MICROBIAL CONTAMINANTS AND LEVEL OF HEAVY METALS CONCENTRATION OF SELECTED VEGETABLES IRRIGATED WITH WASTEWATER IN HARAR TOWN, *KEBELE* 05 VEGETABLE FARM, EASTERN ETHIOPIA

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GETACHEW ALAMNIE

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APPROVAL SHEET

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POSTGRADUATE PROGRAM DIRECTORATE

As thesis research advisors we here by certify that we have read and evaluated this thesis prepared under our direction by **Getachew Alamnie**, entitled, **"Microbial Contaminants and Level of Heavy Metals Concentration of Selected Vegetables Irrigated with Wastewater in Harar Town, *Kebele* 05 Vegetable Farm, Eastern Ethiopia"**. We recommend that it be submitted as fulfilling the Thesis requirements.

Ameha Kebede (PhD)	_____	_____
Major Advisor	Signature	Date
Sissay Menkir (PhD)	_____	_____
Co-advisor	Signature	Date

As member of the Board of Examiners of the M.Sc.Thesis Open Defense Examination, we certify that we have read, evaluated the thesis prepared by Getachew Alamnie and examined the candidate. We recommended that the thesis be accepted as fulfilling the thesis requirement for the degree of Master of Science in Applied Biology.

_____	_____	_____
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_____	_____	_____
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DEDICATION

I dedicate this piece of work to my father Alamnie Achenef and my Mother Enana Jembere**.**

STATEMENT OF THE AUTHOR

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Place: Haramaya University, Haramaya

Date of Submission**:** ----------

BIOGRAPHICAL SKETCH

The author, Getachew Alamnie, was born on September, 1994 (G.C) in Lay Gayint Woreda, South Gondar Zone of Amhara Regional State. He started his education at Ambeshit Elementary School and completed his senior secondary education at Nefas Mewucha Secondary and Preparatory School. Upon successful completion of his high school and preparatory studies, he then joined Wollega University in 2012/2013 and was awarded with B.Sc. degree in Applied Biology in 2015. After his graduation, he directly joined the Postgraduate Program Directorate at Haramaya University for Master of Science Degree in Applied Biology in October 2015.

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ACRONMYS AND ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometry
TAMBC	Total Aerobic Mesophilic Bacterial Count
ANOVA	Analysis of Variance
APHA	American Public Health Association
ATSDR	Agency for Toxic Substances and Disease Registry
CAS	Codex Almentarius Commission
CDC	Center for Disease Control
CFU/g	Colony Forming Units per Gram
FAO	Food and Agriculture Organization
FCC	Fecal Coliform Count
HACCP	Hazard Analysis Critical Control Point
ICMSF	International Commission on Microbiological Specifications for Foods
KIA	Kligller Iron Agar
SPSS	Statistical Package for the Social Sciences
SSA	*Salmonella Shigella* Agar
TCC	Total Coliform Count
WHO	World Health Organization

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ABSTRACT

Food safety issues are of growing concern to consumers globally because of the risks associated with consumption of foods contaminated with heavy metals and pathogenic microbes. In Harar town, kebele 05 vegetable farm is known to produce vegetables irrigated with wastewater. To what extent these vegetables are contaminated with heavy metals and pathogens was not known. Thus, a laboratory based cross sectional study was conducted from October 2016 to January 2017 to assess the extent of heavy metal and microbial contamination of vegetables. Accordingly, a total of 72 samples from four leafy vegetables namely lettuce (Lactuca sativa), spinach (Spinacea oleracea), kale (Brassica carinata)) and cabbage (Brassica oleracea) were examined. The results revealed that the mean values in all vegetables were $9.5x10^7$ CFU/g for total aerobic mesophilic bacterial count, $4.3x10^6$ CFU/g for total coliform and $4.6x10^5$ CFU/g for fecal coliform count. These leafy vegetables were also examined for some pathogenic bacteria (Salmonella, Shigella and Campylobacter species) and infective parasitic stages (Ascaris lumbricoides eggs, Entamoeba histolytica and Giardia lamblia cysts). Salmonella, Shigella and Campylobacter species were isolated in 12.5%, 9.7% and 2.8%, respectively, of all vegetables. Ascaris lumbricoides eggs was the predominant (43.1%) intestinal parasitic stage detected in the present study, followed by Entamoeba histolytica (25%) and Giardia lamblia cysts (15.3%). Lead, Cadmium, and Chromium concentration was determined using atomic absorption spectrophotometry. In all the vegetables, the mean concentrations of Pb, Cd and Cr were 0.17, 0.62 and 1.78 mg/kg, respectively in all vegetables. Cd was found in level more than the maximum limit recommended by FAO/WHO but the level of lead was within the normal range for all vegetables. Chromium was found also within the normal range in all vegetables except in lettuce. The findings of this study have important information on the implications of public health by transmission of pathogenic bacteria and heavy metal among vegetable consumers of Harar town and the surroundings. Thus, it is recommended that the concerned public health authorities need to create awareness in the community and discouraging the use of untreated wastewater for cultivating vegetables.

***Key words**: Heavy metal, Parasites, Pathogenic bacteria, Vegetables, Wastewater*

1. INTRODUCTION

Currently, it has been reported that there is an increasing number of cases of foodborne illnesses mainly linked to eating fresh vegetables (Alhabbal, 2015). Wastewater reuse in irrigation is largely considered an inevitable option to compensate water shortages in developing countries (Sou *et al*., 2011). Municipal wastewater for the irrigation of vegetables by marginal farmers is a common practice in urban and peri-urban ecosystems of many countries (Chang *et al*., 2013). Continuous irrigation of agricultural farms with sewage and wastewater leads to contamination by pathogenic organisms, organic matter, oil, solids and heavy metals accumulation in the vegetables (Sharma *et al.*, 2007). Since the mid-1990s, there have been increasing number of outbreaks of fresh produce associated food-borne illness identified internationally and efforts are being made to resolve these food safety problems (Berger *et al*., 2010).

Human health is affected by pathogens (bacteria, protozoan cysts and helminthes eggs) and other organic and inorganic toxic substances which are likely to exceed health protection standards (WHO, 2006). Pathogens present in contaminated foods may have virulence genes, toxins and enzymes, which lead to pathogenesis (Sahilah *et al*., 2010). Among the main food borne illnesses are diarrheal conditions such as gastroenteritis, typhoid fever and shigellosis (Callejas *et al*., 2011). The study conducted by Sou *et al*. (2011) has demonstrated a very close relation between the consumption of vegetables irrigated with wastewater and many food borne diseases like gastroenteritis, cholera, chemical toxicity etc. Furthermore, preference for eating raw or slightly cooked vegetables to protect heat labile nutrients may increase the risk of food borne infections (Fallah *et al*., 2012).

In developing countries, continued use of untreated wastewater and manure as fertilizers for the production of vegetables is a major contributing factor to contamination that causes numerous foods borne disease outbreak (Johannessen *et al*., 2002). Wastewater generates additional benefits including greater income from cultivation and marketing of high value crops such as vegetables, which create year round employment opportunities (Keraita *et al.*, 2008). Researches on wastewater irrigation have tended to focus on the impacts it has on the health of food consumers and producers, economic implications for producer's livelihoods, and food quality.

However, the biophysical implications (both positive and negative) of wastewater use and management in agricultural ecosystems have received relatively little attention (Qadir *et al.*, 2009). Commonly, wastewater is discharged with little or no treatment in natural water bodies, which can become highly polluted. Such practices and agricultural activities are indeed most common in and around cities but can also be seen in rural communities located downstream of where cities discharge (Drechsel *et al.*, 2006).

Fast population growth, inadequate sanitation and infrastructure are causing serious environmental pollution in Harar town. Wastewater is being generated in the city from domestic, commercial and industrial wastes. The water that comes from the effluents of Harar brewery is being used for irrigation in *kebele* 05 vegetable farm for cultivating vegetables. Many farm households that are irrigating their farmlands with wastewater in this area are not aware of the risks or the potential harmful environmental consequences. Altogether, the situation will put the consumers at high risk of contracting diseases. The magnitude of microbial contaminants and the levels of toxic heavy metals in vegetables grown on wastewater irrigated farms were not known in the study area.

General objective

- To assess microbial contaminants and level of heavy metals concentration of selected vegetables irrigated in *kebele* 05 (*Gomen sefer*) vegetable farm found in Harar town.

The specific objectives were:

1. To determine the levels of indicator bacteria (i.e. total aerobic mesophilic bacterial count, total coliform count and faecal coliform count) in cabbage, spinach, lettuce and kale (*yegurage gomen*) grown on wastewater irrigated farm of *kebele* 05, Harar town
2. To detect the presence of selected bacterial pathogens from these leafy vegetables (*Salmonella*, *Shigella* and *Campylobacter* species)
3. To determine the prevalence of *Ascaris lumbricoides* eggs, *Entamoeba histolytica* and *Giardia lamblia* cysts
4. To determine the levels of some toxic heavy metals (i. e. Lead, Chromium, and Cadmium)

2. LITERATURE REVIEW

2.1. Vegetables

Green leafy vegetables are popular around the world and are important protective food and highly beneficial for the maintenance of health and prevention of diseases (Adebayo-Tayo *et al*., 2012). Vegetable contain various medicinal and therapeutic agent and are valued mainly for their high vitamin and mineral content (Adebayo- Tayo *et al*., 2012). vegetables are also well known sources of useful nutrients in the form of dietary fibers and other phyto nutrients including flavonoids, carotenoids and phenolic compounds that may lower the risk of cancer, heart disease and others illnesses (James and Ngarmsak, 2011).

Raw vegetables may be unsafe, mainly because of the environment under which they are prepared and consumed (Adjrah *et al*., 2013). Therefore, leafy vegetables carry the potential risk of microbiological contamination due to the usage of untreated irrigation water or sewage, inappropriate organic fertilizers or inadequately composted manure, the harvesting, the handling, processing and distributing during the restaurant services (Adjrah *et al*., 2013).

2.2. Microorganisms Associated with Vegetables

The numbers and kinds of microorganisms associated with fresh produce are highly variable. Bacterial pathogens are considered as the most common agents causing food borne diseases and such pathogens involved are *Salmonella* species, *E. coli* O157:H7, *Shigella* species, *Campylobacter* species, *Yersinia* species, *Staphylococcus aureus* and *Listeria* species (Cetinkaya *et al*., 2008).

Wastewater environment is an ideal media for a wide range of microorganisms. The majority is harmless and can be used in biological sewage treatment, but sewage also contains pathogenic microorganisms, which are excreted in large numbers by sick individuals and asymptomic carrier. Bacteria which cause cholera, typhoid and tuberculosis and protozoa which cause dysentery and the eggs of parasitic worms are all found in sewage (Shaaban *et al.,* 2004).

2.3. Evaluation of the Microbiological Quality of Vegetables

The microbiological analysis of vegetables depends majorly on detecting the presence of indicator organisms. Indicator organisms are present in large numbers in vegetables, although they may not necessarily be pathogenic. Their detection suggests that human contamination of the vegetables has occurred and that more dangerous organisms could be present. So to estimate food sanitary quality, the classical approach is based on the search for not only pathogenic microorganisms but also indicator microorganisms (Leclercq *et al.*, 2002).

