Assessment of Microbiological Quality of Raw Vegetables Irrigated with Hasassa River, West Arsi Zone, Oromia Region, Ethiopia

BY

Edao Gelgelu Gemeda

Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's of Science in Applied Biology (Microbiology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

December 2018

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Advisor: Dr. Zerihun Belay

Co-advisor: Dr. Bayissa Chala

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APPROVAL BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by **Edao Gelgelu Gemeda** have read and evaluated his thesis entitled "**Assessment of Microbiological Quality of Raw Vegetables Irrigated with Hasassa River, West Arsi Zone, Oromia region, Ethiopia**" and Examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial Fulfillment of the requirement for Degree of Master of Science in Applied Biology (Microbiology).

_____	_____	_____
Advisor	Signature	Date
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Chairperson	Signature	Date
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External examiner	Signature	Date

DECLARATION

I hereby declare that the work which is being presented in this research entitled as **Assessment of Microbiological Quality of Raw Vegetables Irrigated with Hasassa River, West Arsi Zone, Oromia Region, Ethiopia"** in partial fulfillment of the requirement for the award of the degree of MSc in Applied Biology (Microbiology). It was authentic record of my original work carried out from October 2017 to November 2018 under the advisor of Dr. Zerihun Belay and Co-advisor of Dr. Bayissa Chala, Department of Applied Biology, School of Applied Natural Science, Adama Science and Technology University. Other person or I have not submitted the matter embodied in this for the award of any other degree. All relevant resources of information used in this body have been properly acknowledged.

_____ _____ _____

Name of candidate Signature Date

This MSc Thesis has been submitted for examination with our approval as thesis advisor and co-advisor

_____ _____ _____

Name of Advisor Signature Date

_____ _____ _____

Name of co-advisor Signature Date

Date of submission: November 15, 2018

ADVISOR APPROVAL SHEET

To: Applied Biology Department

Subject: Thesis Submission

This is to certify that the thesis entitled "**Assessment of Microbiological Quality of Raw Vegetables Irrigated with Hasassa River, West Arsi Zone, Oromia Region, Ethiopia**" submitted in partial fulfillment of the requirements for the degree of Master's in Applied Biology (Microbiology), the Graduate program of the department of Applied Biology, and has been carried out by **Edao Gelgelu Gemeda**, with Id. No.GSR/0199/09, under my supervision. Therefore, we recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

_____	_____	_____
Name of Major Advisor	Signature	Date
_____	_____	_____
Name of co-advisor	Signature	Date

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ABBREVIATIONS AND ACRONYMS

AMB .....Aerobic Mesophilic Bacteria

ANOVA.....Analysis of Variance

ARB .....Antibiotic Resistant Bacteria

ARG.....Antibiotic Resistant Genes

ASF.....Aerobic Spore Formers

CFSAN .....Center for Food Safety and Applied Nutrition

CFU .....Colony Forming Units

CLSI.....Clinical and Laboratory Standard Institute

CSA .....Central Statistical Authority

FAO .....Food and Agriculture Organization

FDA .....Food and Drug Administration

HACCP.....Hazard Analysis and Critical Control Point

HACCPTQM.....Hazard Analysis and Critical Control Points-Total Quality Management

