treatment solution, BPW containing *E. coli* was diluted using *E. coli*-free water to reach an OD of 0.4 (corresponding to 3 MacFarland Unit).

d. Comparison between Irrigation Systems

To assess the impact of different irrigation systems on bacterial contamination in both lettuce and soil, two samples were collected from the treated lettuces and their

corresponding soil, and two samples were collected from the control lettuces and their respective soil in each system. The same microbiological testing method as described above was employed for analysis. In the hydroponic system, where no soil was used, the water was tested using the same method described above. However, it was diluted tenfold using autoclaved distilled water before filtration.

In addition, two samples of treated roots and two samples of control roots from each system were collected. The roots were washed to remove soil by soaking in distilled water and then sterilized using 10% sodium hypochlorite. Afterward, 25 grams of each root sample were weighed in a stomacher bag, and 225 mL of BPW was added. The samples were then blended using a stomacher. Serial dilution was performed, and the diluted samples were plated on Rapid *E. coli* 2 agar and incubated for 24 hours at 37 °C.

3. *Statistical analysis*

Using R studio software (R.3.3.0), statistical analysis was performed to compare the average number of colony forming units (CFU) of *E. coli* for each irrigation system based on each sample (lettuce, soil and root). One-way ANOVA was used and then followed by Tukey's Honestly Significant Difference (Tukey HSD) test, and independent sample t-test. Also, one-way ANOVA was performed to compare the level of *E. coli* contamination in different samples (lettuce, water, and soil) between irrigation systems from one side and between samples within the same irrigation system from the other side. Independent samples t-test was used in the cases of analysis between two samples or two irrigation systems. The Kruskal Wallis test was used as a non-parametric alternative to ANOVA followed by Dunn's test. The difference was considered statistically significant for p values < 0.05.

C. Results

1. Screening for *E. coli* and *Salmonella* in Samples from the Demonstration Plots

After conducting microbiological testing of lettuce, soil, and water samples in the 5 demo plots during both the pre-planting and harvest seasons, a noticeable difference in *E. coli* contamination pattern was observed. In D1, which was an open field, during the pre-planting season, water was found to be contaminated by *E. coli*, while no contamination was observed in soil samples. However, during harvesting, no *E. coli* was observed in soil, lettuce, or water. In D2, where cultivation took place in green houses, the water source was found to be contaminated during both the pre-planting and harvest seasons, whereas no *E. coli* contamination was observed in the soil during these seasons. Lettuce was found to be free of *E. coli* contamination during the harvest season. In D3, during the pre-planting season, water samples were found to be contaminated with *E. coli*, while soil samples remained uncontaminated. However, in the harvest season, the contamination pattern shifted. Water samples were no longer contaminated, but both soil and lettuce samples exhibited *E. coli* contamination. In D4, the cultivation took place within greenhouses rather than open fields. Prior to planting lettuce, a microbiological analysis of the soil indicated that soil samples from G1 and G3 were devoid of *E. coli* contamination, while those collected from G2 and G4 tested positive for *E. coli*. Remarkably, the water sample taken before planting tested negative for *E. coli*. As the harvest season approached, a shift in the contamination patterns was observed. In G2 and G4, water, soil, and lettuce samples all tested positive for *E. coli*. In contrast, in G1 and G3, both soil and lettuce samples continued to test negative for *E. coli*. In D5, an open field setting, prior to lettuce planting, the water sample tested negative for *E. coli*, while the soil

sample tested positive. However, during the harvest season, water, lettuce, and soil samples tested positive for *E. coli*. It's noteworthy that the *E. coli* contamination levels were very high, and no Salmonella was detected in any of the plots in the soil, water, or lettuce samples.

Table 1 Microbiological screening of soil, water, and lettuce in the five demo plots during the pre-planting and post harvest seasons. +: presence of *E. coli* ; -: absence of *E. coli*.

	Plot 1					Plot 2				
	Water		Soil		Lettuce	Water		Soil		Lettuce
	Pre-planting	Harvest	Pre-planting	Harvest	Harvest	Pre-planting	Harvest	Pre-planting	Harvest	Harvest
D1	+	-	-	-	-	+	-	-	-	-
D2	+	+	-	-	-	+	+	-	-	-
D3	+	-	-	+	+	+	-	-	+	+
D4 G1/G2 (AUB) G3/G4 (Farmer)	-	+	-/+	-/+	-/+	-	+	-/+	-/+	-/+
D5	-	+	+	+	+	-	+	+	+	+

2. Testing the Effect of Irrigation Systems on *E. coli* Contamination

The contamination of the soil and lettuce irrigated with *E. coli* was assessed to indicate the best irrigation system that would mitigate the contamination. The plants were irrigated using dripping, sprinklers, or hydroponic systems. A significant difference in *E. coli* concentration was observed between the three irrigation systems (Figure 1). The sprinkler irrigation system exhibited the highest level of contamination, followed by the dripping irrigation system, while the hydroponics system demonstrated the lowest level of *E. coli* contamination in lettuce leaves. However, the soil samples were more contaminated

in the sprinkler irrigation system than those collected from the dripping irrigation system (Figure 1).

Testing the lettuce roots collected from the three systems, it was found that no significant difference in *E. coli* contamination between the roots collected from the dripping and sprinkler irrigation systems. On the other hand, roots isolated from the hydroponic irrigation system had the highest *E. coli* contamination compared to the other two systems (Figure 1).

In both the dripping and sprinkler irrigation systems, soil samples were the most contaminated samples compared to lettuce and roots samples. The root and lettuce samples collected from the sprinkler irrigation system had similar levels of contamination whereas in the dripping irrigation system more contamination was observed in the root samples than in the leaf samples. In the hydroponic system, root samples had higher level of *E. coli* contamination than the leaves samples (Figure 1).

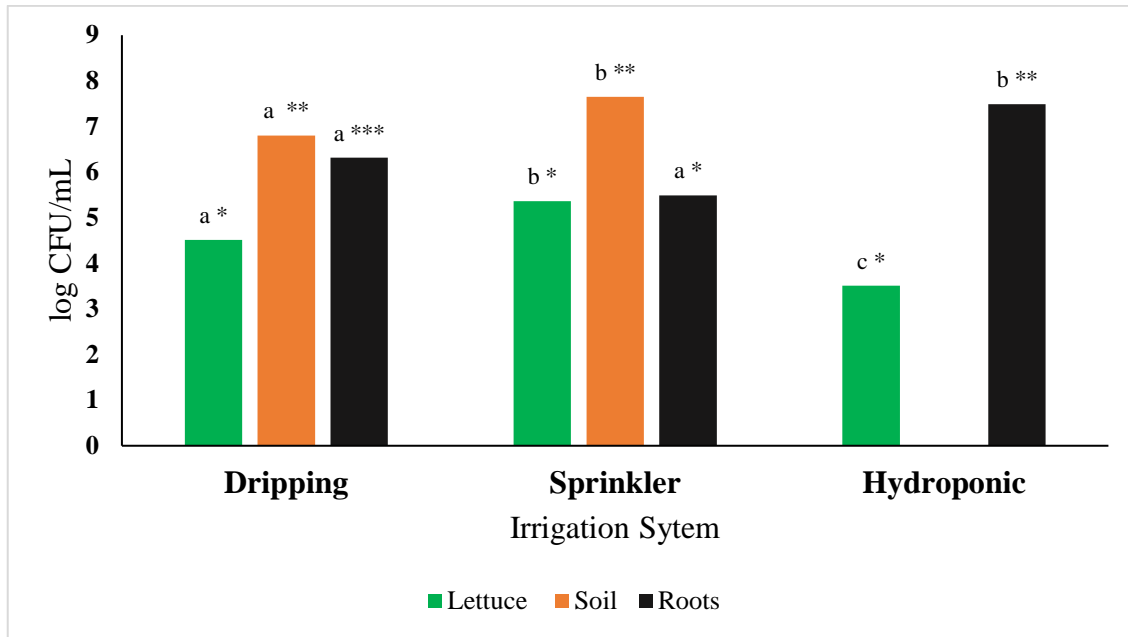


Figure 1 Effect of irrigation systems on *E. coli* contamination

The letters indicate the significant difference of each sample in the different irrigation systems. The asterisks indicate the significant difference between the samples of the same irrigation system.

Within each system comparing the difference in *E. coli* concentrations in the soil, lettuce and roots a significant difference in the pattern of contamination was observed. In the dripping and the hydroponic systems, a significant difference was obtained in the *E. coli* concentration of the roots and lettuce samples with that of the roots being significantly higher. No significant difference in the *E. coli* concentration was observed in the roots and lettuce samples collected from the sprinkler system but a significant difference was observed comparing these two samples to that of the soil which was the highest. For the dripping system, a significant difference was observed between the three collected samples (lettuce, soil, and roots) with that of the soil being the most contaminated with *E. coli* followed by the roots and lastly the lettuce sample (Figure 1).