2.3.1. Total Aerobic Mesophilic Bacteria

According to Tortora (1995), total aerobic bacteria reflect the exposure of the sample to bacterial contamination and, in general, the existence of favorable conditions for multiplication of microorganisms. According to Viswamathan and Kaur (2001), raw vegetables used in salad mixture were contaminated by higher number of total aerobic bacterial count. The load of aerobic mesophilic microorganisms is among those which give an estimate of total viable populations and is indicative of the indigenous microflora and the contamination (Ponce *et al*., 2008).

2.3.2. Total Coliforms

Coliform bacteria belong to the family *Enterobacteriace* and include *Escherichia coli* (*E.coli*) as well as various members of the genera namely *Nitrobacteria, Klebsiella, Citrobacter, Escherichia, Enterobacter,* and probably *Aeromonas* and *Serratia* (Bibek, 2005). The main reason for grouping them together is their many common characteristics. They are all Gram-negative, non-spore forming rods; many are motile, facultative anaerobes resistant to many surface active agents and ferment lactose within 48 h at 32°C or 35°C.

These bacteria originate in the intestinal tract of warm blooded animals and can be found in their wastes. Furthermore, coliform bacteria are relatively simple to identify and are present in much larger numbers than more dangerous pathogens. For this reason the degree of faecal pollution and the presumed existence of pathogens can be estimated by monitoring coliform bacteria (Volk *et al*., 2002).

2.3.3. Faecal Coliforms

Faecal coliforms are subset of a larger group of organisms known as coliform bacteria. Faecal coliform bacteria are indicators of faecal contamination and of the potential presence of pathogens associated with wastewater or sewage sludge. The great diversity of pathogenic microorganisms transmitted by contaminated water and the difficulty and cost of directly measuring all microbial pathogens in environmental samples leads to the use of indicator organisms that may indicate the presence of sewage and faecal contamination (Wade *et al*., 2006).

2.4. Pathogenic Bacteria Associated with Vegetables

Pathogenic bacteria continue to be a major contributor to produce associated foodborne illnesses (Berger *et al.,* 2010). The incidence and frequency of foodborne outbreaks caused by contaminated fresh vegetables is on the increase (Raufu *et al.,* 2014). Vegetables harbour a wide range of microbial contaminants which undermine their nutritional and health benefit thus increasing outbreaks of human infections associated with the consumption of fresh vegetables (Beuchat, 2002).

Surveillance of vegetables has indicated that these vegetables can be contaminated with various bacterial pathogens, including *Salmonella* species, *Shigella* species, *E. coli* O157:H7, *Listeria monocytogenes* and *Campylobacter* at any of several points from the field through to the time of consumption (Ayhan *et al*., 2011). Although most processors and consumers assume that washing fresh vegetables and fruits will reduce the microbial load on their surfaces, but studies have shown that water washing alone is not effective in reducing microbial population on the fresh vegetables (Ayhan *et al*., 2011).

2.4.1. *Salmonella* species

Salmonella is a bacterium that causes infection known as salmonellosis. Several outbreaks of salmonellosis have been traced to contaminated fresh vegetables (Berger *et al.,* 2010). The factors influencing the increase in salmonellosis due to vegetables are changes in agricultural practices, eating habits and increases in the worldwide commerce of fresh produce (Raufu *et al.,* 2014).

Every year, *Salmonella* species contributes 1 million illnesses, 19,000 hospitalizations and 380 deaths in United States (CDC, 2014). Although most *Salmonella* infections cause mild-to-moderate gastroenteritis that usually resolves with or without treatment, some lead to severe invasive infections (e.g., bacteraemia meningitis, and osteomyelitis) which need antibiotic treatments (Chen *et al*., 2013). Invasive *Salmonella* infections can be life threatening, leading to death and are more common in young children, the elderly and the immunocompromised. Controlling *Salmonella* infection could be challenging due to its high tolerance to environmental stresses, widespread distribution, multiple drug resistance, and adaptability (Chen *et al*., 2013).

2.4.2. *Shigella* species

Infections caused by *Shigella* species include bacillary dysentery which is endemic throughout the world and it is responsible for approximately 165 million cases annually, of which 163 million occur in developing countries and 1.5 million in industrialized ones (Sharma *et al*., 2010). Foodborne infections caused by these organisms are of major international health concern. Disease may be caused by any of the 4 *Shigella* species: *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei.* In developing countries, the predominant species is *S. flexneri*, which is characterized by long-term persistence of sublineages in shigellosis endemic regions with inadequate hygienic conditions and unsafe water supplies. More rarely isolated are *S. dysenteriae* and *S. boydii* responsible for large epidemics in the past (Connor *et al*., 2015).

Shigella has become a public health concern because of the development of multiple antimicrobial resistant strains. Shigellosis also leads to the development of some complications like lethal toxic encephalopathy or Ekiri syndrome and haemolytic uraemic syndrome (HUS) (Butler, 2012). Resistance and reduced susceptibility to β-lactam of the antibiotics, especially important is the awareness of the global emergence of multidrug resistant (MDR) *Shigella*, notably the increasing resistance to third generation cephalosporins and fluoroquinolones, and most recently azithromycin (Nüesch-Inderbinen *et al*., 2006).

2.4.3. *Campylobacter* species

Campylobacter is the major bacterial cause of foodborne infection and is the major risk factor of Guillain–Barré syndrome (GBS), a neurological disorder causing muscular paralysis, as a post infection complication. Among pathogenic *Campylobacter* species, *C. jejuni* and *C. coli* are the most frequently associated human infection (Kaakoush *et al*., 2015). Despite the well-known fastidious nature of *Campylobacter*, it is isolated from environmental sources, such as lake, river, sea, and sewage, suggesting that environmental water is a possible vehicle that transmits *Campylobacter* to humans (Silva *et al*., 2011).

2.5. Parasites Associated with Vegetables

Parasitic infections lead to about 300 million severely illnesses with approximately 200,000 deaths occurring in developing countries (Duedu *et al*., 2014). Various parasites have been associated with vegetables including protozoan and helminthes (Daryani *et al*., 2012). Vegetables are used extensively in different parts of the country, but unfortunately people do not know how to consume them properly and the force habit of eating these produces plays a critical epidemiological role in transmitting parasitic foodborne diseases (Alhabbal, 2015). Recovery of parasites from fresh sold vegetables is an indication of the quality of the overall process of cultivation, irrigation and post-harvest handling, and may be helpful in indicating the incidence of intestinal parasites among a local community (Alhabbal, 2015).

2.5.1. *Ascaris lumbricoides*

Ascariasis is an infection with the intestinal nematode *Ascaris lumbricoides* and it is estimated to infect over 800 million people worldwide (Hotez *et al*., 2008). Ascariasis is transmitted through the faecal-oral route; eggs are ingested following contact with contaminated hands, food, soil, or the deliberate act of eating contaminated soil. Infective *A. lumbricoides* eggs can survive, and remain infective for several months, or even for years in soil (Nordin *et al*., 2009).

2.5.2. *Entamoeba histolytica*

Approximately 34 to 50 million symptomatic cases of amoebiasis and 10,0000 deaths occur worldwide each year, making *E. histolytica* second to malaria as a cause of mortality due to protozoan parasites (Ali *et al*., 2003). This parasite is presence in most tropical and subtropical areas of the world, where it causes millions of cases of dysentery and liver abscess each year (Satoskar, 2009). Infection usually occurs by ingestion of water or food contaminated by faecal matter. The cyst wall is dissolved in the upper gastrointestinal tract and excysts within the lumen of the small intestine. Trophozoites of *E. histolytica* are motile forms, which adhere to and invade intestinal epithelial cells which line the gastrointestinal tract. Once penetration of the intestinal mucosa is achieved, dissemination to other organs, extra-intestinal infections, usually the liver can occur. Trophozoites which dwell in the colon multiply encyst and are passed in the stool from where further spread is possible (Clark *et al*., 2000).

2.5.3. *Giardia lamblia*

Giardia, flagellated protozoan, is the most common causative agent of persistent diarrhea worldwide. *Giardia* can be transmitted to a person through contaminated water (Karanisa *et al*., 2006). *Giardia* infections affect the activity of gut enzymes such as lactose disaccharidase, damage the mucosal surface causing shortening of crypts and villi and give rise to overgrowth of bacteria and yeasts in the small intestine (WHO, 2002). Clinical symptoms of giardiasis include diarrhoea, epigastric pain, flatulence, and malabsorption with lactose intolerance, weight loss, weakness, cramps, vomiting, mucus in stool and bloody foul smelling stool (Laurent *et al*., 2005).

2.6. Sources of Water Pollution

The main sources of pollution that enter surface water bodies are industries, municipal solid waste and oily wastes from garages and fuel stations. Organic and inorganic substances which are released into the environment as a result of domestic, agricultural and industrial water activities lead to organic and inorganic pollution (Lim *et al.,* 2010). The inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulphur, phosphate, bicarbonate, ammonium salts and heavy metals (Lim *et al.,* 2010).

2.7. Approaches for Mitigating Risks from Wastewater Irrigation

2.7.1. Conventional Options

Wastewater treatment in designed plants or pond systems has long been considered the ultimate solution for reducing risks in wastewater irrigated agriculture. Wastewater treatment is a risk mitigation method has been widely studied and documented in both developed and developing countries (Patwardhan, 2008).

Indeed, most conventional systems have two treatment systems: primary treatment where suspended solids and organic matter are removed; and secondary treatment for removing biodegradable organics. Tertiary level treatment may also be available, but the aim of tertiary treatment is removal of nutrients and toxic compounds. The processes involved in several conventional treatment systems, except stabilization ponds, are difficult and costly to operate in developing country contexts as they have high energy requirements, need skilled labour and also have high installation, operation and maintenance costs (Metcalf and Eddy, 2002).

2.7.2. Multiple-Barrier Approach

The multiple-barrier approach draws from the Hazard Analysis Critical Control Point (HACCP) concept promoted by the Codex Alimentarius initiative and is based on targeted interventions at key control points along the food chain to achieve a food safety objective (CAC, 2004). The approach, therefore, covers both conventional and non-conventional wastewater treatment methods as well as other health protection measures to meet health targets, be it for the farmer or the consumer. Non-conventional wastewater treatment methods include the use of low cost systems such as on farm ponds, sedimentation traps and bio-sand filters while health protection measures include improved irrigation methods, like drip irrigation, cessation of irrigation before harvesting and produce washing (Keraita *et al.*, 2008).

2.8. Heavy Metals

Heavy metals are generally referred to as those metals which possess a specific density of more than 5 g/cm^{3}and adversely affect the environment and living organisms. These metals are quite essential to maintain various biochemical and physiological functions in living organisms when in very low concentrations; however they become noxious when they exceed certain threshold concentrations (Järup, 2003).

The most common heavy metals in wastewater include arsenic, lead, mercury, cadmium, chromium, copper, nickel, silver, and zinc. Their occurrence and accumulation in the environment is a result of direct or indirect human activities, such as rapid industrialization, urbanization and anthropogenic sources. These elements are usually found as natural components of the earth's crust, as minerals, salts and other compounds that can be absorbed by plants and incorporated into the food chain (Rooney *et al.*, 2006). They can pass into the atmosphere by volatilization and can be mobilized into surface water or groundwater.