HHS .....Health and Human Services

LPS .....Lipopolysaccharides

MDR .....Multiple Drug Resistance

MoWR .....Ministry of Water Resources

TSI .....Triple-Sugar-Iron

TSS .....Total Suspended Solids

USDA .....United States Drug Administration

WHO.....World Health Organization

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ABSTRACT

Vegetables are the fresh and edible portions of herbaceous plants that provide the most important human diet that contains carbohydrates, proteins, vitamins, minerals, and fiber. In contrast to health benefit, there was an increased frequency of pathogen isolation on vegetable products, which may lead to disease in humans. The aim of the present study was to investigate the microbial quality and evaluate incidence of antimicrobial resistance of bacteria isolated from vegetables irrigated with Hasassa River. Irrigation water and vegetable samples (carrot, lettuce, and garlic) were collected from irrigation sites and analyzed for their bacteriological load and presence of any pathogenic microbes, which their resistance pattern was detected following standard procedures. Appropriate serial dilutions of the suspension from 10^{-4}, 10^{-5} and 10^{-6} were spread-plated on a suitable solid media for counts of aerobic Mesophilic bacteria, gram-negative Enterobacteriaceae and Staphylococci, and homogenized samples were heated at 80°C for 10 minutes in a water bath to count aerobic spore formers respectively. The maximum overall mean counts of aerobic Mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, Staphylococci, and total coliform counts were 8.21, 6.58, 6,88, 6.88 log CFU ml^{-1} and >1100 MPN 100 ml^{-1}, respectively. The microflora of vegetable samples was dominated by Bacillus species (21.88%) followed by Corynebacterium spp (12.5%), Lactobacillus spp. (12.5%). Staphylococcus aureus and Salmonella spp. were detected in 21.88% and 15.6% of the samples, respectively. The result of antimicrobial test showed that all the isolates 32(100%) were resistant to Penicillin g, 26(81.25%) to Vancomycin, 23(71.9%) to ampicillin, 15(46.9%) to Chloramphenicol, 15(46.9%) to Erythromycin, and the least 3(9.4%) to Perfloxacin. The present finding revealed that the Hasassa river water used for irrigation had a possible microbial contamination that can be transferred directly or indirectly during pre-harvest, as well as post-harvest handling to fresh vegetables which potentially constitutes a health risk to consumers. Therefore, persons involved in the production chain and consumers should be made aware that contaminated fruits and vegetables eaten raw could be potential vehicles of pathogenic agents.

Keywords: Antibiotic Resistance, Irrigation Water, Pathogen, Vegetables

1. INTRODUCTION

1.1. Background of the study

Vegetables are the fresh and edible portions of herbaceous plants. Fresh and minimally processed vegetables and fruits provide the most important human diet that contains carbohydrates, proteins, vitamins, minerals, and fiber(Lerici *et al*., 2000). Among the plants, vegetables are the excellent sources of minerals and contribute to the RDA (resource description and access) of these essential nutrients (Hanif *et al*., 2006). Fresh vegetables occupy an increasingly important place in the human food supply because of its health-promoting nutritional properties (Cinzia *et al*., 2015). Their role in reducing the risk of lifestyle associated illnesses such as heart disease, diabetes, and cancer has resulted in a further increase in desirability and consumption (Ijabadeniyi, 2010).

In contrast to health benefit, consumption of fresh fruits and vegetables has also been associated to risk for consumers. Jung *et al*. (2014) reported that outbreaks in recent years have been linked to tomatoes, spinach, lettuce and seed sprouts particularly when fresh vegetables are contaminated by foodborne pathogens. Furthermore, surveillance of vegetables has indicated that these foods can be contaminated with various bacterial pathogens, including Shiga toxigenic, *Escherichia coli* (STEC), *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp., *Listeria monocytogenes*, and *Campylobacter* spp. (Adane and Tsehayneh, 2017).

The sources of those pathogens and other microbes traverses the continuum from farm to plant and includes contaminated agricultural water, soil amendments, contaminated harvesting equipment, field workers, processing plants and retail handling(Jung *et al*., 2014). Irrigation is considered as one of the most important transmission modes of enteric human pathogens to vegetable crops (Adane and Tsehayneh, 2017). Irrigation of vegetables with polluted water and untreated wastewater is practiced worldwide (Keratia *et al*., 2007).

This practice is most common in the urban areas of low-income countries, which have no capacity to effectively treat wastewater and face increasing demands for fresh vegetables (Keratia *et al*., 2007). Wastewater provides water and nutrients as important resources for irrigation, but has high levels of pathogens (Keratia *et al*., 2007). The major pathogens associated with the use of highly polluted water are the fecal Coliforms, *E.coli* and eggs of

some Helminthes such as *Ascaris lumbricoides*, *Trichuris trichura*, *Hymenolepis diminuta*, *Fasciola hepatica* and *Strongyloides* whose resistant eggs can be found in the wastewater (Samuel *et al*., 2013).

Desta and Diriba, (2016) In Jimma Town reported that the microflora of vegetables and irrigation river samples was dominated by *Bacillus species* (32.7%) followed by Enterobacteriaceae (25%) and Micrococcus (16%) and pathogen such as *Staphylococcus aureus* and *Salmonella* spp. were detected in 24.0% and 20.7% of the samples, respectively. Water from the river that received both human and animal waste disposal poses a health risk due to contamination with all microorganisms of human and animal intestinal habitat.