D. Discussion

In this study five different demo plots were chosen around the LRB in Lebanon. The reasons behind choosing this geographical location were: (1) previous studies that showed the high levels of microbiological and heavy metals contamination (M. A. Halablal et al., 2010; Haydar et al., 2014b), and (2) its significant agricultural value (Jalkh et al., 2020). In this area, conventional agricultural practices have been used for ages when no GAPs are applied (McKelvey, 2021). Fertilizers and pesticides have been dumped randomly without any limitations or consideration of the potential hazards that might be imposed. Excessive use of non-fermented manure has been the choice of farmers for years, which rises concerns about contaminating the soil with pathogenic bacteria that might reach the produce and thus exposing the consumers to potential health risks and threatening food safety (Tien et al., 2017). The scenario was observed in the soil collected from D4 and D5 where the samples tested positive for *E. coli* (Table 1). In addition the main sources of irrigation water in the LRB are either the river water directly or shallow wells dogged in the nearby area (FAO & IHE Delft, 2019). Its noteworthy that the water used was found to be contaminated with *E. coli* and many other pathogenic bacteria without any treatment (Holtmeier, 2019). These results were consistent with our findings, were water collected from D1, D2 and D3 before planting found to be contaminated with *E. coli* (Table 1). Sources of *E. coli* in water are either disposal of untreated wastewater into water resources, including rivers, or *via* runoff from agricultural fields amended with manure (Daou et al., 2018; FAO & IWMI, 2017; Manyi-Loh et al., 2018). Based on several studies, it was found that *E. coli* concentrations in the summer increase to reach its peak in autumn season (Mohammed et al., 2019). This was consistent with our results where water samples tested positive for *E. coli* only during the pre-planting season in D1 and D3 (D1 and D3 started in summer and

finished in winter), and only during the harvest season of D4 and D5 (D4 and D5 started in winter and finished in summer) (Table 1). This might be due to many reasons, (1) increase in temperature leads to the evaporation of water and thus concentration of the *E. coli* and the nutrients (Petersen & Hubbart, 2020; Yang et al., 2020), (2) *E. coli* prefers warm temperatures (*E. coli* can grow at temperatures ranging from 10°C to 40°C but its optimum growth temperature for *E. coli* is the same as that of the body which is 37°C) (Percival & Williams, 2014)., (3) increase in domestic use of water in summer thus more sewage disposal (Geara-Matta et al., 2010), (4) dilution of river water in winter due to rainfall (Huang et al., 2020; Maphanga et al., 2022) and (5) extremely low temperature might stress the bacteria causing VBNC (Ramamurthy et al., 2014).

What comes first, the egg or the chicken? Trying to trace the origin of *E. coli* was a similar scenario. But since *E. coli* are natural inhabitants of the intestinal tract, it was suggested through, microbial source tracking, that the route of its transmission to the environment was through fecal deposition (Ishii & Sadowsky, 2008). Therefore, *E. coli* can be considered as an indicator of fecal contamination in the environment with the host considered as its primary habitat (van Elsas et al., 2011). A recent concern had arisen after the emergence of the naturalization concept where *E. coli* has been proven to become naturalized to soil, sand, sediments, and algae in the environment. Hereby considering *E. coli* as the only indicator of fecal contamination became an issue (Ishii & Sadowsky, 2008). At the molecular and genetic levels, it was found that *E. coli* isolated from the soil was genotypically different than that isolated from the colon of warm-blooded animals in the same area. Different *E. coli* isolates coming from the soil had genotypically identical DNA fingerprints which were totally distinct from the animal-borne ones in the same area suggesting that the former *E. coli* was not deposited into the soil from the feces of these

animals (Ishii et al., 2006). In our study this scenario was observed in demo plots (in G2 and G4 of D4 and in D5) where water was *E. coli* free whereas the soil was contaminated

Although *E. coli* is a non-spore forming bacterium, some strains have the ability to enter a dormant state where it is Viable But None Culturable (VBNC) (X.-H. Zhang et al., 2020). In this state, these cells maintain viability while being unable to be cultured on standard laboratory media. For instance, in an experiment involving *E. coli* O157:H7 derived from manure, direct microscopic counts revealed significantly higher cell numbers than those obtained through plating on a selective medium (Semenov et al., 2007). This state can be triggered due to different types of stresses such as extreme temperatures (such as -4°C) and the presence of toxic heavy metals (Cu, Pb, Hg and Cd) (Klein & Alexander, 1986). Knowing that LRB is exposed to extreme temperatures (low during winter and high during summer) and is contaminated with HM, this may explain the unjustified absence of *E. coli* in soil and water when possible sources of contaminations were detected (samples contaminated during the pre-planting season and not contaminated during the harvest period and vice versa in soil water samples of D3, water samples of D4 and D5) (Table 1).

As for the Romain lettuce, this type of produce is planted in the soil what exposes it to the animal feces and contaminated water (Wolters, 2022). Lettuce is also affected by the harvest season, for instance, the fall lettuce harvest in coastal California is associated with a higher likelihood of *E. coli* outbreaks (Leonard et al., 2021). The consumption of *E. coli* contaminated lettuce imposes a potential food safety risk, since this type of produce is consumed raw without any boiling or cooking to kill the bacteria and washing it alone isn't enough to remove the bacteria (CDC, 2021). In our study, lettuce in three out of five demo plots were found to be contaminated with *E. coli*. In plots where soil samples tested positive

for *E. coli* during the harvest season, lettuce samples also tested positive. Which means that *E. coli* might have transmitted from soil to lettuce.

Agricultural water has been defined as a major risk factor in the contamination of leafy crops eaten raw as salads (EFSA, 2014). So, we established in a greenhouse three irrigation systems to track the *E. coli* contamination from one side and to check which system will mitigate the *E. coli* contamination. For the treated lettuce samples, *E. coli* isolated from water sample collected from LRB and inoculated in water to get 10^8 CFU/mL concentration. Lettuce samples collected from the hydroponic system were the least contaminated with *E. coli* suggesting that it's the best irrigation system compared to the other two systems (Figure 1). Since *E. coli* was directly sprayed on the leaves in the sprinkler irrigation system, the *E. coli* levels were the highest. The lettuce samples might be the least contaminated due to the presence of the root border cells that play a role in plant defense mechanisms against pathogens (Hawes et al., 2000). Due to the high levels of nutrients and organic matter in the soil, in addition to the warm temperatures *E. coli* levels in can survive in the soil for long period of time establishing a commensal relationship that, thus in absence of predation and competition, it maintains stable populations in soil. This was consistent with our results where soil samples were the most contaminated with *E. coli* (NandaKafle et al., 2018; Petersen & Hubbart, 2020).

E. Conclusion

In conclusion, this study opens new horizons in understanding the intricate dynamics of *E. coli* contamination in agricultural environments, particularly in the vulnerable LRB in Lebanon. By shedding light on the seasonal variations, sources of contamination, and the potential adaptation of *E. coli* in the environment, we aim to pave

the way for innovative strategies in mitigating risks to both public health and environmental sustainability. The findings underscore the urgency of adopting responsible agricultural practices and efficient water management systems to address the challenges posed by *E. coli*, offering new perspectives for future research and practical interventions. As we navigate these new horizons, there is an opportunity for transformative approaches that safeguard food safety and promote ecological resilience in agricultural landscapes.

CHAPTER III

INFLUENCE OF HEAVY METALS ON THE VIABILITY OF *ESCHERICHIA COLI* ISOLATED FROM WATER, PLANT AND SOIL

A. Introduction

Foodborne diseases may result from bacterial or chemical contaminants present in food. Over the past years, intensive studies led to identifying *Escherichia coli* as a major cause of foodborne illnesses (Organization & Nations, 2018). According to the CDC, vegetables and other food products, including lettuce, are the common cause of *E. coli* outbreaks (*Division of Foodborne, Waterborne, and Environmental Diseases | CDC*, 2023). One of the major sources of contamination of leafy greens by *E. coli* is from the growing environment. Specifically, the soil and irrigation water can harbor *E. coli* originating from wild animals' fecal matter, untreated manure, and household sewage water (Canada, 2017; Commissioner, 2020; Liu et al., 2013).

Moreover, studies have also shown that the soil and irrigation water are contaminated with Heavy Metals (HM) that may be, in fact, one of the sources of lettuce chemical contamination (Eissa et al., 2018; Ferri et al., 2012) urban and industrial effluents, excessive doses of manure, fertilizers, pesticides and mining being major sources of HM in the soil (Alengebawy et al., 2021). When agricultural fields are irrigated with wastewater containing HM, these latter accumulate in the soil and become bioavailable to crops with time (Toze, 2004). In fact, when the levels of HM in the water and soil were below the maximal limit, their concentrations in lettuce were also low (Akrong et al., 2012; Mekonen et al., 2022; Somda et al., 2019).

In Lebanon, the agricultural sector is responsible of 60% water usage with 45% of irrigated lands relying on surface water as a primary source (Boretti & Rosa, 2019). Farmers depend on pumping ground water by digging wells for irrigation (Khoury et al., 2014). Numerous investigations have been conducted over the years to assess the biological and chemical quality of the river water, particularly due to Lebanon's ongoing solid waste mismanagement issues. Notably, a study by Dagher et al. in 2021 (Dagher et al., 2021b) revealed the presence of *E. coli* in 96.29% of the tested samples. Furthermore, the river water was found to have high concentrations of HM (Borjac et al., 2020; Haydar et al., 2014a; Shaban, 2021). The leaching of fertilizers and the dump of sewage into the rivers increased the contamination (Saadeh et al., 2012). Hereby, water and soil contamination impose a critical problem on the agricultural sector and thus food safety in Lebanon (Borjac et al., 2020).