2.9. Heavy Metal Contamination of Vegetables

Heavy metal contamination of the food items is one of the most important aspects of food quality assurance (Khan *et al.,* 2009). Utilization of sewage water in urban areas deserves special attention as it is making environment quite unsuitable for human health as well as animals and plants are also affected by heavy metals toxicity. Waste water irrigation may lead to the accumulation of heavy metals in agriculture soils and plants. Soil gets polluted due to waste water irrigation and absorbed minerals settle in edible tissue of the vegetables (Sharma *et al.,* 2007).

Various sources of heavy metals include soil erosion, natural weathering of the earth's crust, mining, industrial effluents, urban runoff, sewage discharge, insect or disease control agents applied to crops, and many others. The contamination chain of heavy metals almost always follows a cyclic order: industry, atmosphere, soil, water, foods and humans (Mapanda *et al.,* 2005).

2.10. Uptake of Heavy Metals by Leafy Vegetables

Heavy metal concentrations vary among different vegetables, which may be attributed to a differential absorption capacity of vegetables for different heavy metals (Singh *et al*., 2010). Also up take of heavy metals by crops may be done through absorption from contaminated soils through roots or by deposition on foliar surfaces (Jassir *et al*., 2005). Uptake through roots depends on many factors such as the soluble content of heavy metals in soil, soil pH, plant growth stages as well as type of crops, fertilizers and soil (Sharma *et al*., 2006). Among soil properties, soil pH had the greatest impact on desorption and bioavailability of heavy metals, because of its strong effects on solubility and speciation of heavy metals both in the soil as a whole and particularly in the soil solution. Apart from soil pH, organic matter was also one of the most important properties affecting heavy metal availability in soils for retaining heavy metals in an exchangeable form (Zeng *et al*., 2011).

2.11. Risk of Heavy Metals to Human Health

Toxic heavy metals entering the ecosystem may lead to bioaccumulation, particularly by eating fruits and vegetables (Kashif *et al*., 2009). Heavy metals are not biodegradable, have long biological half-lives and have the potential for accumulation in the different body organs leading to unwanted health effects (Nabulo *et al*., 2011). Most heavy metals are extremely toxic, and because of their solubility in water, contamination may readily reach toxic levels. Food chain contamination is one of the most important pathways for the entry of these toxic pollutants in to the human body (Wang *et al*., 2011).

The increasing demand for food and food safety has drawn the attention of researchers to the risks associated with consumption of contaminated food products, particularly heavy metals in vegetables (Uwah *et al*., 2011). The excessive content of these metals in food is associated with a number of diseases, especially with cardiovascular, kidney, nervous as well as bone diseases. In developing countries like Ethiopia, limited data are available on heavy metals in food products. Some data have been reported for leafy vegetables (Yirgaalem *et al*., 2012; Fisseha, 2002).

2.11.1. Lead (Pb)

Lead occurs naturally as a sulfide compound and is a soft bluish-white, silvery grey, metal that melts at 327.5^{o}C (Budavari, 2001). Once it is released into the environment, it persists. Lead toxicity causes reduction in the haemoglobin synthesis, disturbance in the functioning of kidney, joints, reproductive and cardiovascular systems and chronic damage to the central and peripheral nervous systems (Ogwuegbu and Muhanga, 2005). Lead is also well-known neurotoxin and its accumulation in the skeleton and its mobilization from bones during pregnancy and lactation causes exposure to fetuses and breastfed infants (ATSDR, 2007). It can also reduce cognitive development and intellectual performance in children and damage kidneys and reproductive system (Qin and Chen, 2010).

2.11.2. Cadmium (Cd)

Health risk due to heavy metal contamination of soil has been widely reported (Muchuweti *et al.,* 2006). Cadmium is becoming an increasing health concern in wastewater irrigated agriculture, especially due to its association with damage to kidneys and bones and its potential carcinogenic nature (Suruchi and Khanna, 2011). Heavy metals are present in vegetables and irrigation water as a consequence of human activities involving unmanaged utilization of herbicides, chemical fertilizers and manures in the catchment area of the irrigation water source (McBride, 2003).

2.11.3. Chromium (Cr)

Chromium is a trace metal that occurs in several forms in the environment. The most important are the trivalent Cr (III) and hexavalent Cr (VI) species. Chromium (Cr) can cause skin ulcers and nasal septum perforations. While both Cr (III) and Cr (VI) can be toxic to plants and animals, Cr (III) toxicity occurs in higher concentrations, and this form is actually an essential nutrient to human and other animals. Cr (VI), on the other hand, is toxic in much lower concentrations and also tends to be more mobile and bio-available than Cr (III) in surface and subsurface environments. Naturally occurring chromium is almost always present as Cr (III), though relatively few data are available describing speciation of Cr in natural waters. Coal plants and waste incinerators can also release Cr (VI) to the environment (Adriano, 2001).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

Harar town is the capital city of Harari People National Regional State and is located in eastern part of Ethiopia. Geographically, it is located at 9^0 18' 43''N latitude and 42^0 07' 23''E' longitude. The elevation of the town is approximately between 1800 and 2200 meters above sea level (masl). *Kebele* 05 vegetable farm is found within *Abadir* woreda which is using water for irrigation from different sources including Harar beer brewery and domestic water; and its geographical position is suitable for getting the entire water source that drains from the surrounding villages. Major vegetables grown in this area include cabbage (*Brassica oleracea),* lettuce (*Lactuca sativa*), Spinach (*Spinacea oleracea*), kale (*Brassica carinata*), carrot (*Daucus carota*) and beet root (*Beta vulgaris*).

3.2. Study Design

A laboratory based cross sectional survey was conducted from October 2016 – January 2017 to assess the microbial contaminants and level of heavy metals concentration on the main selected leafy vegetables [Lettuce (*Lactuca sativa*), Cabbage (*Brassica oleracea*), Spinach (*Spinacea oleracea*), and kale (*Brassica carinata*)] that are grown in Harar town, *kebele* 05 vegetable farm. The samples were regularly collected within three week from October 2016 – November 2016 and analysed for indicator bacteria (total aerobic mesophilic bacteria, total coliform bacteria and faecal coliform bacteria), for detection of *Salmonella*, *Shigella* and *Campylobacter* species and for detection of *Ascaris lumbricoides* eggs, *Giardia lamblia*, and *Entamoeba histolytica* cysts. The vegetable samples were also analysed for concentrations of the heavy metals such as cadmium (Cd), lead (Pb), and chromium (Cr) using Atomic Absorption Spectrophotometry (AAS).

3.3. Sample Collection

A random sampling procedure was adopted to collect the vegetable samples. A total of 72 samples comprising four vegetable types were collected from *kebele* 05 (*Gomen Sefer*) vegetable farm found in Harar town. All samples were collected aseptically in a disinfected universal ice box and transported to Haramaya University for microbiological and heavy metal analysis.

3.4. Microbiological Analysis

The edible portions (leaves) of these vegetables were washed with running tap water. After draining for 1 min, the vegetables were aseptically transferred to a sterile stomacher bag for stomaching. Vegetables that were too large for stomaching were first cut into smaller pieces with a sterile scalpel.

For bacteriological analysis, 25 grams of leafy vegetable samples were aseptically removed from each vegetable sample using a sterile scalpel. Each twenty five gram of vegetable sample was weighed and homogenized in 225 ml of sterile 0.1% (w/v) bacteriological peptone water blended in a sterile blender for 2-3 minutes under sterile conditions (Biniam and Mogessie, 2010). The homogenate was used as a source of microbial inoculum for determining the total aerobic mesophilic bacterial count, total coliform count and faecal coliform count and for detection of some selected pathogenic bacteria (*Salmonella, Shigella* and *Campylobacter* species). To check the sterility of the media each agar medium and broth used in this study was prepared by pouring into two Petridish and two test tubes respectively, at the same time and same analysis environment with the test vegetable samples. In addition to this, parasitological analysis was also undertaken for the vegetable samples using standard procedures.

3.4.1. Total Aerobic Mesophilic Bacterial Count (TAMBC)

Total aerobic mesophilic bacterial count for all the vegetable samples were determined by standard plate count method as described by APHA (Downes and Ito, 2001). A total of ten sterile test tubes were dispensed with 9 ml of bacteriological peptone water as a diluent (Girmaye *et al*., 2014).

Ten fold serial dilutions of 10^{-1} to 10^{-10} were prepared from 1ml of the stomacher bag suspension with 0.1% buffered bacteriological peptone water using disposable pipettes. Then a sterile molten agar was dispensed into petridish and allowed to solidify by leaving the petridish stand on the horizontal surface of the biological safety cabinet. After complete solidification, 0.1ml from each dilution (10^{-1} to 10^{-10}) was spread plated on to standard plate count agar medium (Himedia, M091A, India) in duplicates. Then the medium was carefully mixed by rotating the petridish and inoculated by spread plate method. All the petridish were inverted and incubated in an incubator at 37 ^{o}C for 24 h.

After the incubation period duplicate plates were selected from the appropriate dilution that contained 30-300 colonies per plate. Colonies on both plates were counted and averages of the two counts were taken to calculate the total aerobic mesophilic bacterial count and were expressed as CFU/g (Hannan *et al*., 2014).

3.4.2. Total Coliform Count (TCC)

Appropriate serial dilutions of the samples were prepared in 0.1% buffered bacteriological peptone water as in section 3.4.1.; 0.1 ml from each dilution (10^{-1} to 10^{-6}) was plated on Eosin Methylene Blue Agar (Levine) medium in duplicates. The plates were incubated at 37 ^{o}C for 24-48 h. After incubation plates with colonies between 30-300 were used for determining TCC (Roberts and Greenwood, 2003).

3.4.3. Faecal Coliform Count (FCC)

From appropriate dilutions of the samples prepared in section 3.4.2.; 0.1ml aliquots were spread plated in duplicates on Eosin Methylene Blue (EMB) agar plates by spread plate technique. Inoculated EMB agar plates were incubated at 44.5 ℃ for 24 h. After 24 h of incubation, red colonies surrounded by reddish zone of precipitated bile were counted as faecal coliforms. Duplicate plates were maintained and the average of the colonies was taken. The mean numbers of colonies counted were calculated and expressed as colony forming units per gram (CFU)/g (Asha *et al*., 2014).

3.5. Detection of pathogenic Bacteria

3.5.1. Detection of *Salmonella* species

Twenty five grams of each washed vegetable sample was thoroughly mixed in 225 ml buffered peptone water and the suspension was incubated at 37 °C for 24 h for the metabolic recovery and proliferation of cells (pre-enrichment). After incubation 1ml of culture were transferred into separate tubes each containing 9 ml Selenite Cystein Broth for selective enrichment and incubated at 37 ºC for 24 h (Biniam and Mogessie, 2010). After the selective enrichment a loop full of the samples was streaked onto *Salmonella-Shigella* (SS) agar (selective agar) and incubated at 37 ºC for 24 h. typical *Salmonella* colonies appeared colourless with black centers were suspected as *Salmonella* (Abakpa *et al*., 2015). Suspected *Salmonella* colonies on SSA were further subcultured on to nutrient agar for biochemical tests.