It is observed that Hasassa River is the primary source of water for a range of activities such as recreation, bathing, washing clothes, and household utensils, small-scale agricultural irrigation, car washing, and other uses. The deterioration of the quality of Hasassa River because of discharge of municipal wastes and urban runoff has been well observed.

However, in Hasassa River, no databases on microbial quality of irrigated vegetables and irrigation water have been compiled to date. Therefore, this study was aimed at investigating the composition of pathogenic and other bacteria of some fresh vegetables irrigated with Hasassa River, in southeastern Ethiopia.

1.3. Objectives of the study

1.3.1. General objective

To investigate the microbial quality of raw vegetables and evaluate the incidence of antimicrobial resistant bacteria isolated from vegetable irrigated with Hasassa river, in west Arsi zone

1.3.2. Specific objectives:

- To determine the bacteriological load of some fresh vegetables irrigated with Hasassa River.
- To evaluate antibiotic resistance pattern of the isolates in the irrigation water and vegetable samples.
- To evaluate the distribution of multiple drug resistance bacteria in the study area.

2. REVIEW OF RELATED LITERATURE

2.1. Use of irrigation water in Ethiopia

Water is considered as an essential resource for irrigation. This is an intentional action made by human to apply water for growing crops, especially during dry seasons where there is a shortage of rainfall. Water applications to crop fields are of various types. The most commonly used and most ancient type is surface irrigation methods through the usage of gravity forces. Nowadays, modernized irrigation systems are mostly used which works based on the pressurized energy system. The sprinkler and drip irrigation systems are of this type of water application systems. Irrigation in Ethiopia is considered as a basic strategy to alleviate poverty and hence food security. It is useful to transform the rain-fed agricultural system, which depends on rainfall into the combined rain-fed, and irrigation agricultural system. This is believed to be the most prominent way of sustainable development in the country. However, the development of irrigation practices in Ethiopia has to be investigated to seriously know the history of irrigation emergence and its subsequent developments (Gebremedhin and Asfaw, 2015).

2.2. Ways of irrigation development in Ethiopia

Makombe *et al*. (2011) noted that irrigation development is a key for sustainable and reliable agricultural development, which leads to overall development in Ethiopia. Irrigated agriculture is being practiced under smallholders, medium and large-scale farming. According to Ministry of Water Resources of Ethiopia (MoWR, 2002), irrigation development in Ethiopia is classified based on the size of the command area, in three types:

1. Small-scale irrigation systems (<200 Hectares)

2. Medium-scale irrigation systems (200-3,000Hectares)

3. Large-scale irrigation systems (>3,000Hectares)

This classification system is the most common in Ethiopia. Accordingly, 46% of proposed irrigation developments are in the small-scale irrigation category.

According to Makombe *et al*. (2011), the irrigation development in Ethiopia is also classified based on the uses of a mix of the history of establishment, management system and the nature of the structures as Follows:

1. **Traditional schemes**: These are small-scale irrigation systems, which usually use diversion weirs made from local material and needs annual maintenance. The canals are usually earthen and the community manages the schemes.
2. **Modern schemes**: These are small-scale irrigation systems with more permanent diversion weirs made from concrete that do not require annual maintenance. They are mostly community-managed and the primary and secondary channels are made of concrete.
3. **Public**: These are large-scale operations constructed and managed by the government. Sometimes these schemes support out-growers (smallholder farmers who have farms near the large–scale schemes).
4. **Private**: These are privately owned systems in mechanized farms, which need a highly intensive operation. These two classification systems are the most used classification system in Ethiopia among others. This systematic classification has been used by different stakeholders in the sector