Although numerous initiatives were undertaken in Lebanon to evaluate soil, irrigation water, and lettuce contamination with *E. coli* and HM, we couldn't find studies done to understand the relationship between those two co-existing contaminants. It is important to mention that metals are essential for different biological pathways in bacteria where they act as cofactors for enzymes involved in cellular energy production and growth (Loutet et al., 2015). At specific concentrations, HM become toxic to microorganisms (Ngwewa et al., 2022) and are responsible for reducing microbial diversity (Tsai et al., 2005). Furthermore, recent studies showed that the increase in HM contamination might drive the increase in Anti-Microbial Resistance (AMR) (Bazzi et al., 2020) or might inhibit it (Khaira et al., 2022). Therefore, this study aims at comprehending the impact of various HM concentrations on the viability of three *E. coli* isolates and testing the AMR of those isolates. In this study, it was hypothesized that a resistance mechanism against high

concentrations of HM in the environment has been developed by *E. coli*, potentially leading to the acquisition of AMR genes.

B. Material and Methods

1. Isolates collection and storage

From the Central Bekaa area, in an agricultural land located near the Litani River Basin (LRB) a highly contaminated area (Haydar et al., 2022; Mcheik et al., 2018), irrigation water, lettuce, and soil samples were collected and tested for the presence of *E. coli*. The microbiological testing was done using Rapid *E. coli* 2 agar (Bio-rad USA) after enrichment in Buffered Peptone Water (BPW) (Himedia, India). The suspected colonies were confirmed using the biochemical tests of the Api20E strips (Biomerieux, France). Finally, purification on Mac Conkey (Himedia, India) and storage in glycerol stocks at -80°C were done for investigation.

2. Preparation of the treatments

a. Preparation of the isolates

From the stored stocks, the isolates were revived by streaking on Rapid *E. coli* 2 agar and incubating at 37°C, overnight. For each isolate, two to three colonies were then used to inoculate 1000 mL of BPW and incubated at 37°C with constant shaking. The Optical Density (OD) using the spectrophotometer was measured at 600nm to determine the concentration of the inoculum by comparing the results to a pre-prepared growth curve. Two solutions were then prepared:

- BPW containing 10^8 CFU/mL of the isolate, corresponding to OD of 0.4
- BPW containing 10^4 CFU/mL of the isolate, corresponding to OD of 0.2

b. Preparation of the HM solutions

Four HM salts (copper II sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), MERCK), nickel sulfate (NiSO_4 UNI-CHEM), zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, MERCK) and manganese sulfate ($(\text{MnSO}_4 \cdot \text{H}_2\text{O})$, FLUKA) were used to prepare 500 mL stock solutions of 1000ppm using autoclaved distilled water. The amount of metal salt used was calculated using the following equation:

$$\text{amount of metal salt} = \frac{\text{molecular weight of metal salt}}{\text{molar mass of target metal}}$$

From the initial 1000ppm HM stock, a serial dilution using BPW was prepared. For Cu, Mn, and Zn, seven treatments were prepared containing respectively 2, 10, 20, 40, 100, 140 and 200 ppm dilutions whereas 20, 40, 100, 140, 200, 240 and 300ppm treatment solutions were prepared respectively for Ni.

c. Preparation of the HM solution containing *E. coli*

To prepare the solutions containing HM and *E. coli* together, 25 mL of the HM solution was added to 25 mL of the isolate broth forming 50 mL of BPW containing *E. coli* at a certain concentration (10^4 or 10^8 CFU/mL) and HM at a specific concentration from the previously prepared HM solutions to obtain for each HM seven treatments (1, 5, 10, 20, 50, 70 and, 100 ppm for Zn, Mn, and Cu, and 10, 20, 50, 70, 100, 120, and 150ppm for Ni) at 2 different *E. coli* concentrations (10^2 or 10^4 CFU/mL). A control formed of 25ml of isolate broth mixed with 25ml of BPW without HM in a sterile urine cup was prepared for comparison.

3. Bacterial behavior assessment

To assess the effect of the HM on the behavior of the bacteria, two different methods were used. First, the absorbance at 600 nm was measured at T_0 and at T_{10} after 10

hours of treatment, and the difference in OD was calculated and compared to the control. Second, the growth curve of the bacteria was obtained using a plate reader (Fisher, Canada) for 20 hours. The growth curves were compared to the control and to each other. The survival of bacterial cells was confirmed by culturing the bacteria on Mac Conkey agar after 10 hours of treatment. To do that, a serial dilution was done for each treatment using BPW and the 10^{-9} and 10^{-10} dilutions were plated in duplicate on Mac Conkey agar. The viable count was evaluated after incubation at 37°C for 24h.

4. Synergy between the four HM treatments

To investigate the response of *E. coli* to various HM treatments, a Principal Component Analysis (PCA) was conducted. The PCA involved the comparison of the four HM over durations of 5 to 15 hours all ranging from 10 ppm to 100 ppm. This allowed for the observation of correlations between these variables. This analytical approach aimed to identify patterns and trends in *E. coli*'s response to various HM concentrations, aiding in a comparative assessment of the treatments.

5. Antimicrobial susceptibility tests

Kirby-Bauer disc diffusion method was done to test the antimicrobial susceptibility of *E. coli* isolates. The results were interpreted based on the Clinical Laboratory Standards Institute (CLSI) guidelines. The antimicrobials used are β Lactams, Aminoglycosides, Quinolones, Tetracyclines and Macrolides. The list used is as follow Cefuroxime (Cxm30, 30 μg , BIO-RAD), Ciprofloxacin (Cip5, 5 μg , BIO-RAD), Fosfomycin (Fos200, 200 μg , BIO-RAD), Amikacin (AKN30, 30 μg , BIO-RAD), Cefepime (FEP30, 30 μg , BIO-RAD), Trimethoprim + Sulfamethoxazole (SXT25, 1.25 + 23.75 μg , BIO-RAD), Levofloxacin

(LVX5, 5µg, BIO-RAD), Gentamicin (GMN10, 10µg, BIO-RAD), Piperacillin + Tazobactam (TZP 110, 100 + 10 µg, BIO-RAD), Tetracycline (TET30, 30µg, BIO-RAD) and Meropenem (MEM10, 10µg, BIO-RAD).

When the visual opacity of the *E. coli* inoculum reached 0.5 McFarland Standard, a loop of the inoculum was evenly spread on MacConkey agar plates in five different directions. The antibiotic discs were then dispensed onto the plates that were left to completely dry before being incubated for 24h at 37°C. The standard breakpoints (resistant, intermediate, or susceptible) produced by CLSI guidelines were used to interpret the measured zone diameters.

6. Statistical analysis

Statistical analysis was performed using R studio software (R.4.3.0) to perform statistical analysis on water, lettuce and soil samples treated with various concentrations on Cu, Mn, Zn and Ni. One-way ANOVA was used followed by Tukey's Honestly Significant Difference (Tukey HSD) test and the Kruskal-Wallis's test as a non-parametric alternative to ANOVA followed by Dunn's test. Differences were considered statistically significant for p values < 0.05.

C. Results and Discussion

From the field near the LRB, *E. coli* isolates were found in the irrigation water, lettuce, and soil samples. At 10⁴CFU/mL concentration, the three *E. coli* isolates were indifferent to the presence of HM. The comparison of the growth curves over the period of 20 hours (Data not shown) showed no significant differences between the different treatments and the control. However, when the starting concentration dropped to

10^2 CFU/ml of *E. coli* in the presence of the same concentrations of HM, it was observed that these bacteria exhibited various responses to the treatments. According to several studies, the density of a bacterial culture or population may also influence the toxicity of HM (Yu et al., 2011). Regardless of the treatment, the growth curves of the treated *E. coli* at 10^4 CFU/ml were similar to that of the control thus when the bacterial concentration was high, *E. coli* became insensitive to the presence of HM.

1. Effects of the HM on low concentrations of E. coli

The initial concentration of *E. coli* was 10^2 CFU/ml. Each isolate was treated with the different concentrations of HM that were 1, 5, 10, 20, 50, 70 and 100 ppm for Zn, Mn, and Cu, and 10, 20, 50, 70, 100, 120 and 150ppm for Ni. The difference in absorbance between T_{10} and T_0 was calculated. In parallel, the behavior of *E. coli* in the presence of the different HM was assessed through the growth curves obtained over a period of 20h of treatment.

a. Treatment with Copper (Cu)

Treatment with Cu showed different profiles when comparing the three isolates (Figure 1). The soil isolate was affected by the Cu at all concentrations from 1 ppm to 100 ppm, whereas the water and lettuce isolates, had similar behaviors. When comparing to the control, the water isolate started to be slightly affected by the presence of Cu at 50 ppm and above, whereas the lettuce isolate showed no significant differences. The soil isolate, as mentioned before was significantly different at all the concentrations.