3.5.2. Detection of *Shigella* species

Twenty five grams of each washed vegetable sample was thoroughly mixed in 225 ml buffered peptone water and the suspension was incubated at 37 °C for 24 h for the metabolic recovery and proliferation of cells (pre-enrichment). After incubation 1ml of culture were transferred into separate tubes each containing 9 ml Selenite Cystein Broth for selective enrichment and incubated at 37 ºC for 24 h (Biniam and Mogessie, 2010). After the selective enrichment a loop full of the samples was streaked onto *Salmonella-Shigella* (SS) agar (selective agar) and incubated at 37 ºC for 24 h. *Shigella* species characteristic showing colourless colonies on SSA plates were selected; subcultured on to nutrient agar and further identified using biochemical tests (Swagato *et al*., 2015).

3.5.3. Detection of *Campylobacter* species

Twenty five grams of each vegetable samples was thoroughly mixed in 225 ml of buffered peptone water and incubated at 42 ^{o}C for 20 h (pre-enrichment). One ml of the suspension was pipetted to 9 ml of Bolton broth base (selective enrichment broth) and was incubated at 42 ^{o}C for 24 h. A loop full of the sample suspension was streaked directly onto *Campylobacter* blood free charcoal-based selective medium, specifically mCCDA (modified cefperazone charcoal deoxycholate agar) which is selective for the isolation of *Campylobacter* species. After inoculation, the medium was kept in gas jar containing *Campylobacter* gas pack systems to maintain microaerophilic condition. The gas pack system containing the inoculated Petri-plates was incubated at a temperature of 42 ^{o}C for 48 ± 2 h under microaerobic conditions (Cinzia *et al*., 2015). After 48 hours, growth of *Campylobacter* was identified by colonial morphology. Typical mucoid, spreading and convex colonies were taken as suspect colonies and sub-cultured on to nutrient agar plates. Biochemical tests were also used for identification of *Campylobacter* species from sub-cultured colonies.

3.6. Biochemical Tests for the Identification of Pathogenic Bacteria

Standard biochemical tests were performed to identify pathogenic bacteria isolated from vegetable samples. The tests include Kliglller iron agar test, Motility test, Indole test, Citrate utilization test, Urease test, and Catalase test (Swagato *et al*., 2015).

KIA (Kligller Iron Agar) test: Using sterile loop, presumptive positive sub-cultured colonies were inoculated into test tubes containing klligler iron agar (KIA) and stabbed the center of the medium to the bottom of the tube and then streaked the surface of the agar slant. The caps were left on loosely and the tubes were incubated for 18-24 hours at 35^{o}C in an incubator. After incubation when the bacteria ferment glucose, enough acid was produced and red butt turned to yellow/acidic with or without production of gas and black precipitate and the slant remains red/alkaline, but bacteria incapable of fermenting the carbohydrates were not change the colour of the medium.

Motility test: was performed as, sub-cultured colonies were inoculated in to test tubes containing motility agar and incubated at 37 ºC for 24 hr, bacteria that typically showed diffuse and hazy growths spreading from the line of inoculation were regarded as motile.

Urease test: The suspected subcultured colonies were streaked in to the entire slant surface of test tubes containing urea agar and the butt did not stab and it served as a color control incubated at 37 ºC for 24 hr. Urease production was indicated by a bright pink color on the slant.

Indole test: Test tubes containing tryptophan broth was inoculated with the test organisms and incubated for 18 to 24 h at 37 ºC. After incubation drops of Kovac's reagent was added down the inner wall of the tube. A positive Indole test was indicated by the formation of a pink to red color ("cherry red ring") in the reagent layer on top of the medium within seconds of adding the reagent.

Citrate utilisation test: Suspected subcultured colonies were inoculated in to test tubes containing Simmons citrate agar and incubated at 37 ºC for 24 hr and the bacteria that utilise citrate were produced intense blue colour and that could not utilise citrate were not change the colour of the medium (remained green).

Catalase test: with an inoculating loop, a portion of a colony from the recently subcultured plate was mixed with a drop of 3% hydrogen peroxide (H_2O_2) on a clean glass slide. Development of bubbles was considered as catalase positive.

For *Campylobacter* species all the above biochemical tests were performed and the incubation of cultures was under microaerophilic condition at 42 ºC.

3.7. Parasitological Analysis of Vegetable Samples

In the laboratory, 100g of each fresh vegetable sample was chopped into small pieces and put into a clean beaker containing 250 ml physiological saline solution (0.85% NaCl) enough to wash the samples. After removing fragments of the vegetable sample from the washing saline using clean forceps, it was kept for 24 hours to allow sedimentation to take place. Following sedimentation, the top layer of the washing solvent was carefully discarded leaving 5 ml of the suspension that contained the sediment. This was finally centrifuged at 2000 rotations for 5 minutes using a centrifuge (Dada and Olusola-Makinde, 2015). After centrifugation, the supernatant was carefully siphoned off without shaking, and discarded; and the remaining residue was agitated gently by hand in a drop of physiological saline solution for further distribution of the cysts and eggs in the residue. Then the residue was mounted on a slides, stained with Lugol's iodine solution and examined by compound light microscope using 10X and 40X objectives (Daryani *et al.,* 2008) for the presence of *A. lumbricoides eggs* and cysts of *E. histolytica* and *G. lamblia* (Girmaye and Fikadu, 2014). Three slides were prepared for each sample to increase the chance of parasite detection.

3.8. Determination of Heavy Metals in Leafy Vegetable Samples

3.8.1. Instruments and Apparatus

Ceramic pestle and mortar were used for grinding and homogenizing dried vegetable samples according to its type; digital analytical balance and dry heat oven were used for weighing and drying the samples, respectively. Flame atomic absorption spectrometry (Model 210/211 VGP, USA) equipped with deuterium background and hallow cathode lamp of each of metals was used to detect the absorbance of the metals (Pb, Cr and Cd) using air acetylene flame. Borosilicate volumetric flasks were used during dilution of samples and preparation of metal standard solutions. A refrigerator was used to store the samples which were digested till analysis. In addition funnels and whatman filter paper were used to filter the undissolved particles.

3.8.2. Chemicals and Reagents

All the chemicals that were used were, of high purity analytical grade. Distilled water was used for sample preparation, dilution and rinsing apparatus prior to analysis. Cadmium nitrate (Cd $(NO_3)_2$), Lead nitrate (Pb $(NO_3)_2$) and Potassium dichromate ($K_2Cr_2O_7$) were used to prepare standards; 70% HNO_3, 30% H_2O_2 and 38% HCl were used for digestion of samples.

3.8.3. Calibration Curve

In this study, a calibration curve was constructed in order to determine the concentration of the experimental vegetable samples for lead, cadmium and chromium. Series of standard solutions were prepared using the stock solutions and diluted with distilled water to obtain five working standards for each metal ion of interest. In this study a total of three metals were analyzed using flame atomic absorption spectrophotometer. All the three metals (Pb, Cr and Cd) were analyzed by the absorption mode of the instrument. The concentrations and measured absorbance data for each set of standard metal ion solutions were used to construct the calibration curve then the unknown concentration of the vegetables were calculated using the equation from the standard calibration curve.

3.8.4. Preparation of Vegetable Samples

All samples were collected and stored in sterile ice box and brought to the laboratory for preparation. Then they were thoroughly mixed to give a composite sample as representative fraction of the vegetables. About 1kg of the edible portions (leaves) of these vegetables [(Cabbage (*Brassica oleracea*), Lettuce (*Lactuca sativa*), Spinach (*Spinacea oleracea*) and kale (*Brassica carinata*)] were prepared for heavy metal (lead, chromium and cadmium) determination. The bruised or rotten portions were removed. In the laboratory, collected vegetable samples were washed with tap water and then with double distilled water to eliminate adsorbed dust and particulate matters. Samples were then cut and chopped into small pieces using scissor in order to facilitate drying. The samples then air dried for five to six days and further dried in a hot air oven at 50-60 ^oC for 24 hr to remove moisture and maintain constant mass and at the end of drying, the oven was turned off and left over night to enable the sample cool to room temperature (Adugna *et al*., 2015). Finally, the dried samples were ground into powder using acid-washed mortar and pestle.

3.8.5. Digestion of Vegetable Samples

A mixture of 0.5 g of homogenized powdered vegetable samples and 10 ml of HNO_3-HCl-H_2O_2 (8:1:1, v/v/v) were added into a borosilicate flask. The mixture was heated at 120 ^oC over 3 h on a hot plate. After the completion of the digestion, the bright yellowish solutions were transferred into 50 ml of volumetric flak. Each digestion tube was rinsed with distilled water to collect any possible residue, and added to the volumetric flask which made up to volume with distilled water. Then dilute samples were stored in 100 ml plastic bottles until analysis. Each vegetable sample was digested and analysed in triplicate to increase the precision of the result. The blank solution was prepared by taking a mixture of 8 ml HNO_3, 1 ml HCl and 1 ml H_2O_2 and treating similarly as that of the samples (Tadele *et al*., 2015). Then the heavy metals (Cr, Pb, and Cd) were analysed by atomic absorption spectrophotometry. All concentrations of metals were expressed in mg/kg of dry weight.

3.9. Data Analysis

In this study, all statistical analyses were computed using SPSS software version 20 for heavy metal and microbiological analyses. Descriptive statistics such as mean, percentage and frequency were used to describe the extent of pathogenic bacteria, infective parasitic stages and the loads of indicator bacteria and also Microsoft Excel was used to draw calibration curves and graphs. As the level of microbial and heavy metal contamination might vary with vegetable types, ANOVA was used to test the existence of significant difference between means. In statistical analyses, confidence level was held at 95% and $P<0.05$ (at 5% level of significance) for microbial contamination and at 99% and $P<0.01$ (at 1% level of significance) for heavy metal determination were considered as significant.

4. RESULTS AND DISCUSSION

4.1. Bacterial Contaminants in Leafy Vegetable Samples

Table 1 shows the percentage of vegetable samples contaminated with indicator bacteria from *kebele* 05 vegetable farm in Harar town. The highest percentage was obtained for total aerobic mesophilic bacteria (100%) as demonstrated by its occurrence in all vegetable samples analyzed. Obviously such a result is expected as our oxygenated environment is largely occupied by aerobic microflora. Similar to this result, Girmaye *et al*. (2014) reported that total aerobic mesophilic bacteria (100%) occurrence in all vegetable samples collected from Melka Hida and Wonji Gefersa farms around Adama town (Ethiopia) and Getachew and Desalegn (2015) reported that all vegetable samples (100%) were contaminated with total aerobic mesophilic bacteria in Nekemte town, Ethiopia.

Table 1: Percentage of positive vegetable samples for indicator bacteria

	Leafy Vegetables									
Indicator organisms	Lettuce (N=18)		Spinach (N=18)		Kale (N=18)		Cabbage (N=18)		Total (N=72)	
	F	%	F	%	F	%	F	%	F	%
TAMB	18	100	18	100	18	100	18	100	72	100
TCB	18	100	17	94.4	16	88.9	18	100	69	95.8
FCB	17	94.4	17	94.4	15	83.3	16	88.9	65	90.3

TAMB=Total aerobic mesophilic bacteria, N=Number of examined samples, TCB=Total coliform bacteria, FCB=Fecal coliform bacteria, F=Frequency of positive samples, %=Percentage of positive samples, Kale=*Yegurage gomen* (Ethiopian local leafy vegetable) (*Brassica carinata*)

It can be understood that there was an improper pre-harvest contamination of vegetables in this selected study area. Pre-harvest contaminations with the indicator organisms can arise from irrigation water, improperly composted manure used as fertilizer, fecal contamination from human and domestic animals.