2.3. Challenges of irrigation

As water used for irrigation may contain a high concentration of human excrement, there is an increased risk that consumers may contract a foodborne infection when ingesting these irrigated crops. Except for the possible pathogenic bacteria that may be contained and the health risk this entails for agricultural workers and consumers alike, wastewater may contain various chemicals in amounts that may retard the growth or even harm the crops (Marijke, 2010). The foremost challenge for public agencies in developing countries is to determine the appropriate scale at which treatment is possible and viable with a particular emphasis on the separation of industrial and domestic wastewater to facilitate the likelihood of safe reuse. The optimal treatment strategy will vary with the economic and institutional capacities, wastewater sources and constituents, and should preferably consider the requirements of reuse than standards, which are difficult to maintain (Qadir *et al*., 2010)

2.4. Microbial contaminants in wastewater

Bacterial pathogens occur in large quantities in wastewater because they are excreted in the feces of colonized individuals. In addition to large concentrations of bacterial pathogens as a whole, wastewater also contains antibiotic-resistant bacteria. Antibiotic-resistant bacteria are bacteria that have acquired resistance, or are inherently resistant, to antimicrobials that would otherwise limit their growth or kill them. Antibiotic-resistant bacteria present a threat to human health because they limit treatment options, often cause more serious infections than their antibiotic-susceptible counterparts cause, and are more likely to cause mortality. The concentrations of antibiotics released into wastewater are often high enough to exert selective pressures to favor the proliferation of resistant strains of microorganisms. The combination of high concentrations of microorganisms, nutrients, and antibiotics found in wastewater makes it a favorable environment for bacterial growth and horizontal transfer of resistance genes (Rachel, 2010).

The major microbial pathogens in wastewater are bacteria, viruses, fungi and protozoan parasites. Bacteria pathogens are mostly present in feces and a wide variety can be present in wastewater due to fecal contamination. The discharge of untreated or inadequately treated wastewater into the environment can have negative impact on human health due to the release of pathogenic microorganisms into water, which could lead to serious health diseases. Water that is contaminated with microbial pathogens is a medium for several water borne diseases, such as cholera, typhoid fever, shigellosis, salmonellosis, campylobacteriosis, giardiasis, cryptosporidiosis and Hepatitis A. Several pathogenic organisms in contaminated water are the basic causes of gastrointestinal illnesses in human. Some of the pathogens are known to cause several outbreaks of diseases by releasing toxins in the human body (Tomilola *et al*., 2014)

2.4.1. Pathogens in irrigation water

A wide range of microbial pathogens has been found in water and can be transferred to crops during irrigation. Okafo *et al*. (2003) recovered *E. coli*, *Salmonella* spp. and *Vibrio* spp. From irrigation stream water in Nigeria and found that these microbes were also present on the irrigated plants. Aquatic plants and sediments can help pathogen survival in the open irrigation

system, whereas in case of pipe-based irrigation systems pathogens can survive through biofilms.

Survival of pathogens in the water and surrounding environment is mainly dependent on factors such as nutrient availability, temperature, organic matter content, competition with other microorganisms, pH and radiation. It has been shown that *E. coli* can survive for up to 300 days in autoclaved, filtered river water at 4 °C. Use of contaminated water for irrigation of crops is considered to be responsible for several outbreaks of disease following consumption of such crops. Bottom sediment could be one of the major reservoirs of pathogenic microorganisms as it provides nutrient availability and protection from UV sunlight (Mehboob, 2014).

For example, Pachepsky *et al*. (2011) showed that faecal Coliforms are multiple-fold higher in sediments than in the water column. Therefore, to obtain maximum decontamination in water treatment, it is recommended that the total suspended solids (TSS) content be reduced before treatment. Biofilms can be formed in the water circuit by certain pathogens in order to persist and survive. Important pathogens including *E.coli* O157:H7, Salmonella, *Listeria monocytogenes*, and, Cryptosporidium oocysts, *Vibrio* spp. and *Yersinia* Spp. have been found in different irrigation systems. These can subsequently mix with the irrigation water and may reach the plant surface (Mehboob, 2014).

2.4.2. Microbial indicators in wastewater or foodborne transmission and also human infected with these organisms by direct contact with carriers

Indicator organisms in wastewater are organisms whose presence suggests the presence of a pathogen in wastewater. The density of an indicator organism is always associated with health hazards and several sources of pollution. It is indicated that for an organism to qualify as an indicator organism of a particular pathogen, it must be continuously and totally related to the source of the pathogen and be abundant enough to provide appropriate and exact mass concentration of the level of pathogen in relation to high risk of illness. Indicator organism should have resistant ability to disinfectants, environmental stress and toxic materials that may be present at the source of the pathogen. Bacteria indicators are the most important indicators of faecal contamination. These indicators include members of the Enterobacteriaceae family,

which the total and the fecal Coliforms and *E. coli*. Other bacterial indicators are the fecal streptococci (Streptococcus and Enterococus) and Clostridium(Tomilola *et al*., 2014).