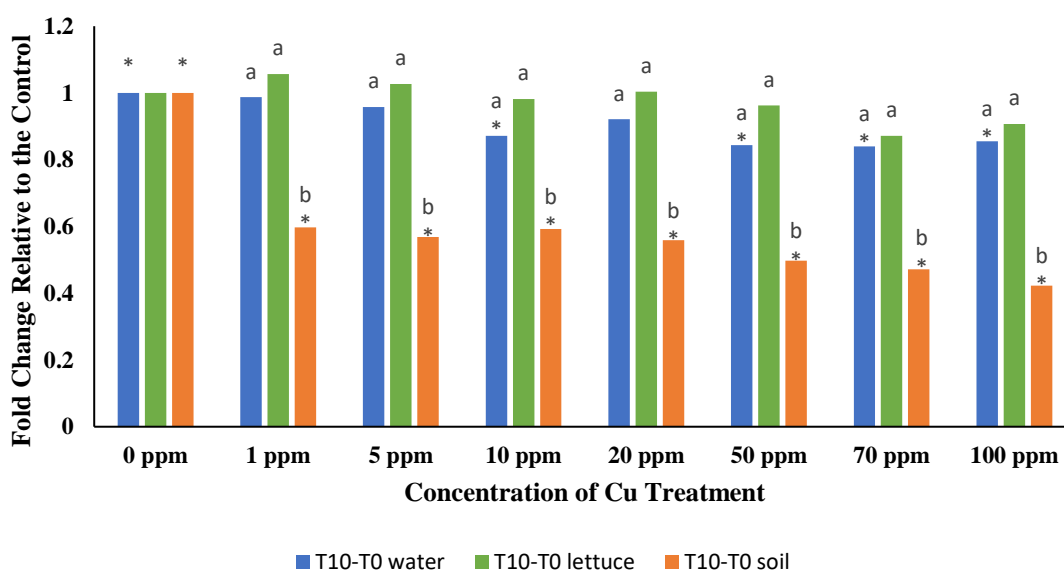


Figure 2 Effect of Cu on the growth of *E. coli* isolated from water, lettuce, and soil. The O.D at T0 was subtracted from the O.D at T10 to get the difference in the growth of bacteria and compare it to the control. The * are used to indicate the significance between the control of each isolate and its corresponding treatments. The letters represent the significant effect of each treatment on each bacterium that compares the effect of each treatment on the three isolates.

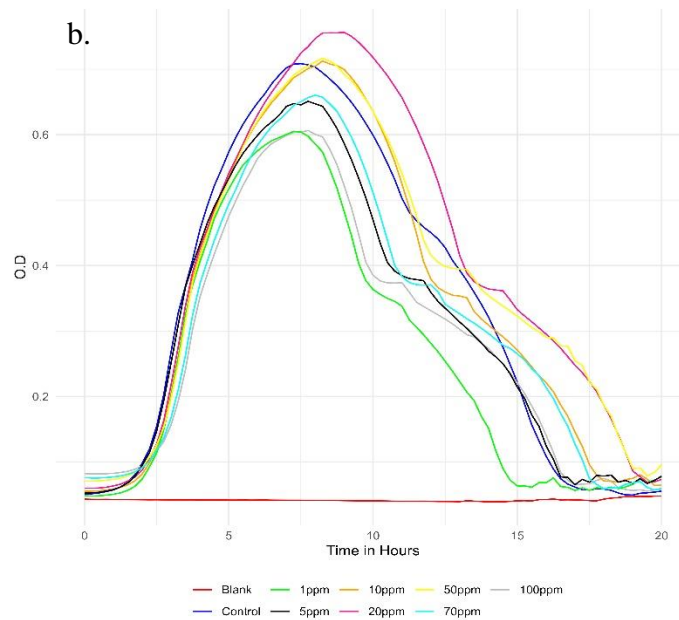
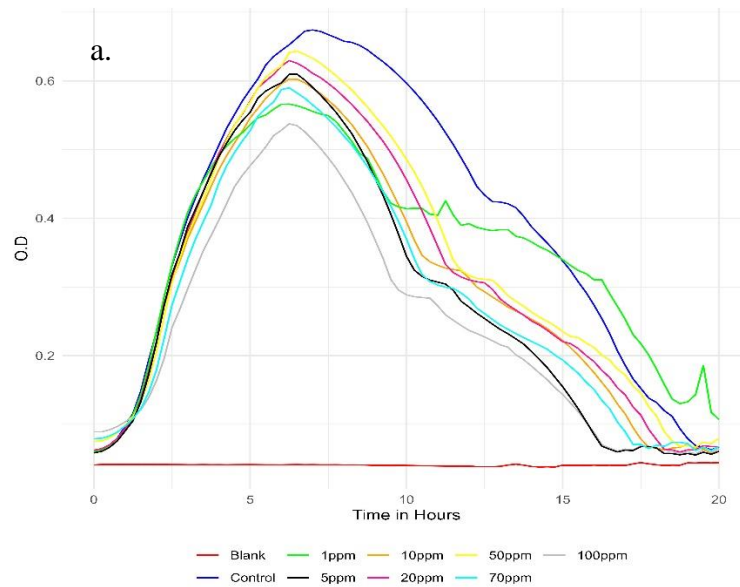
To better understand the behavior of *E. coli* in the presence of Cu, the growth curves were plotted during a period of 20h (Figure 3). During the early stages (till 5h for water, 7h for lettuce and 6h for soil), our study found that Cu treatment did not exert a noticeable impact since no significant differences were noted in the behavior of the isolates. The differences were found starting the late exponential phase (5h for water, 7h for lettuce and 6h for soil) where all isolates showed a dose-dependent response with increasing Cu concentrations. In Fact, when comparing the maximal concentrations of the water isolate, the peak of the control reached the highest OD at 0.67. When treated with Cu, the peaks increased from 0.56 OD to 0.64 OD in the presence of 1 to 50 ppm Cu, then decreased to reach 0.53 at 100 ppm. Accordingly, the same behavior was observed during the death phase. Consequently, water isolate was affected by the presence of Cu since even at low concentrations the peaks were lower than the control. The scenario of increase than

decrease could be from the ability of the isolate to accommodate the presence of Cu to reach a point of toxicity (70 ppm) that lead to the significant decrease in the growth rate. In lettuce, a similar pattern was observed where the peaks of the curves increased with higher concentrations from 1 to 20 ppm, to decrease afterwards. It should be mentioned that the 10 and 50 ppm had a same maximum as the control. The 20 ppm treatment induced the growth of bacteria to reach an OD of 0.75 which was above that of the control (0.70 OD) (Figure 3-b).

In soil, the Cu played a role of an inducer. As the concentration of the treatment increased, the growth increased till it reached its maximum at 20 ppm to decrease afterward and reach the same level of the control at 100 ppm (Figure 3-c).

Therefore, the response to Cu treatment was complex and isolate-specific. The water isolate displayed increased sensitivity to higher Cu concentrations, which is consistent with microbial responses to environmentally toxic metal ions in the literature (Reyes-Jara et al., 2016). In contrast, the lettuce isolate displayed a different response profile. The soil isolate, in contrary, exhibited a unique response pattern distinct from both the water and lettuce isolates. The differences in the isolates' profiles are justified by the studies proposing a complex role for Cu in microbial pathogenesis. In fact, Cu is considered as an essential co-factor for specific microbial enzymes on one hand, and has a potential utility in limiting pathogen proliferation *in vivo* on the other hand (Samanovic et al., 2012). In the case of *E. coli*, the elevated Cu levels pose toxicity to the microorganism (Nies, 1999). In Lebanon, studies showed that 0.01 to 0.2 ppm of Cu was present in irrigation water (Haydar et al., 2022) concentrations at which *E. coli* wouldn't be affected, 9 ppm in Lettuce (Fadel et al., 2017, 2019) and 6.25 to 35 ppm in soil (Mohamad Darwish et al., 2022). Reconciliation at which the growth of *E. coli* may be induced. In

conclusion, *E. coli* isolates from lettuce exhibited a greater degree of resilience to Cu treatment. However, soil and water isolates were affected at different concentrations. Thus, the MIC (Minimum Inhibitory Concentration) of Cu are 1ppm and 50 ppm for the soil and water isolates respectively.



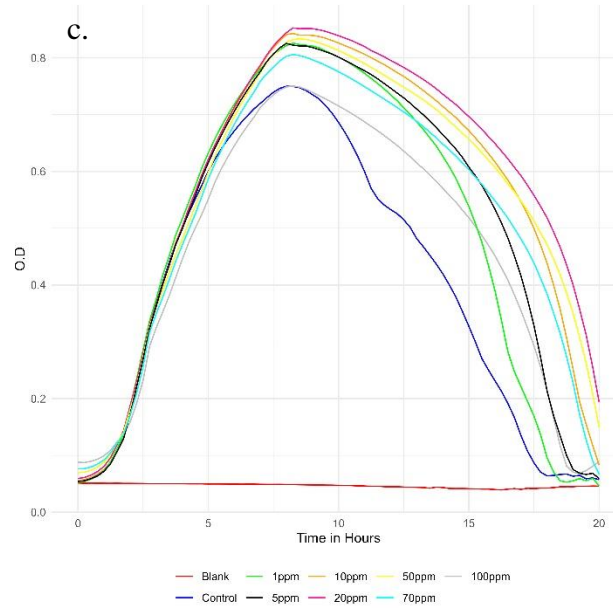


Figure 3 Behavior of the *E. coli* isolated from a. water b. lettuce, c. soil, and treated with different concentrations of Cu over a period of 20 hours.

b. Treatment with Manganese (Mn)

Treatment with varying Mn concentrations produced distinct responses in *E. coli* isolates, demonstrating isolate-specific effects. The soil-derived *E. coli* isolate exhibited a unique trend, with higher Mn concentrations promoting increased viability. Conversely, the water and lettuce isolates were resistant to Mn treatment where the difference in growth between T10 and T0 didn't show any significant differences compared to each other and to the control (Figure 4).