The mean values for TAMBC, TCBC, and FCBC were $9.5x10^7$, $4.3\ x10^6$, and $4.6\ x10^5$ CFU/g, respectively, for all positive samples (Table 2). Indicator bacterial count showed that the total aerobic mesophilic count, total coliform count and fecal coliform counts ranged from 1.1×10^6 to 9.2×10^8, 6.2×10^4 to 9.9×10^6 and 1.0×10^4 to 4.2×10^6 CFU/g, respectively.

Table 2 : Mean, maximum and minimum values (CFU/g) of indicator bacteria among the tested vegetable samples

Indicator organisms		Vegetable types				
		Lettuce	Spinach	Kale	Cabbage	For all positive samples
TAMBC	Mean	$5.1x10^{7\ b}$	$2.9x10^{8\ a}$	$7.0x10^{6\ b}$	$3.4x10^{7\ b}$	$9.5x10^7$
	Minimum	$5.4\ x10^6$	$3.7\ x10^7$	$1.1\ x10^6$	$1.0\ x10^7$	$1.1\ x10^6$
	Maximum	$8.8\ x10^7$	$9.2\ x10^8$	$7.7\ x10^7$	$9.7\ x10^7$	$9.2\ x10^8$
TCBC	Mean	$5.1\ x10^{6\ a}$	$4.9\ x10^{6\ a}$	$5.1\ x10^{5\ b}$	$6.2\ x10^{6\ a}$	$4.3\ x10^6$
	Minimum	$2.2\ x10^5$	$7.4\ x10^5$	$1.0\ x10^5$	$6.2\ x10^4$	$6.2\ x10^4$
	Maximum	$9.7\ x10^6$	$9.1\ x10^6$	$2.5\ x10^6$	$9.9\ x10^6$	$9.9\ x10^6$
FCBC	Mean	$3.4\ x10^{5\ bc}$	$9.2\ x10^{5\ a}$	$5.4\ x10^{4\ b}$	$4.8\ x10^{5\ c}$	$4.6\ x10^5$
	Minimum	$5.5\ x10^4$	$2.0\ x10^5$	$1.0\ x10^4$	$1.0\ x10^5$	$1.0\ x10^4$
	Maximum	$9.0\ x10^5$	$4.2\ x10^6$	$1.3\ x10^5$	$9.9\ x10^5$	$4.2\ x10^6$

[a-b-c] Means with different superscript letters across the row for the same parameter do significantly differ ($P<0.05$), TAMBC = Total aerobic mesophilic bacterial count, TCBC = Total coliform bacterial Count, FCBC = Faecal coliform bacterial count, Kale= *Yegurage gomen* (*Brassica carinata*)

The mean total aerobic mesophilic bacterial counts for the vegetables were $5.1x10^7$ (lettuce), $2.9x10^8$ (spinach), $3.4x10^7$ (cabbage) and $7.0x10^6$ CFU/g (kale). This result was in connection with Razzaq *et al*. (2014) who reported that the average total aerobic mesophilic count of cabbage was $3.0x10^7$ CFU/g.

In this study, total aerobic mesophilic bacterial counts ranged from $1.0x10^7$ to $9.7x10^7$ CFU/g for cabbage, $5.4x10^6$ to $8.8\ x\ 10^7$ CFU/g for lettuce, $3.7\ x\ 10^7$ to $9.2\ x\ 10^8$ for spinach and $1.1\ x\ 10^6$ to $7.7\ x\ 10^7$ for kale *(yegurage gomen)*. In connection with this result, Viswanatha and Kaur (2001) from India indicated that total aerobic mesophilic bacterial count for cabbage and lettuce were found to be $2.8x10^6$ to $1.2x10^8$ and $1.3x10^7$ to

2.3×10^7CFU/g, respectively. But it is lower than that of the study conducted by Girmaye *et al*. (2014) who reported that the TAMBC for spinach, lettuce and cabbage were in the range of 2.0×10^8 to 2.2×10^8, 1.6×10^8 to $1.7\times10^8$and 8×10^7 to 9.3×10^7CFU/g, respectively from vegetable samples collected from Melka Hida and Wonji Gefersa farms irrigated with wastewater around Adama Town (Ethiopia).

The data showed that there was a highly significant difference ($p<0.0001$) in the average counts of TAMBC between spinach and the other vegetable types (Table 2, Appendix Table 1, 2). This investigation additionally showed that the vegetable samples collected from this farm were heavily contaminated by total aerobic mesophilic bacteria. Moreover, all the bacterial counts recorded in this study exceeded the recommended levels by the International Commission on microbiological Specifications for Food (ICMSF, 1998) standards (10 to 100 coliforms CFU/g, 10 faecal coliform CFU/g and 4.9×10^6 aerobic counts CFU/g) wet weight vegetables.

Total coliform levels ranged from 2.2×10^5 to 9.7×10^6 CFU/g for lettuce, 7.4×10^5 to 9.1×10^6 CFU/g for spinach, 1.0×10^5 to 2.5×10^6 CFU/g for kale and 6.2×10^4 to 9.9×10^6 CFU/g for cabbage. In contrast to this study, Nma and Oruese (2013) revealed that vegetables in Port Harcourt, metropolis (Nigeria), total coliform counts ranged from 3.4 $\times10^5$ to 5.6×10^5 CFU/g for cabbage and from 3.4×10^5 to 4.0×10^5 CFU/g for lettuce Furthermore, the mean value for cabbage in the present study (6.2×10^6 CFU/g) was higher than that of the data reported by Razzaq *et al*. (2014) who demonstrated that the average total coliform count in cabbage was 9.0×10^2 CFU/g.

According to Viswanathan and kaur (2001) who also reported that the total coliform counts of vegetables ranged from 1.0×10^6 to1.0×10^9 CFU/g, value which was higher than the present study (6.2×10^4 to 9.9×10^6 CFU/g). There was highly significance difference ($P<0.0001$) between kale (*yegurage gomen)* to the other vegetable types (cabbage, lettuce and spinach) (Table 2, Appendix Table 1, 2). In this study, the fecal coliform counts for lettuce, spinach, kale, and cabbage samples collected from the study site ranged from 5.5 $\times10^4$ to 9.0×10^5, 2.0×10^5 to 4.2×10^6, 1.0×10^4 to 1.3×10^5, and 1.0×10^5 to 9.9×10^5 CFU/g, respectively.

In line with this Girmaye *et al*. (2014) reported that the fecal coliform counts of cabbage, lettuce and spinach samples grown in wastewater ranged from 5.2×10^5 to 5.7×10^5, 2.3×10^5 to 3.1×10^5 and 2.2×10^5 to 3.7×10^5 CFU/g from Melka Hida and Wonji Gefersa farms, respectively around Adama Town (Ethiopia). The mean fecal coliform values of all the four vegetable samples exceed the ICMSF recommended level of 10 fecal coliform g-1 fresh weight. The results of the analysis of variance for fecal coliform count showed that there was a significant difference amongst vegetable types (Table 2, Appendix Table 2).

4.2. Detected Pathogenic Bacteria in Leafy Vegetable Samples

A total of seventy two (72) samples of leafy vegetables from wastewater irrigated farm were examined for *Salmonella*, *Shigella* and *Campylobacter species* from Harar town, *kebele* 05 vegetable farm. These selected pathogenic bacteria were detected from four different leafy vegetable types (Lettuce, Spinach, Kale and Cabbage) with the overall prevalence of *Salmonella* species (12.5%), *Shigella* species (9.7%) and *Campylobacter* species (2.8%) (Table 3). The presence of *Salmonella* species in each vegetable type was 22.2% (4/18), 16.7% (3/18), 11.1% (2/18), 0% in lettuce, spinach, cabbage and kale, respectively.

Table 3: Prevalence of selected pathogenic bacteria in some leafy vegetables irrigated with wastewater in Harar town, *kebele* 05 vegetable farm

Leafy Vegetables	Number of examined samples (N)	Detected Pathogenic bacteria					
		Salmonella species		*Shigella* Species		*Campylobacter* specie	
		F	%	F	%	F	%
Lettuce	18	4	22.2	3	16.7	1	5.6
Spinach	18	3	16.7	1	5.6	0	0
Kale	18	0	0	2	11.1	1	5.6
Cabbage	18	2	11.1	1	5.6	0	0
Total	72	9	12.5	7	9.7	2	2.8

F=Frequency of positive samples, %=Percentage of positive samples, Kale=*Yegurage gomen* (*Brassica carinata*) (Ethiopian leafy vegetable)

Of the 72 leafy vegetable samples 9 (12.5%) had *Salmonella* species which was in connection with the results of Nma and Oruese (2013) who demonstrated that the frequency of occurrence of *Salmonella* species associated with vegetables in Port Harcourt metropolis (Nigeria) was (13.6%); but higher than the study conducted by Tambekar and Mundhada (2006), Abadias *et al*. (2008) and Oliveira *et al*. (2011) who reported that 5.8%, 0.7% and 1.2%, respectively, of the total vegetable samples contaminated by *Salmonella* species. In contrast to this result, Adjrah *et al*. (2013) and Moayed *et al*. (2013) did not detect *Salmonella* species in any of the vegetable samples examined.

The higher detection of this pathogen from produce might be related to the large surface area of these vegetables exposed to contact with the effluent/point source contaminants from irrigation water and the type of irrigation system used. Farmers in this study area employ the surface irrigation system. The existence of *Salmonella* in these vegetables may be due to the use of waste water for irrigation purpose. *Salmonella* is an important foodborne pathogen and its prevalence in fresh food poses a threat to humans.

Shigella species was isolated in 9.7% (7/72) of the 72 leafy vegetable samples and its presence in each vegetable type was 16.7% (3/18), 5.6% (1/18), 11.1% (2/18), and 5.6% (1/18) in lettuce, spinach, kale and cabbage, respectively. This result (9.7%) was higher than the findings of Nma and Oruese (2013) who reported that the frequency of *Shigella* species was 2.5%, from Port Harcourt, metropolis (Nigeria); Tambekar and Mundhada, (2006) from India indicated that *Shigella species* was isolated in 3.4% of vegetable samples; Wahla and Devi (2015) also revealed that the percentage of occurrence of *Shigella* species was 5% from salad vegetables in India and Cinzia *et al*. (2015) revealed that from 125 vegetable samples collected from Italy, *Shigella* species were not detected at all.

Among all leafy vegetables analyzed for pathogenic bacteria 2.8% were contaminated by *Campylobacter* species. This result was in connection with; Park and Sanders (1992) who reported that *Campylobacter* species was detected in 1.6–3.3% of vegetables tested and, Kumar *et al*. (2001) who reported low isolation rates of *Campylobacter* in 2 (3.6%) of 56 fresh vegetables.

In the present study, low detection rate of *Campylobacter* species compared to other pathogenic bacteria (*Salmonella* and *Shigella* species) from vegetables may be because of the fact that these bacteria are microaerophilic food borne pathogens; survive poorly on plants, perhaps because of lack of microsites with sufficiently low oxygen concentrations in that habitat.

According to PHLS (2000) guide line in 25 grams of raw vegetables, pathogenic bacteria should not be detected. However, pathogenic bacteria were detected in leafy vegetable samples. Generally, the results of this study also confirmed that the level of contamination was high in vegetables that were irrigated with wastewater in Harar town, *kebele* 05 vegetable farm. It was also shown that thorough washing was not sufficient to reduce pathogen levels to safe limits in leafy vegetable types studied.