Total Coliforms - Organisms that form part of the total Coliforms group are defined as being aerobic or facultative anaerobes. They are gram and oxidase negative, catalase positive and have the ability to ferment lactose at 35ºC with the formation of acid and gas as products. These organisms are rod-shaped and do not possess the ability to form Endospores. This group includes various genera such as Klebsiella, Escherichia, Citrobacter and Serratia (Marijke, 2010).

Intestinal Enterococci and Faecal Streptococci - These Gram- positive, catalase negative cocci are homofermentative, facultative anaerobic organisms. The most well-known human pathogens of this genus include, *Streptococcus pyogenes* and *Streptococcus pneumoniae*, and several studies have reported the isolation of these organisms from frozen vegetables, making them a threat worth mentioning (Marijke, 2010).

Spore-forming bacteria - Members of the genera *Bacillus* and *Clostridium* are Gram-positive, spore-forming rods. *Bacillus cereus* has been implicated in foodborne outbreaks linked to sprouts and this comes as no surprise as the organism is ubiquitous to plant material and soil. Of the genus Clostridium, two species are of great concern where fresh produce is implicated. *Clostridium perfringens* is often found in the Faeces of humans and animals and has been isolated from river water on occasion. The neurotoxigenic *Clostridium botulinum* is the etiological agent for a condition known as botulism. These anaerobic bacilli are often isolated from improperly processed canned foods, where they proliferate and produce the deadly neurotoxin, botulin. Although this foodborne infection is rare it claims many lives when outbreaks occur (Marijke, 2010).

2.5. Potential sources of contamination in environment

The main sources of contamination of vegetables are from soil, water and air(Marijke, 2010). These issues highlight the important role of the growing environment as a source of contamination in fresh produce production. Although not part of the local environment as such, tools and equipment used in horticulture may also be considered as potential sources of

contamination, e.g. cross-contamination via the knives used in harvesting lettuce heads or spring onions.