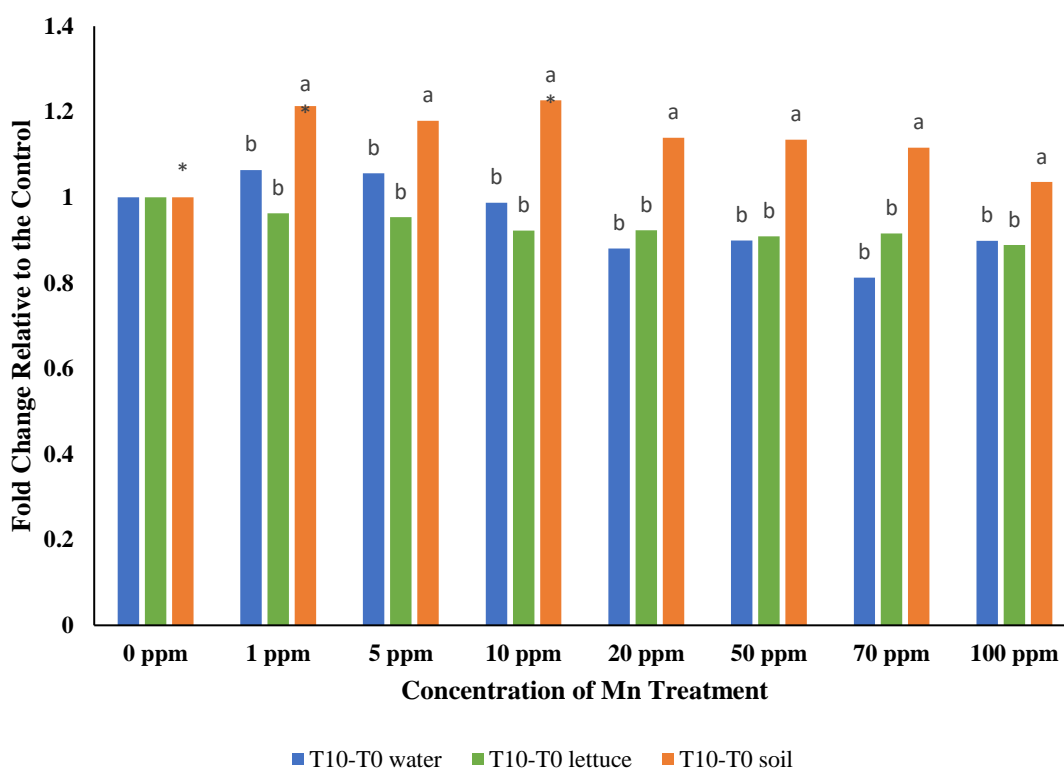
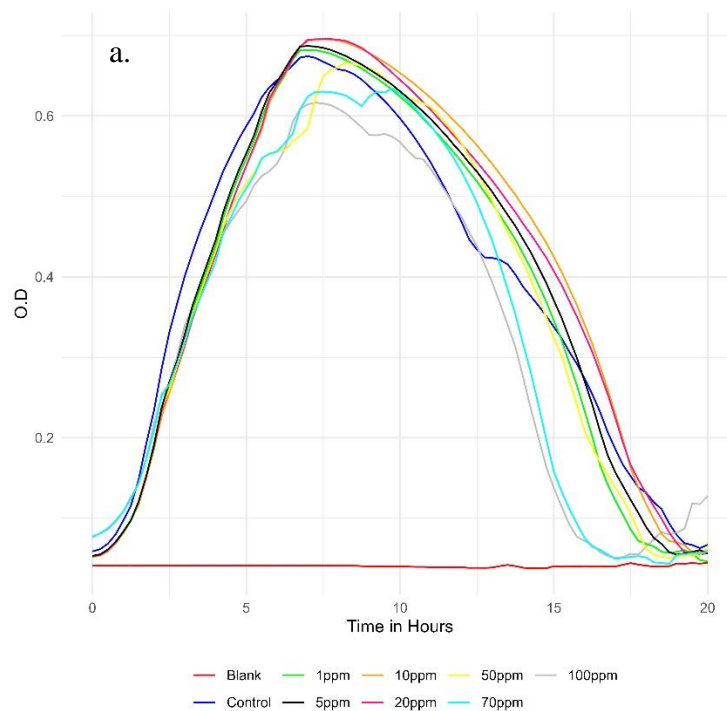


Figure 4 Effect of Mn on the growth of *E. coli* isolated from water, lettuce, and soil. The O.D at T0 was subtracted from the O.D at T10 to get the difference in the growth of bacteria and compare it to the control. The * are used to indicate the significance between the control of each isolate and its corresponding treatments. The letters represent the significant effect of each treatment on each bacterium that is comparing the effect of each treatment on the three isolates.

When comparing the behavior over a period of 20h, no visible difference was evident in the growth patterns of the three distinct *E. coli* isolates concerning their responses to Mn treatments, during the lag and early exponential phases (till 7h for water and lettuce and 9h for soil) (Figure 5). After that phase, the water isolate treated with Mn displayed consistent growth from 1 to 50 ppm compared to the control. Only at 70 and 100 ppm, *E. coli* was significantly affected (Figure 5-a). For lettuce and soil, Mn induced the isolates' growth where the curves were higher than the control in a dose-dependent manner. The peaks reached their maximum at 50 ppm before decreasing (Figure 5-b-c). Comparing the three isolates, the water isolate emerges as the most sensitive to Mn treatment where

the 70 ppm was considered as the MIC. In contrary, lettuce and soil isolates were resilient as the presence of this metal induced the growth. In Lebanon, a study quantified the level of Mn in water (Haydar et al., 2022), soil and lettuce(Fadel et al., 2017) where it reached 1.17 ppm, 354 ppm and 72.8 ppm respectively. Those concentrations were enough to induce the growth of *E. coli* in those environmental samples. In the case of *E. coli*, Mn is an unnecessary element since its uptake typically occurs when Fe is limited, as it can function as a substitute cofactor for Fe-dependent enzymes. This Mn-induced stress can be seen as an antagonist to Fe metabolism, ultimately leading to the depletion of cellular Fe levels in *E. coli* (Kaur et al., 2014; Martin et al., 2015). This explained in our study the induced growth in most cases and the stress at higher concentrations in water isolate (70 and 100 ppm).



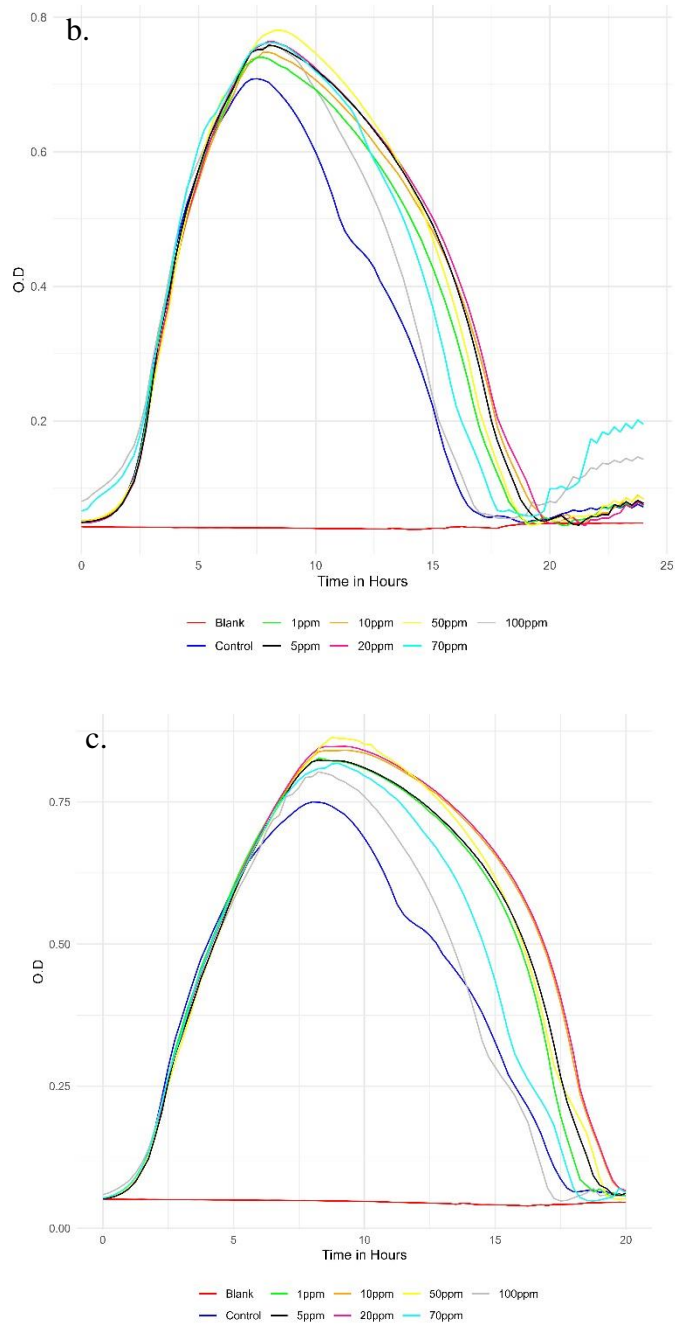


Figure 5 Growth curve of the *E. coli* isolated from a. water b. lettuce c. soil and treated with different concentrations of Mn.

c. Treatment with Zinc (Zn)

Treatment with Zn also showed isolate-specific behavior (Figure 6). No significant difference was observed upon the treatment of the water isolate at all concentrations when compared to the control. The lettuce isolate on the other hand, was affected by Zn where even at low concentrations (1 ppm) Zn had a bacteriostatic effect that is considered as MIC. However, soil-derived *E. coli* isolate was resilient, showcasing survival below the 10-ppm threshold. A treatment of 20 ppm was enough to prevent the bacteria from growing making it an MBC (Minimal Bactericidal Concentration) since less than ten colonies were detected upon culturing on agar. This behavior aligns with findings from studies investigating isolate-specific responses of bacterial strains to Zn and Cu exposure (Ji & Silver, 1995).