4.3. Parasitological Analysis of Leafy Vegetables

Infective parasitic stages in this study (*A. lumbricoides* eggs, *E. histolytica* and *G. lamblia* cysts) were detected. As can be seen from Table 4, the percentage of detection of *Ascaris lumbricoides* eggs, *Entamoeba histolytica* and *Giardia lamblia* cysts were 43.1%, 25% and 15.3%, respectively in all vegetable samples.

Table 4: Percentage of positive samples for parasites detected in spinach, lettuce, kale, and cabbage

Detected parasitic stages	Leafy vegetables									
	Lettuce (N=18)		Spinach (N=18)		Kale (N=18)		Cabbage (N=18)		Total (N=72)	
	F	%	F	%	F	%	F	%	F	%
A. lumbricoides eggs	8	44.4	9	50	4	22.2	10	55.6	31	43.1
E. histolytica cyst	6	33.3	1	5.6	5	27.8	6	33.3	18	25
G. lamblia cyst	3	16.7	4	22.2	1	5.6	3	16.7	11	15.3

F=Frequency of positive samples, N=Number of samples examined, %=Percentage of positive samples, kale=*Yegurage gomen* (*Brassica carinata*)

Ascaris lumbricoides eggs was the most prevalent parasitic stage, followed by *Entamoeba histolytica*, and *Giardia lamblia* cysts. In contrast, Abougrain *et al*. (2010) reported that eggs of *Ascaris lumbricoides* was detected in 68% (85/126) of vegetables examined a proportion that was greater than the present finding. In addition, Al-Binali *et al*. (2006) reported the detection of *A.lumbricoides* in 16% of leafy vegetables; Daryani *et al*. (2008), reported the prevalence of 25% and 29% for pathogenic parasites in vegetables of markets and gardens, respectively with *A. lumbricoides* eggs being detected in 2% of samples examined in Iran; And Shahnazi and Jafari-Sabet (2009) reported that the rates of contamination with *Ascaris lumbricoides* eggs in vegetables in Iran was 2.3% which were lower than the present result. In line with this, study done in Shahrekord (Iran) had shown that *Ascaris lumbricoides* eggs were the most predominant parasitic stages in vegetables (Fallah *et al*., 2012).

In the present study, among protozoan parasites detected from vegetables was a cyst of *Entamoeba histolytica* (Table 4). It was detected in 25% (18/72) of fresh leafy vegetables examined including 33.3% (6/18) of lettuce, 5.6% (1/18) of spinach, 27.8% (5/18) of kale and 33.3% (6/18) of cabbage samples from this farm. In contrast to this study, Damen *et al*. (2007) isolated *E. histolytica* (14%) from different vegetables in Jos (Nigeria), which showed lower value than the current finding. The presence of the *Entamoeba* species in the vegetable samples could be the result of inappropriate agricultural practices; during cultivation, when cultivated vegetables come in a direct contact with soil and water that is contaminated with human and animal feces (Silva *et al*., 2014).

Giardia cyst was also detected in 15.3% (11/72) of all leafy vegetables examined in the present study (Table 4). These included 16.7% (3/18) of lettuce, 22.2% (4/18) of spinach, 5.6% (1/18) of kale and 16.7% (3/18) of cabbage. As can be seen from Table 4 *Giardia lamblia cyst* was the least prevalence among the other parasites detected. However, it was higher than the study reported by Erdogrul and Sener (2005), Abougrain *et al*. (2010) and Ali and Ameen (2013) who revealed that the prevalence of *Giardia lamblia* cysts attached to vegetables in some developing countries ranging from 3% to 10%.

Occurrence of more than one parasite per sample in this study reflects the possibility of multi faecal contamination of vegetables which could lead to multiple parasitic infections in human. It might also indicate the persistence of intestinal parasitic infection in the area (Tamirat *et al*., 2014). The presence of infective parasitic stages poses a greater health risk from handling and consuming the contaminated vegetables. The differences were seen in the relative abundance of the parasites found in the vegetables.

4.4. Heavy Metal Determination in Vegetables

The concentrations of lead, cadmium and chromium in vegetables (leaf) were determined (Table 5). The concentrations of Lead, Chromium and Cadmium in leafy vegetable samples ranged from 0.04 to 0.3 mg/kg, 0.7 to 3.2 mg/kg, and 0.3 to 1.0 mg/kg, respectively, with mean concentrations of 0.17, 1.78 and 0.62 mg/kg, respectively.

Table 5: Mean concentration of three purposively selected heavy metals in leafy vegetables cultivated at Harar town, *kebele* 05 vegetable farm in terms of mg/kg dry weight (Mean±SE)

Leafy vegetables	Detected heavy metals		
	Lead (Pb)	Chromium (Cr)	Cadmium (Cd)
Lettuce	0.27±0.03[a]	2.60±0.3[a]	0.50±0.06[bc]
Spinach	0.06±0.01[b]	2.23±0.03[ab]	0.37±0.03[c]
Kale	0.13±0.03[ab]	0.77±0.03[c]	0.67±0.07[b]
Cabbage	0.23±0.03[a]	1.53±0.03[b]	0.93±0.03[a]
For all vegetables	0.17±0.03	1.78±0.2	0.62±0.07
Maximum limit	0.3***	2.3***	0.2***

SE=standard Error of the mean

[a-b-c] Means with different superscript letters down the column for the same parameter with in vegetable types do significantly differ (P<0.01)

Kale (*Yegurage gomen*)
***Source: FAO/WHO (2011)

Vegetable species differ in their ability to take up and accumulate heavy metals, even among varieties within the same species (Säumel *et al*., 2012). A study conducted by, Muchuweti *et al*. (2006) who reported that vegetables grown on land amended with sewage sludge presented a health risk for humans, in Zimbabwe. In addition, Adekunle *et al*. (2009) found that Pb concentration in leafy vegetables exceeded recommended values for three cities in Nigeria.

4.4.1. Lead

The mean concentrations of Pb were 0.27 mg/kg, 0.06 mg/kg, 0.13 mg/kg and 0.23 mg/kg in lettuce, spinach, kale and cabbage, respectively. The mean value of Pb reported in this study (0.17±0.03 mg/kg) for all vegetable samples was lower than the data obtained by, Sharma *et al*. (2006) who reported that the Pb concentration in leafy vegetables grown in industrial areas of Varanasi (India) was 17.54–25.00 mg/kg.

In addition, Muchuweti *et al*. (2006) reported that the level of Pb in vegetables irrigated with wastewater from Zimbabwe was 6.77 mg/kg. The highest mean value of Pb in the present study was in lettuce (0.27 mg/kg) and the lowest mean was in kale, but all vegetables were below the maximum limit (0.3 mg/kg) recommended by FAO/WHO (2011). In contrast to this study, Kumar *et al*. (2009) and Farooq *et al*. (2008) who reported that Pb concentration was above limited level in leafy vegetables grown in vicinity of an industrial area of Jaipur city (India) and Faisalabad (Pakistan), respectively.

The present result showed that the level of Pb ranged between 0.04 to 0.3 in all vegetables, and 0.2 to 0.3 mg/kg for lettuce, 0.04 to 0.07 mg/kg for spinach, 0.1 to 0.2 mg/kg for kale (*yegurage gomen*) and 0.2 to 0.3 mg/kg for cabbage. A study conducted by Kumar *et al.* (2009) who reported that the concentration of lead in lettuce ranged from 2.3 to 5.3 mg/kg which is greater than the present result (0.2 to 0.3 mg/kg). Study conducted by, Yirgaalem *et al*. (2012) who reported that Pb levels in vegetables varying from 0.11 to 0.89 mg/kg which was greater than the present result.

4.4.2. Cadmium

The mean concentration of cadmium (Cd) in lettuce, spinach, kale and cabbage was 0.50, 0.37, 0.67, and 0.93 mg/kg, respectively. The concentrations of Cd was significantly difference ($P < 0.0001$) amongst vegetable types (Table 5, Appendix Table 5).

In all the vegetable samples analyzed, cadmium concentration ranged between 0.30 to 1.00 mg/kg. In contrast, Odai *et al*. (2008) reported that the concentration of cadmium in vegetables grown on waste dumping sites was 0.68–1.78 mg/kg in Kumasi (Ghana). The accumulation of elevated concentration of Cd in all vegetables might be attributed to the use of water from contaminated source for cultivation. All vegetable types contained cadmium concentration levels were exceeded the recommended maximum value for leafy vegetables of 0.2 mg/kg.

Similarly, Levels exceeding the maximum limit of leafy vegetables were reported for cadmium in studies conducted by Sharma *et al*. (2007), Yirgaalem *et al*., (2012) and Farooq *et al*. (2008), in Harare (Zimbabwe), Addis Ababa (Ethiopia) and Faisalabad (Pakistan), respectively. This may be because of cadmium is easily absorbed and translocate to shoots of vegetables (Mumba *et al*., 2008).

4.4.3. Chromium

In this study the chromium concentration ranged from 0.7-3.2 mg/kg for all vegetables. This shows that chromium levels are generally within normal range almost in all vegetable samples, except lettuce (2.60 mg/kg) as shown in (Table 5).

Similarly, Fisseha (1998) reported that the concentration of Cr was highest in lettuce at Peacock vegetable farm Addis Ababa (Ethiopia). In addition, Sharma *et al*. (2006) demonstrated that the heavy metal contents in different vegetables grown in the lands irrigated by wastewater and noted the concentration of Cr to be within the safe limits. In line with the present finding, Farooq *et al*. (2008) reported that Cr was within the permitted limits in leafy vegetables grown in vicinity of an industrial area of Faisalabad (Pakistan).

5. SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1. Summary

This was aimed to investigate the microbial contaminants and level of heavy metals concentration on vegetables irrigated with wastewater in *kebele* 05 vegetable farm (*Gomen sefer*) in Harar town. So a laboratory based cross sectional survey was conducted from October 2016 to January 2017 to know the contaminants to selected leafy vegetables. All the mean values for vegetables were $9.5x10^7$ for total aerobic mesophilic bacterial count, $4.3x10^6$ CFU/g for total coliform count, and $4.6x10^5$ CFU/g for fecal coliform count. The aerobic mesophilic bacterial count was highest in spinach and lowest in kale (*Brassica carinata*). The highest total coliform count was recorded in cabbage and the lowest from kale. The highest faecal coliform count was also recorded in spinach and the lowest also from kale. There were also significance differences between vegetable types for indicator bacteria.

From the isolated pathogenic bacteria, *Salmonella* species was highly isolated followed by *Shigella* species and *Campylobacter* species in all vegetable types. Pathogenic bacteria were detected in vegetable samples in the study area which were above the PHLS (Public Health Laboratory Service) guidelines which indicated that pathogenic bacteria should not detect in twenty five grams of vegetable samples. *Shigella* species was highly detected in lettuce and low detection rate in spinach and cabbage and also *Campylobacter* species was isolated from lettuce and kale but not detected in cabbage and spinach. *Ascaris lumbricoides* eggs was the predominant intestinal parasite in the present work with the highest score frequency in cabbage, followed by *Entamoeba histolytica* and *Giardia lamblia* cysts of the vegetable samples examined.