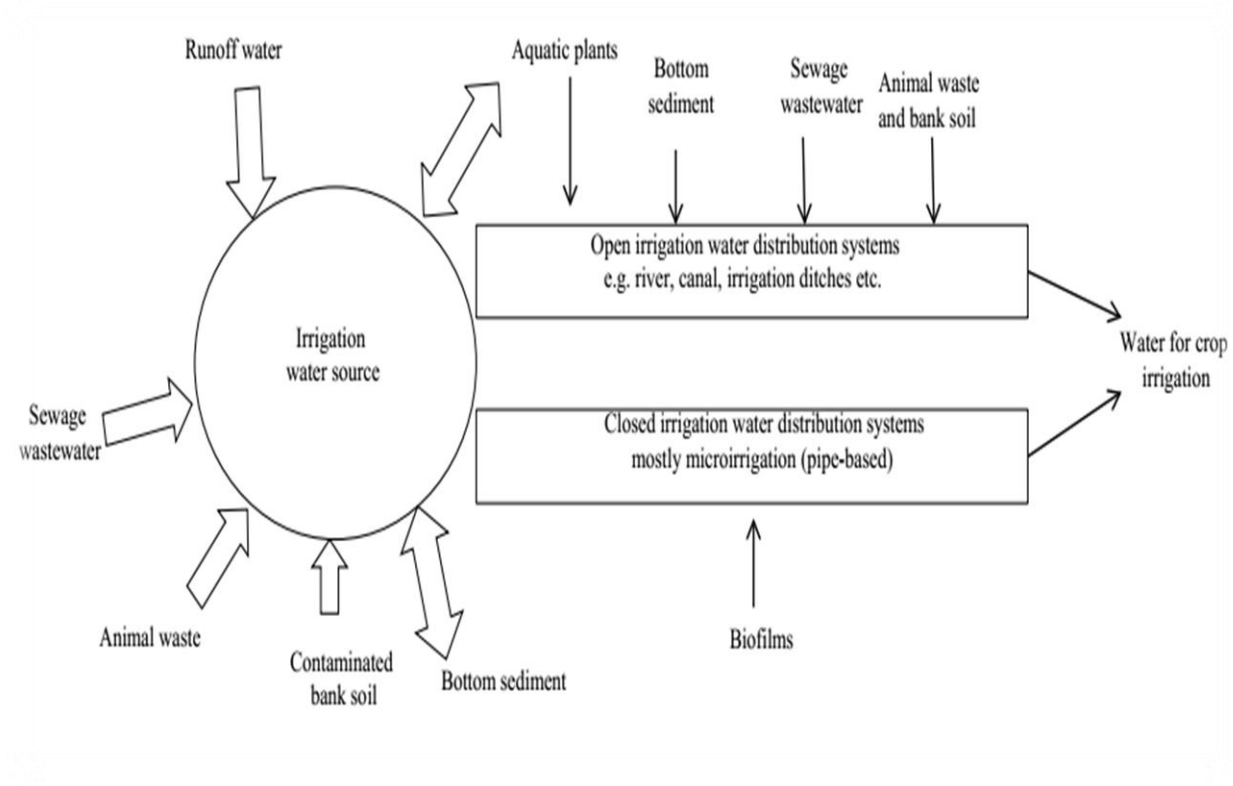


Figure 1.Potential factors affecting the microbiological quality of irrigation water sources and irrigation water distribution systems(Pachepsky et al., 2011).

2.5.1. Fertilizer from human and animal waste

Fertilizer can be an expensive input to production. As wastewater can be a good source of nutrients or plants, it is often sought after. Similarly, other cheap sources of fertilizer, including animal and poultry manure, are widely used, often without appropriate composting, which means they are also a source of microbial hazards. However, apart from this use, organic waste is often applied to agriculture land as an economical means of treatment and reuse. While such land may not necessarily be used for horticulture, such activities still present an opportunity for the introduction of potential contaminants into the environment (FAO/WHO, 2008).

2.5.2. Worker health and hygiene

Following some of the recent outbreaks linked to fresh produce, such as those caused by viruses in berries and green onions, the potential role of workers in contamination of fresh produce has been highlighted. Worker hygiene is affected by the availability and accessibility of wash and comfort stations on the farms. Another issue is the presence of sick workers or children in the fields or packing facilities, which is often linked to the economic needs, demographics or the culture of the workers. This again highlights the breadth of issues that need to be considered when addressing the problems linked to fresh produce, and hence the need to take a broad, multidisciplinary approaches(FAO/WHO, 2008).

2.5.3. Consumption patterns and practices

In recent years, there has been an increase in the consumption of fresh produce. This is happening in both developed and developing countries. In addition, consumers are eating raw products, and products that were traditionally cooked before consumption is now being eaten raw. Differences also exist among regions in terms of preparation and consumption practices. For example, baby corn is cooked in Thailand but often eaten raw in many western countries. Baby spinach is a very popular raw salad vegetable in North America but is cooked before consumption in many other parts of the world. The challenges identified above suggest that there was not be a unique solution to minimize the risks associated with microbiological hazards in fresh produce. Clearly, the whole food chain needs to be taken into consideration and the challenges at each step need to be addressed according to the characteristics of each product(FAO/WHO, 2008)

2.6.Pathogen pathways into plants

Pathogens can enter vegetable plants and become internalized, that is, colonize some plant tissues. Early studies suggested that *E. coli* could be transported into the edible part of lettuce from soil through root system or that Salmonella New port could be transported from contaminated roots to the aerial parts of Romaine lettuce seedlings depending on the developmental stage of the plant. However, more studies that are recent have not confirmed these results. *E. coli* was found in root tissue but not in shoot tissue of spinach plants grown on inoculated soil (Pachepsky *et al*., 2011).