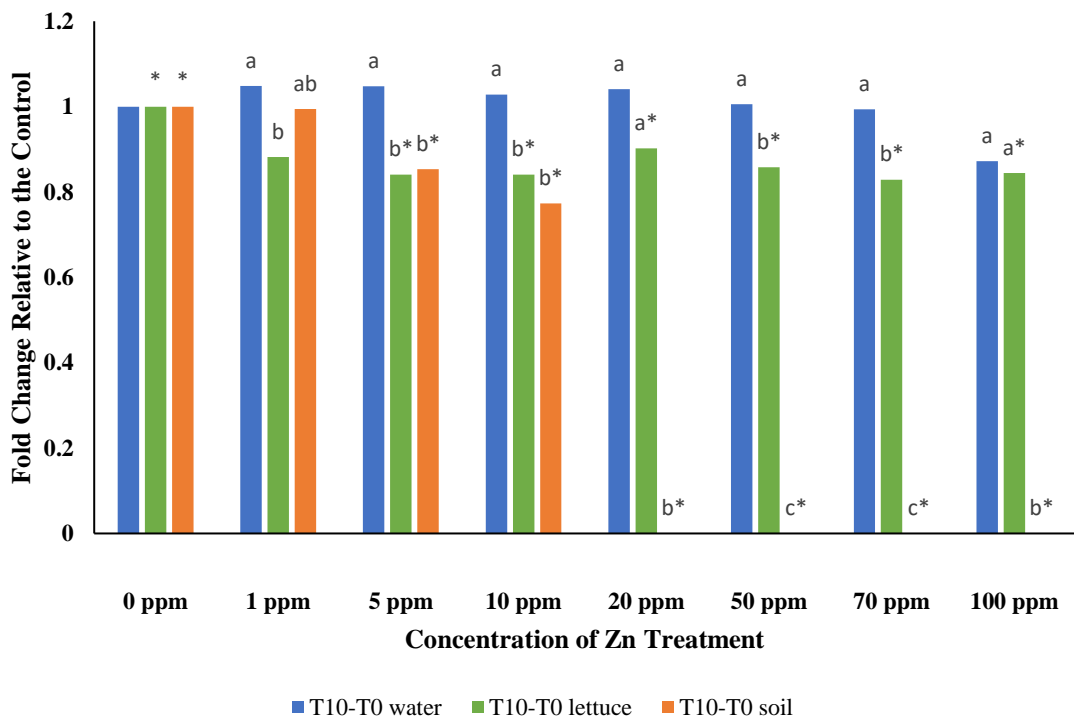
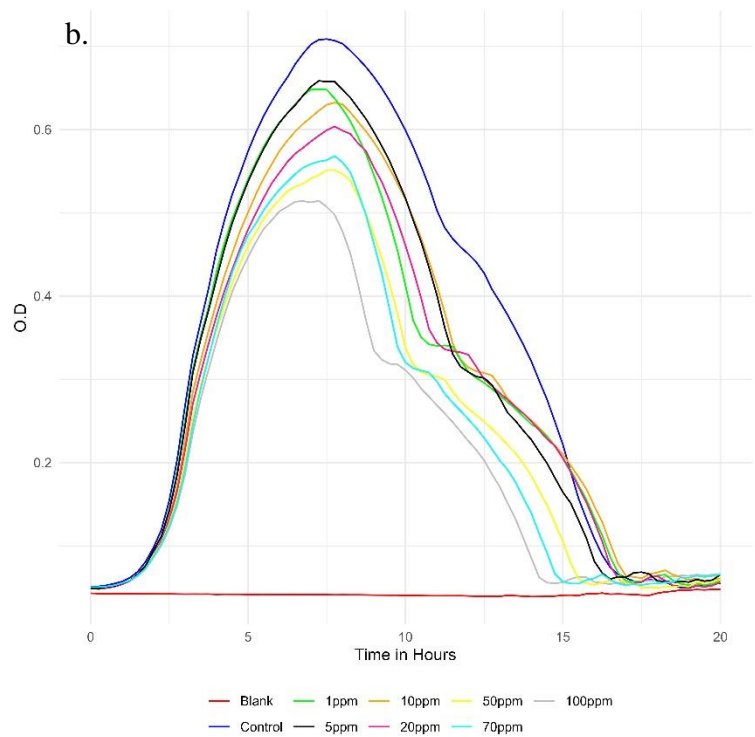
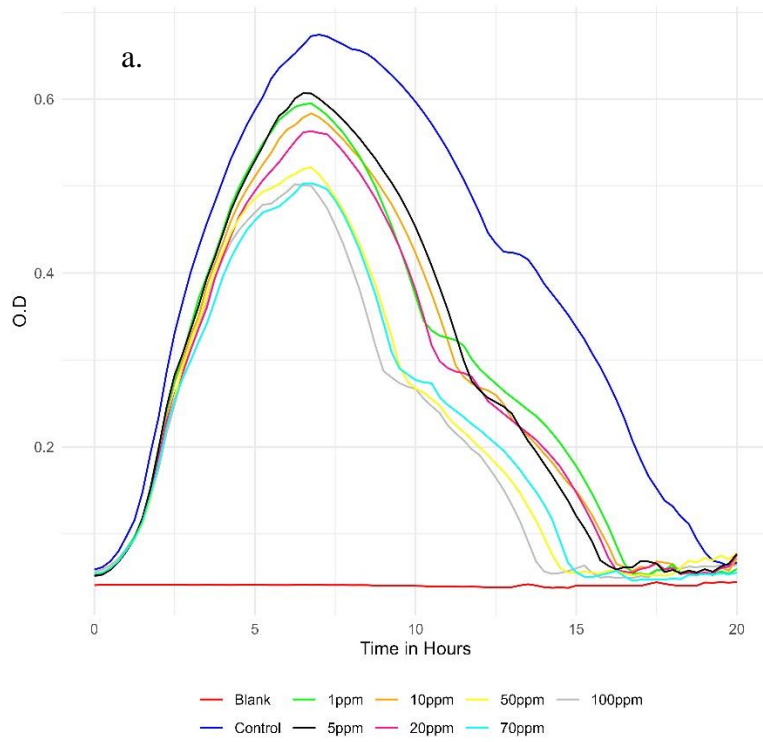


Figure 6 Effect of Zn on the growth of *E. coli* isolated from water, lettuce, and soil. The O.D at T0 was subtracted from the O.D at T10 to get the difference in the growth of bacteria and compare it to the control. The * are used to indicate the significance between the control of each isolate and its corresponding treatments. The letters represent the

significant effect of each treatment on each bacterium that is comparing the effect of each treatment on the three isolates.

Interestingly, when comparing the behavior of the three isolates over a period of 20h, all the isolates were affected by the Zn treatment in a concentration-dependent manner (Figure 7). The growth rate decreased with the increase of concentration during the late exponential phase and beyond. These observations align with existing literature on the dualistic nature of Zn, which can exert both essential and toxic effects on microorganisms, depending on its concentration (Bamberger et al., 1993). Zn is an essential trace element required for all living organisms due to its pivotal roles in structural, enzymatic, and regulatory functions within proteins and enzymes (Hamzah Saleem et al., 2022; Sturikova et al., 2018). However, excessive Zn levels can lead to toxicity, characterized by competition with the absorption of other essential elements, the generation of reactive oxygen species, and contributing to heavy metal toxicity, ultimately inhibiting bacterial growth (Kabasakal et al., 2009; Li et al., 2019; Y. Y. Zhang et al., 2022). The adaptability of the isolates to higher Zn concentrations echoes the adaptability of certain microbial populations to heavy metal exposure, a phenomenon well-documented in microbial ecology (Hussain et al., 2022; Khouri et al., 2014).