Vegetable types were also examined for heavy metal (Cadmium (Cd), Chromium (Cr) and lead (Pb)) concentration by the absorbance mode of reading in an instrument atomic absorption spectrophotometry. Cadmium was more than the maximum limit recommended by FAO/WHO in all leafy vegetables but the level of lead was generally within the normal range in all samples. Similarly chromium was also generally within the normal range in all vegetables except lettuce.

5.2. Conclusion

As most farmers irrigate different leafy vegetables before harvesting them with the same water again and again, it is likely that there is pre-harvest contamination of vegetables. This water can easily spread pathogenic bacteria, parasites and toxic heavy metals from the contaminated source to leafy vegetables. Data obtained from the microbial analysis suggested that, there were more risks from consumption of vegetables. These were illustrated by high contamination of vegetables by indicator bacteria, pathogenic bacteria, and parasites from this farm.

The high contaminations of vegetables with total and fecal coliforms suggest high risk of acquiring infectious diseases through the consumption of vegetables. The occurrence of such indicator microorganisms is an indication of the contamination of the vegetables with faecal matter derived from humans and other animals. The current result exceeds the International Commission on Microbiological Specifications for Foods (ICMSF) recommended level for indicator bacteria in vegetable samples. This indicates that the leafy vegetables in the present study site which were cultivated using wastewater had poor microbiological quality. The higher number of coliforms also indicates that pathogenic bacteria may exist. High total coliform bacteria levels are known to be related to lack of proper hygiene practice in and around the farms and the quality of water used for irrigation.

The results of this study showed that vegetables cultivated in Harar town, *kebele* 05 vegetable farm may posed a great public health problems, due to the presence of pathogenic bacteria like *Salmonella* species, *Shigella* species, *and Campylobacter* species, despite the vegetables did not show any visible signs of contamination. This study is a preliminary, on-going research; it showed the presence of organisms that have serious public health significance in this site. High numbers of pathogenic bacteria in raw consumed vegetables would lead to the consumer’s illness with symptoms of the particular or combined microbial presence. Washing of vegetables with just water is inadequate to remove all contaminating pathogens. The contaminating pathogens are responsible for various types of enteric diseases as well as serious intoxications in human.

This finding also raised the concern of public health being at high risk of infection with amoebiasis, giardiasis and ascariasis. Biologically, the highest health risk is for helminth infections compared with other pathogens because helminthes persist for longer periods in the environment, host immunity is usually low to non-existent, and the infective dose is small.

Wastewater irrigation can also lead to accumulation of heavy metals in the soil and consequently into the vegetables, exposing the human population of the area and surrounding community to serious health risks. It may be concluded that irrigation by untreated sewage water and industrial effluents are the main reasons for accumulation of heavy metals in vegetables.

Generally, the results of the present study revealed that heavy metal and microbial contamination of vegetables in varying magnitude among vegetables in the study area which may lead to public health crisis.

5.3. Recommendations

Based on the findings of this study the following recommendations are forwarded:

- ✓ This finding suggests that further work should be done to determine the heavy metal levels of the soil to determine the transfer factor.
- ✓ The finding of this study indicates the potential microbiological risks of leafy vegetables that are eaten raw should be properly washed with salt or vinegar before eating.
- ✓ A powerful monitoring program is needed to provide reliable information about the current water quality before its use in a large scale in agriculture.
- ✓ Awareness should be given to the farmers of the area regarding the serious consequences of using contaminated wastewater for irrigation purpose
- ✓ This finding also suggests further work to be undertaken about the content of heavy metals and pathogens in the irrigation water in the area

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7. **APPENDIX**

Appendix Table 1: Mean comparisons among vegetable types for indicator bacteria

Mean comparisons between vegetable types for total aerobic mesophilic bacterial count (TAMBC)

Vegetable types	Lettuce		Cabbage		Kale	
	Mean difference	P-value	Mean difference	P-value	Mean difference	P-value
Spinach	237516666***	0.000	254933333***	0.000	281853889***	0.000
Lettuce			17416667	0.607	44337223	0.193
Cabbage					26920556	0.428

Mean comparisons between vegetable types for total coliform count (TCC)

Vegetable types	Lettuce		Cabbage		Kale	
	Mean difference	P-value	Mean difference	P-value	Mean difference	P-value
Spinach	225242	0.797	1278575	0.148	4365584***	0.000
Lettuce			1053333	0.226	4590826***	0.000
Cabbage					5644159***	0.000

Mean comparisons between vegetable types for fecal coliform count (FCC)

Vegetable types	Lettuce		Cabbage		Kale	
	Mean difference	P-value	Mean difference	P-value	Mean difference	P-value
Spinach	582706***	0.002	442871***	0.020	870519***	0.000
Lettuce			139835	0.453	287813	0.131
Cabbage					427648***	0.029

Mean comparisons significant at the 0.05 levels are indicated by ***

Appendix Table 2: Analysis of variance (ANOVA) results for TAMBC, TCC and FCC)

Total aerobic mesophilic bacterial count					
Source	DF	SS	Mean square	F value	Prob
Vegetable types	3	9.17	3.06	29.83	< 0.0001
Total coliform count					
Vegetable types	3	3.09	1.03	15.41	< 0.0001
Fecal coliform count					
Vegetable types	3	6.39	2.13	7.55	0.0002

Appendix Table 3: Biochemical test results for *Salmonella*, *Shigella* and *Campylobacter* species

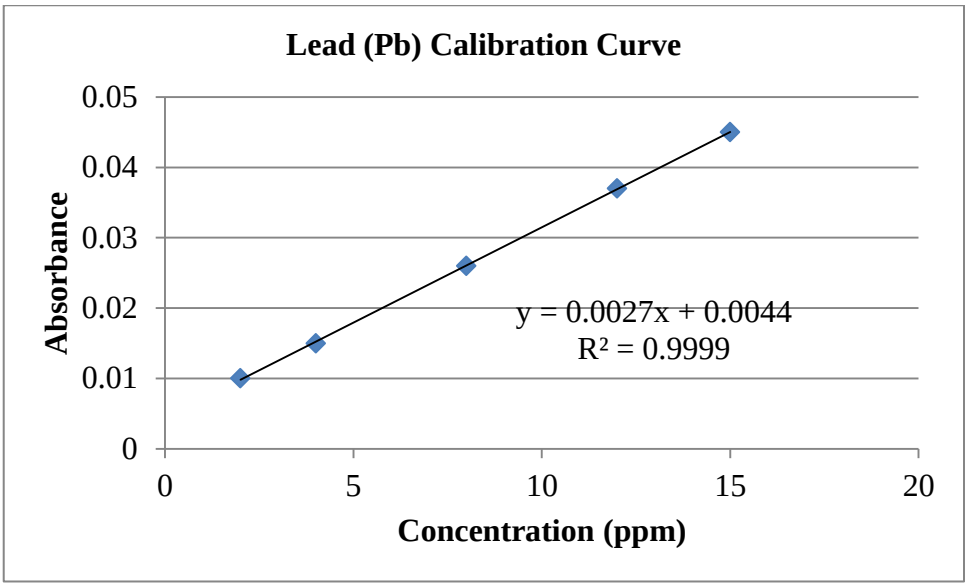
Date: _____

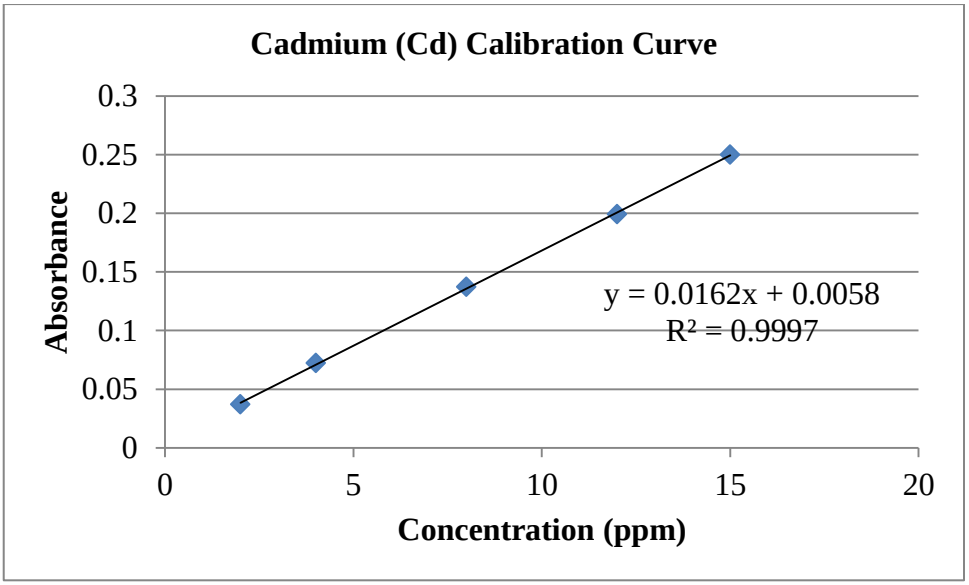
Biochemical tests

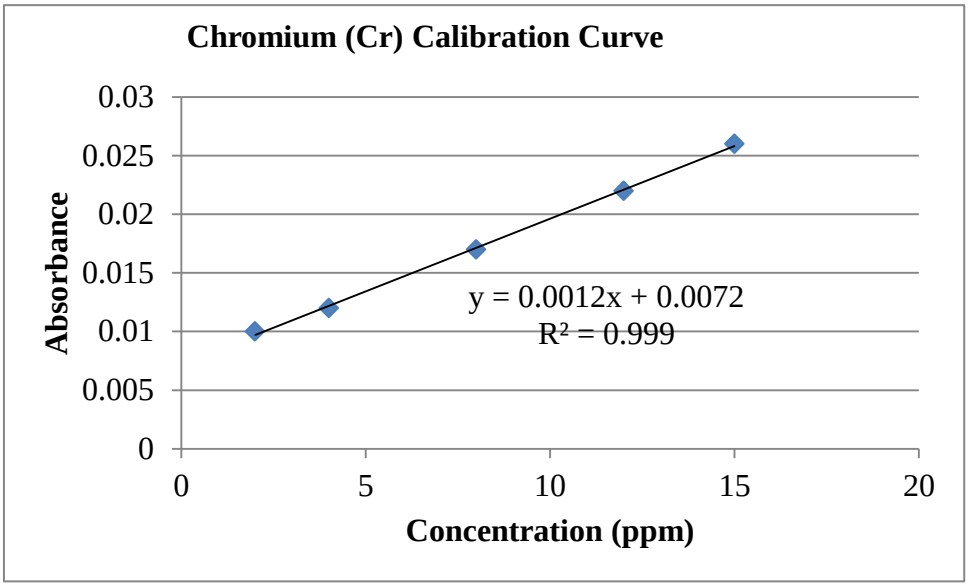
Isolated bacteria		KIA test				Catalase	Motility	Indole	Citrate utilization	urease
Salmonella species	Isolated code	Slant	Butt	Gas	H_2S					
	Lettuce 3	Red	Yellow	+	+	+	+	-	+	-
	Lettuce 9	Red	Yellow	-	+	+	+	-	+	-
	Lettuce 12	Red	Yellow	+	-	+	+	-	-	-
	Lettuce 17	Red	Yellow	+	+	+	+	-	+	-
	Spinach 2	Red	Yellow	+	-	+	+	-	+	-
	Spinach 5	Red	Black	-	+	+	+	-	+	-
	Spinach 14	Red	Yellow	+	-	+	+	-	+	-
	Cabbage 6	Red	Black	-	+	+	+	-	+	-
	Cabbage 9	Red	Yellow	+	+	+	+	-	-	-
Shigella species	Lettuce 4	Red	Yellow	-	-	+	-	+	-	-
	Lettuce 10	Red	Yellow	-	-	+	-	-	-	-
	Lettuce 17	Red	Yellow	-	-	+	-	-	-	-
	Spinach 13	Red	Yellow	-	-	+	-	+	-	-
	Kale 15	Red	Yellow	-	-	+	-	+	-	-
	Kale 8	Red	Yellow	-	-	+	-	-	-	-
	Cabbage 9	Red	Yellow	-	-	+	-	+	-	-
Campylobacter species	Lettuce 16	Red	Red	-	-	+	+	-	+	+
	Kale 15	Red	Red	-	-	+	+	-	+	+