Erickson *et al*. (2010) found that internalization of *E. coli* and Salmonella via the root system does not occur or is an extremely rare event. Pathogens may enter aerial portion of plants through stoma, scar tissue, or wounds because of irrigation water contacting leaf surfaces or from raindrop splashes from the soil surface (Pachepsky *et al*., 2011). Guo *et al*. (2001) observed migration of Salmonella from soil directly into the stem scar tissue of green tomatoes. Irrigation Waters as a Source of Pathogenic microorganisms in Produce: Wound surfaces seemed to be suitable for *E. coli* to enter iceberg lettuce tissues and promoted its survival. Stomata cavity was the preferential port of entry of *E. coli* internalization to the vegetable leaf in experiments of Gomes *et al*. (2009) with four different varieties of lettuce. A possible pathway from soil to stomata along the wet stem surface has not been explored to date.

2.6.1. Adherence to plants

Any pathogen may reach plant surfaces via irrigation water; however, the potential for adherence is both strain and plant specific. For example, strain-specific properties of *Salmonella* (curli and cellulose) affected its ability to enter parsley plants from contaminated irrigation water. Substantial differences in survival on and in tomato plants were observed for various serotypes of Salmonella Guo *et al*. (2001). Plants also differ in their propensity to become contaminated with pathogens when irrigated with contaminated water. Quantitative risk assessment models for the use of reclaimed water show that risk varies between crops, with lettuce found to pose a higher risk than cucumber, but comparable to that of broccoli and cabbage.

The prevalence of total *E. coli* was significantly higher in both organic and conventional lettuce than in any other produce varieties. When *E. coli* and *Clostridium perfringens* were added to irrigation water that was supplied in furrows and in drippers, microorganisms were detected on the surfaces of cantaloupe and lettuce, but were never recovered on the bell peppers. Those crops whose edible parts develop on the ground surface, such as lettuce and parsley, were more contaminated with Salmonella than those that grow above the soil surface, like tomatoes and pimento. There are indications that differences between cultivars may influence the extent of contamination from irrigation water to different levels (Pachepsky *et al*., 2011).

2.6.2. Effect of the concentration

The evidence for whether the initial concentration of pathogens in irrigation water is critical for produce contamination is mixed. For example, work of Webb *et al*. (2008) shows a positive relationship between the *E. coli* O157:H7 concentrations and incidence on spinach. Whereas no *E. coli* O157:H7was detected on spinach plants spray-irrigated with water with 10^2 *E. coli* O157:H7 CFU ml^{-1} in that study, the pathogen was found at higher concentrations of 10^4 CFU $ml^{-1}$and the incidence increased when the concentrations were increased to 10^6 CFU ml^{-1}. Salmonella were undetectable by MPN analysis on spinach plants during a 6-week study with plants irrigated repeatedly with water containing 10^3 CFU ml^{-1}. Salmonella persisted at a level of 10^4 CFU ml^{-1}after 24 h after plants were irrigated with very high levels of the pathogen (10^6 CFU ml^{-1}). These results imply lower concentrations correspond to lower incidence of contamination (Pachepsky *et al*., 2011).

2.7.Pathogens associated with fresh fruits and vegetables

There are a number of food borne microbial pathogens associated with the consumption of fresh fruits and vegetables that can cause illness or death among consumers who eat contaminated produce. The major foodborne microbial pathogens that may be found in fresh produce, including *Cyclospora cayetanensis*, *Escherichia coli O157:H7*, *Hepatitis A*, *Listeria monocytogenes*, *Norovirus*, *Salmonella* spp., and *Shigella* spp. (HHS, FDA, USDA, CFSAN, 2008).

Cyclospora: This pathogen is commonly characterized by watery diarrhea, loss of appetite, weight loss, abdominal bloating and cramping, low-grade fever, nausea, vomiting, and fatigue. Contaminated water used for irrigation and pesticide application and poor worker hygiene has been suggested as the most likely causes of contamination. Cyclospora outbreaks have been linked to fresh raspberries, mesclun lettuce, and basil or basil-containing products (HHS, FDA, USDA, CFSAN, 2008).