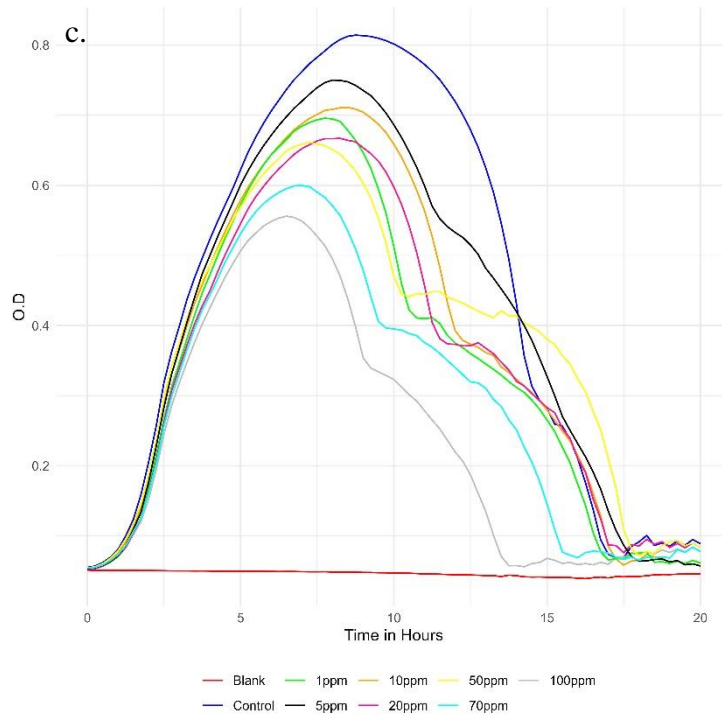


Figure 7 Growth curve of the *E. coli* isolated from a. water b. lettuce c. soil and treated with different concentrations of Zn.

d. Treatment with Nickel (Ni)

The case of Ni treatment is similar to the three other HM as its effect was isolate-dependent. The growth of each isolate was inhibited at a different concentration, where the recorded MIC values were 50, 20 and 150 ppm for water, lettuce and soil isolates respectively (Figure 8). It was interesting to mention that the lettuce isolate maintained a constant behavior at all concentrations whereas the water and soil isolates decreased directly after the MIC treatment. In fact, at 150 ppm treatment, the decrease reached 0.7, 0.25 and 0.62-fold-change for the water, lettuce, and soil isolates respectively.

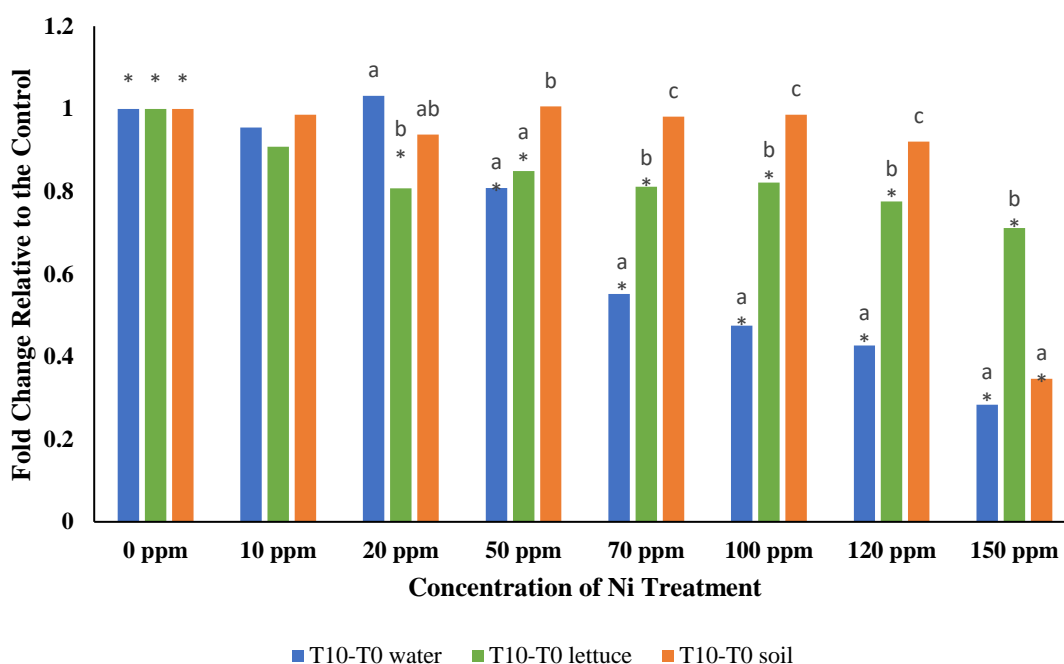
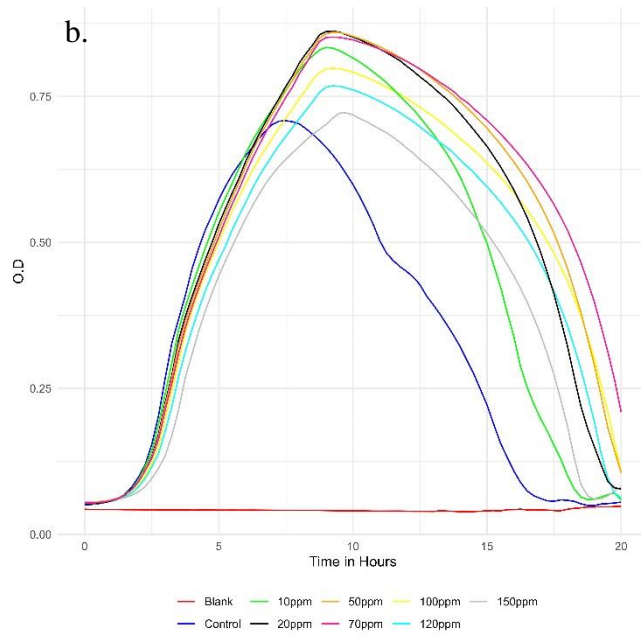
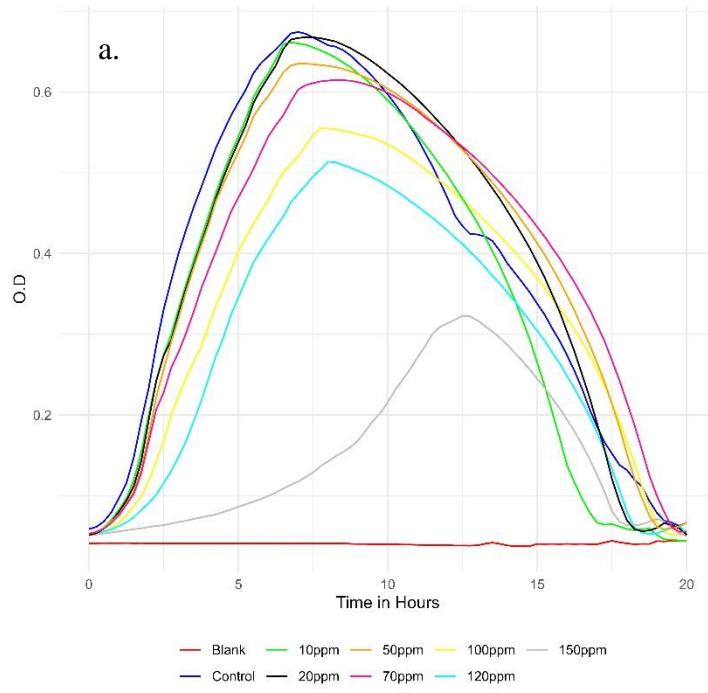


Figure 8 Effect of Ni on the growth of *E. coli* isolated from water, lettuce, and soil. The O.D at T0 was subtracted from the O.D at T10 to get the difference in the growth of bacteria and compare it to the control. The * are used to indicate the significance between the control of each isolate and its corresponding treatments. The letters represent the significant effect of each treatment on each bacterium that is comparing the effect of each treatment on the three isolates.

When comparing the behavior for 20h, water and soil isolates had similar growth patterns where the concentration of Ni and the peaks of the curves were inversely proportional (Figure 9-a-c). At high concentrations (above 100 ppm), these isolates displayed an extended lag phase before acclimating and entering the exponential phase, which had a steeper slope and reached the stationary phase earlier than the control. Note that the observed sensitivity of the soil isolate to Ni is consistent with previous studies on metal toxicity in soil-dwelling microorganisms (Rashid et al., 2023; Washington – Hughes et al., 2018; Zhao et al., 2020). For the lettuce isolate, the growth curves showed the resilience of the *E. coli* that took more time to complete its cycle (10h) with the presence of Ni at all concentrations, whereas the control arrived to its peak after 6h (Figure 9-a). The significant impact of Ni on lag phase duration underscores the potential physiological

alterations induced by elevated Ni levels, shedding light on the dynamic interactions between microbial communities and environmental Ni contamination (Pishchik et al., 2021). The latter article highlighted the effect of a combined interaction between plants and bacteria to resist the Ni toxicity where resistance genes might be present to overcome this stress. Ni has been reported as a natural component of soil and water and its concentration in the Earth's crust is about 0.008% (Kumar et al., 2021). It is an essential nutrient for microorganisms, where some enzymes are Ni dependent. According to the WHO, the safe limit of Ni in plants should be 1.5 ppm, and 75–150 ppm in soil (Bhatnagar & Awasthi, 2000). *E. coli* is a facultative anaerobic bacterium that requires the activity of Ni-containing hydrogenase to promote anaerobic growth thus deficiency of the specific Ni transport system leads to a hydrogenase-minus phenotype and slowed down the fermentative growth in the *nik* mutant. An addition of small quantities (17.607 ppm) of Ni can reverse the effect of such mutation but the addition of higher concentration of Ni inhibited the growth of *E. coli* (Wu et al., 1994).

These findings underscore the importance of considering the specific microbial responses to different metals and concentrations, as such variability can have substantial ecological implications (Gadd, 2010; Naylor et al., 2022; Wyszowska et al., 2005).