KIA=Kligller Iron Agar, (+) = positive reaction, (-) = negative reaction, H_2S=Hydrogen sulphide production

Appendix Table 4: Standard calibration curves for absorbance of heavy metals (Pb, Cd, and Cr)







Appendix Table 5: Analysis of variance (ANOVA) for heavy metal concentration

Lead					
Source	DF	SS	Mean Square	F Value	Prob
Vegetable type	3	0.08	0.03	10.39	0.004
Cadmium					
Vegetable type	3	0.54	0.18	23.85	0.0002
Chromium					
Vegetable type	3	5.90	1.97	28.08	<0.0001

Mean comparisons for heavy metals (Pb, Cr and Cd) among vegetable types

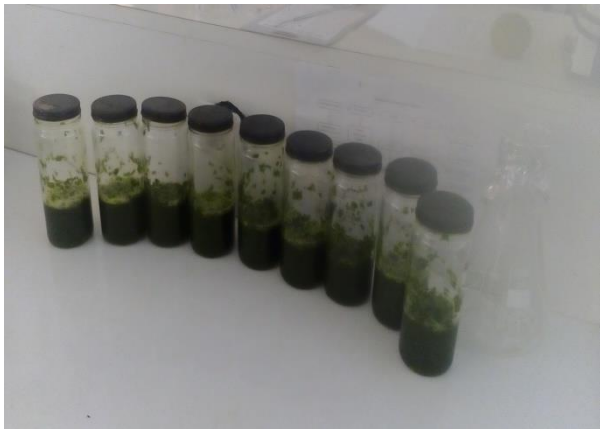
Lead						
Vegetable types	Lettuce		Cabbage		Kale	
	Mean difference	P value	Mean difference	P value	Mean difference	P value
Spinach	0.2066667***	0.001	0.1733333***	0.003	0.733333	0.115
Lettuce			0.0333333	0.44	0.133333	0.012
Cabbage					0.1	0.042
Kale						

Chromium						
Vegetable types	Lettuce		Cabbage		Kale	
	Mean difference	P value	Mean difference	P value	Mean difference	P value
Spinach	0.3666667	0.128	0.7	0.012	1.4667***	<0.0001
Lettuce			1.0666667***	0.001	1.83333***	<0.0001
Cabbage					0.7666667***	0.008
Kale						

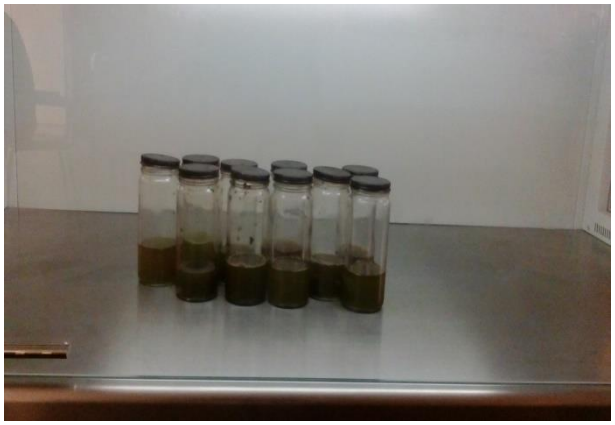
Cadmium						
Vegetable types	Lettuce		Cabbage		Kale	
	Mean difference	P value	Mean difference	P value	Mean difference	P value
Spinach	0.1333	0.96	0.5667***	<0.0001	0.3***	0.003
Lettuce			0.4333***	<0.0001	0.1667	0.46
Cabbage					0.2667***	0.005
Kale						

Mean comparisons significant at the 0.01 levels are indicated by ***

Photographs during infective parasitic stage detection

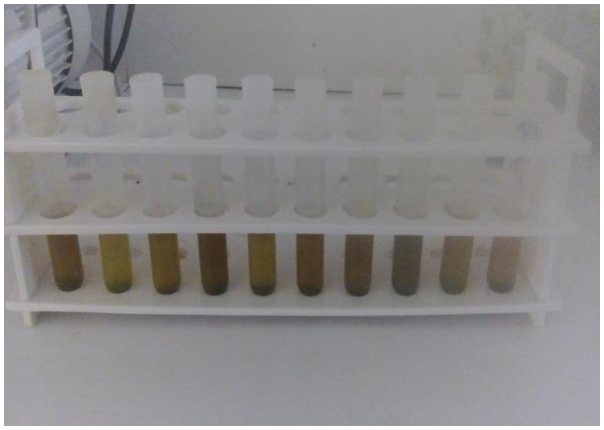


Crushed vegetables washed with PSS



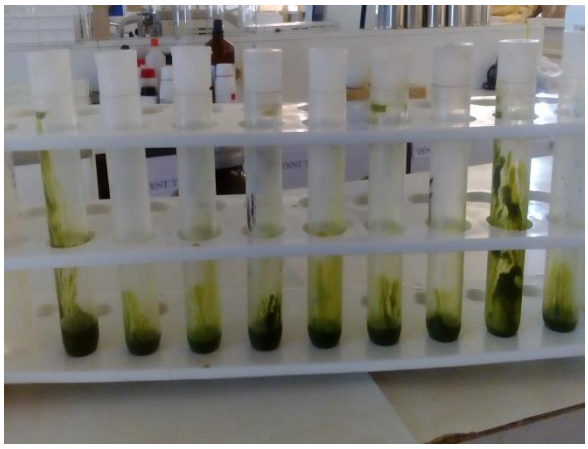
Vegetables after 24 hr sedimentation with PSS

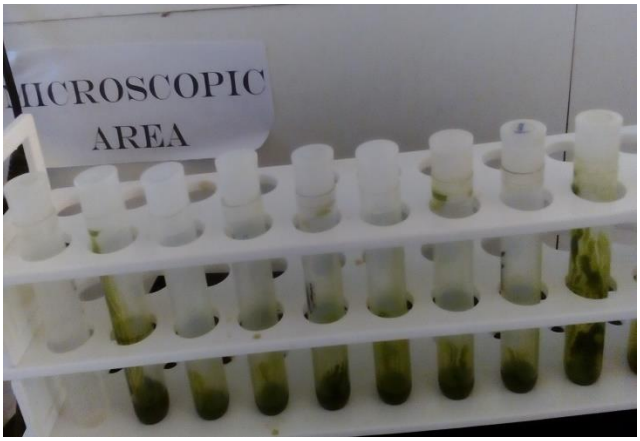
PSS= Physiological Saline Solution (0.85% NaCl)



5 ml of sediments before centrifugation

5 ml of sediments after centrifugation

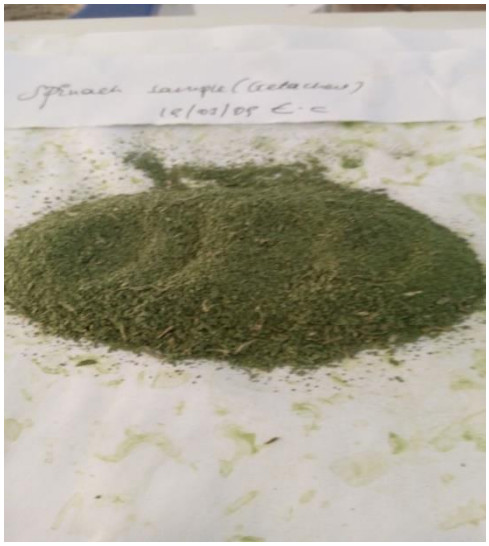




Centrifuged residue with siphoned supernatant mixed with a drop of physiological saline to increase the chance of distribution of the parasites infective stage

Photographs during vegetable preparation for heavy metal analysis

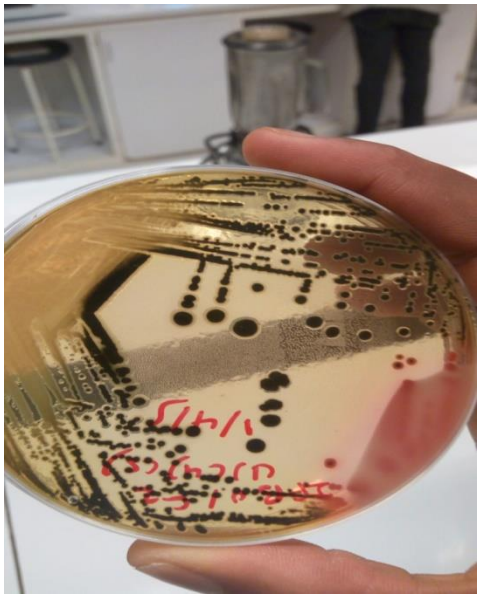
Leafy Vegetable samples during drying



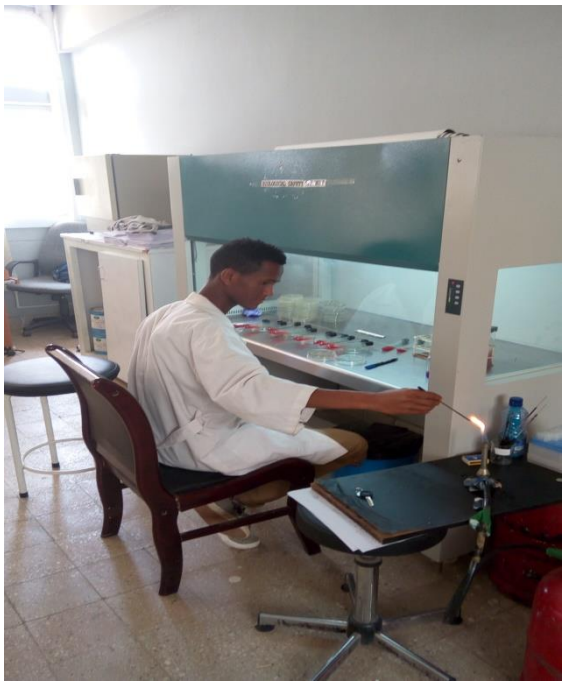
composite spinach sample during drying

During determination of heavy metals (Pb, Cd, and Cr) by an instrument called Atomic Absorption Spectrophotometry (AAS) at HU, central laboratory

Photographs during some pathogenic bacteria detection (*Salmonella* spp, *Shigella* spp and *Campylobacter* spp)



Suspected *salmonella* colony on SSA sub cultured colony on nutrient agar



During biochemical test to confirm sub cultured pathogenic bacterial colony