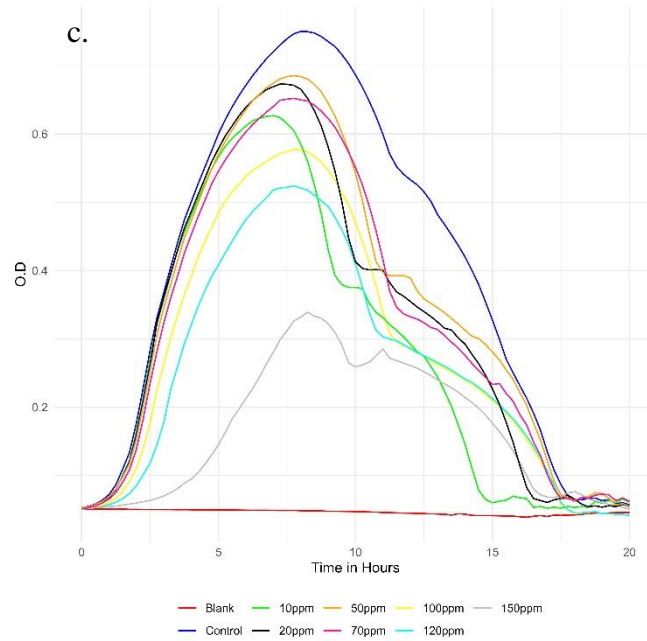
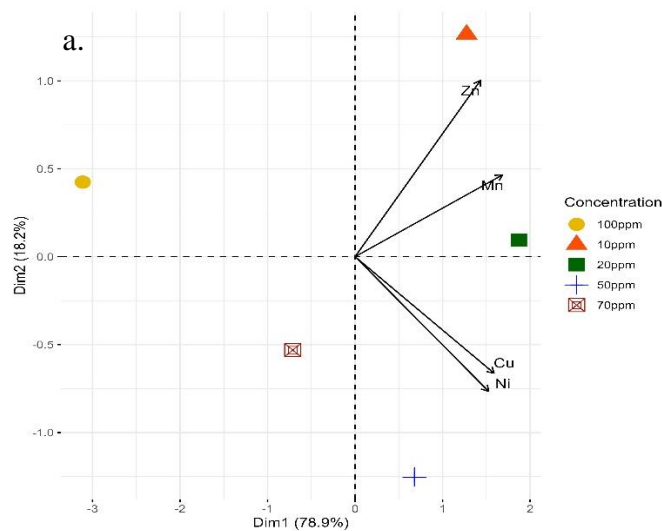


Figure 9 Growth curve of the *E. coli* isolated from a. water b. lettuce c. soil and treated with different concentrations of Ni.

2. Relationship between the HM and *E. coli* isolates

Principal Component Analysis (PCA) was conducted to analyze the interrelationships among Zn, Mn, Cu, and Ni concentrations ranging from 10 to 100 ppm in water, lettuce, and soil. The goal was to discern patterns and associations within the multivariate dataset encompassing these essential elements in various environmental compartments. The PCA revealed distinct clusters and trends based on concentration levels and environmental matrices. In water isolate, Cu and Ni had a pronounced relationship around 50 ppm, while Zn and Mn showed a less pronounced correlation at 10 ppm suggesting independent sources or influences on their presence (Figure 10-a) Regarding lettuce isolate, Cu and Zn displayed a notable correlation between 10 and 20 ppm, potentially implying shared uptake mechanisms or physiological interactions within the bacterial system, while Mn and Ni demonstrated a divergent pattern at 20 and 50 ppm, suggesting their separate bioavailability or uptake pathways in *E. coli* (Figure 10-b). In the

soil samples, Mn, Zn and Cu demonstrated a strong positive correlation, possibly indicating similar sources or mechanisms of accumulation at around 20 ppm. Conversely, Ni exhibited a weaker association, hinting at their differing behaviors or origins in the soil (Figure 10-c). Overall, the PCA provided valuable insights into the nuanced interactions and behavior of these trace elements across the studied environmental conditions. These variations highlight the importance of considering both the specific metal and its concentration when assessing microbial responses in different environments (Dahlin et al., 2005; Kapahi & Sachdeva, 2019; Ma et al., 2016; Nies, 2003; Pande et al., 2022).



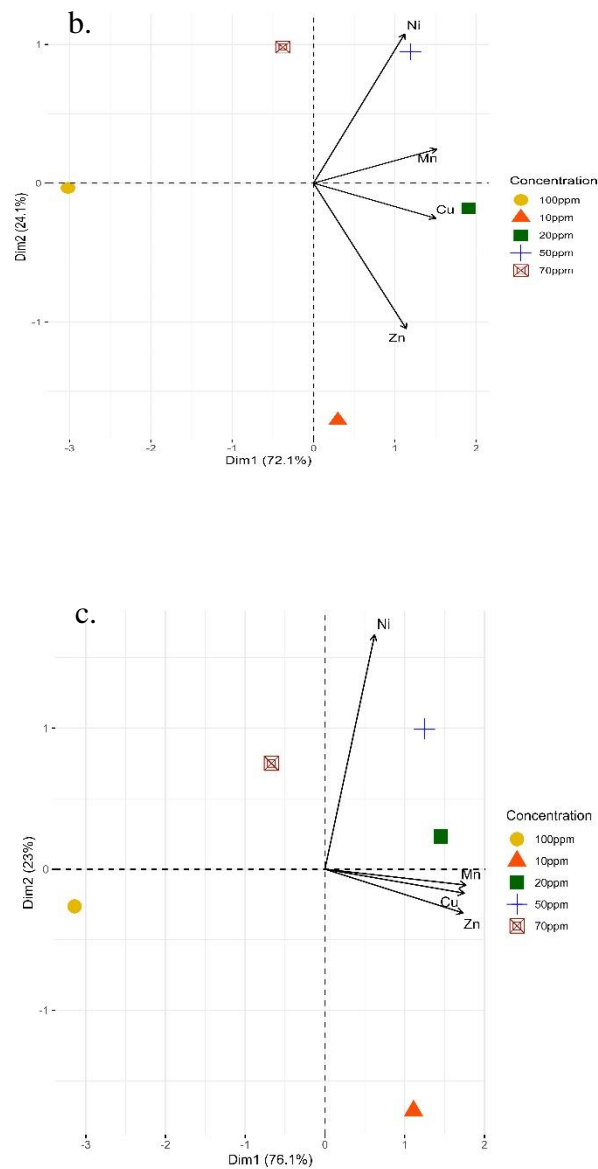


Figure 10 Interrelationships among heavy metals and their impact on *E. coli* isolated from a water, b lettuce, and c soil.

3. Antibiotic resistance

The three isolates were tested against 11 antibiotics (Table 2). Results showed that the *E. coli* isolated from lettuce, showed multi drug resistance against Gentamicin, Trimethoprim-Sulfamethoxazole and Levofloxacin. The other two isolated from water and soil were only resistant to Gentamycin. A similar case was observed in a study published in 2022, when two scenarios were also observed. Some bacteria showed additional

resistance when treated with HM, when others were sensitive to HM treatment (Urban-Chmiel et al., 2022). Although our three isolates exhibited resistance to Gentamicin, a recent study was conducted to assess the water quality in Lebanon, and showed that only 0.5% of the tested *E. coli* isolates were resistant to gentamicin and 32% of the isolates exhibited resistance to trimethoprim-sulfamethoxazole (Dagher et al., 2021b).

Table 2 Antimicrobial effect of different antibiotics against the 3 *E. coli* isolates

ANTIBIOTIC	WATER	LETTUCE	SOIL
CXM30	Sensitive	Sensitive	Sensitive
CIP5	Sensitive	Sensitive	Sensitive
FOS200	Sensitive	Sensitive	Sensitive
AKN30	Sensitive	Sensitive	Sensitive
FEP30	Sensitive	Sensitive	Sensitive
SXT25	Sensitive	Resistant	Sensitive
LVX5	Sensitive	Resistant	Sensitive
GMN10	Resistant	Resistant	Resistant
TZP110	Sensitive	Sensitive	Sensitive
MEM10	Sensitive	Sensitive	Sensitive
TET30	Sensitive	Sensitive	Sensitive

D. Conclusion

In conclusion, this study shed light on a critical issue in the LRB of Lebanon, where the high contamination of *E. coli* and HM in the water and soil poses a significant threat to food safety and public health through produce. By investigating the impact of the four HM on the behavior of the three *E. coli* isolates and assessing their antibiotic resistance, we have uncovered valuable insights. The distinct responses of the isolates to HM exposure and to antibiotics underscored the complexity of the interaction between environmental contaminants and bacterial behavior. These findings emphasize the importance of continued research and proactive measures to mitigate the risks posed by contaminated water sources in agricultural regions, ultimately safeguarding both the environment and public health.

CHAPTER IV

CONCLUSION

In conclusion, our study in the vulnerable LRB of Lebanon unveils the intricate dynamics of *E. coli* contamination in agricultural settings. By illuminating seasonal variations, contamination sources, and potential bacterial adaptation, we charted new paths for innovative strategies to mitigate risks to public health and environmental sustainability. Urgency was underscored for adopting responsible agricultural practices and efficient water management, presenting novel perspectives for future research and interventions. Navigating these new horizons offers an opportunity for transformative approaches that safeguard food safety and promote ecological resilience in agricultural landscapes. Additionally, our investigation in the LRB highlighted the pressing threat of *E. coli* and HM contamination, emphasizing the critical need for ongoing research and proactive measures. The distinct responses of *E. coli* isolates to HM exposure and antibiotics underscore the complexity of environmental contaminants on bacterial behavior. These insights emphasized the importance of continuous efforts to mitigate risks from contaminated water sources, ensuring the safety of produce and protecting both the environment and public health.

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