Escherichia coli O157:H7: Most *Escherichia coli* strains are nonpathogenic (they suppress harmful bacterial growth), and are commonly found in the intestines of all animals, including humans. However, there are a minority of strains such as serotype O157:H7 that may cause human illness. *Escherichia coli O157:H7* is a life-threatening bacterium that produces large

quantities of potent toxins that can cause severe damage to the lining of the intestines. Human illness associated with *Escherichia coli O157:H7* infection may include hemorrhagic colitis, hemolytic uremic syndrome (HUS), or thrombotic thrombocytopenic purpura (TTP). HUS largely affects young children and is the leading cause of acute renal failure in children. E. coli O157:H7 outbreaks have been linked to fresh produce (such as spinach and leafy greens) (HHS, FDA, USDA, CFSAN, 2008).

Listeria monocytogenes: This bacterium causes listeriosis, a serious disease in pregnant women, the elderly, and those with weakened immune systems. Listeriosis is characterized by flu-like symptoms, including persistent fever, and can be accompanied by gastrointestinal symptoms such as nausea, vomiting, and diarrhea. Listeriosis outbreaks have been linked to raw vegetables (HHS, FDA, USDA, CFSAN, 2008).

***Salmonella* spp**. typical symptoms of salmonellosis are nausea, vomiting, abdominal cramps, fever, mild diarrhea, and headache. Salmonella outbreaks have been linked to fresh produce (HHS, FDA, USDA, CFSAN, 2008).

***Shigella* spp**. Contamination by this pathogen has often been associated with poor personal hygiene of food workers. Typical symptoms include abdominal pain, cramps, diarrhea, fever, vomiting, and blood, pus, or mucus in stools. Shigellosis outbreaks have been linked to shredded lettuce, potato salad, green onions, and parsley (HHS, FDA, USDA, CFSAN, 2008).

2.8. Risks from microbiological contaminants

The result of following study reveals the presence of some highly pathogenic bacteria and fungi on raw fruits and vegetables purchased from market. These microbes are generally associated with some acute to chronic diseases. For example, Pseudomonas is an opportunistic human pathogen and a common food spoiler. Micrococcus can lead to many infections like bacteremia, septic shock, septic arthritis, endocarditis and meningitis. Corynebacterium is well known pathogen for diphtheria and Streptococcus is responsible for pneumonia. Staphylococcus is generally related to gastrointestinal infections because of its ability to produce very strong toxins. The group members of Enterobacteriaceae are widely known for intestinal infection and diarrhea (Ankita *et al*., 2014).

For consumption-related risks, this includes, for example, the amount and frequency of Vegetable intake, information about the person, like age, and needs to consider the dose response function of individual pathogens. Seidu *et al*. (2008) evaluated the annual risks of rotavirus and Ascaris infections for consumers of lettuce irrigated with the different water qualities after postharvest handling and of farmers using different irrigation water qualities. The risks of Ascaris and rotavirus infections recorded for consumers of irrigated lettuce were attributed mainly to the contamination of the lettuce on farm and to a lesser extent on postharvest handling. Such contamination was attributed to common irrigation practices involving watering cans and the application of fresh poultry manure (Amoah *et al*., 2008).

Current on farm arrangements do not allow any time lapse for pathogen die-off between the last irrigation/manure application and harvesting attention should also be given to soil contamination. To reduce the risk for consumers, an analysis of the marketing and contamination pathways from irrigation water source to farm as well as whole sale market, retail and consumer outlets showed key entry points for risk-reducing interventions. These are (a) the farm, where by far the most contamination takes place and (b) the kitchen, where most salads are eventually prepared for sale/consumption (Drechsel *et al*., 2014).

2.8.1. Risks to consumers of vegetables eaten uncooked bacterial infections and helminthes infections

There are several studies of the risk of specific bacterial disease associated with consumption of vegetable crops irrigated with untreated wastewater. The most important microbes causing infections or epidemics through drinking water include the bacteria *Campylobacter* spp., *Escherichia coli*, *Salmonellas.*, *Shigella* spp., *Vibrio cholera* and *Yersinia enterocolitica*, viruses such as: adeno, entero-, hepatitis A- and E-, noro-, sapo- and rotaviruses and the protozoa: *Cryptosporidium parvum, Dracunculus medinensis, Cyclospora cayetanensis, Entamoeba histolytica, Giardia duodenalis* and *Toxoplasma gondii* (Ari, 2015).

Blumenthal *et al*. (2003) in Mexico indicated that there was a two-fold or greater risk of Diarrheal disease associated with medium or high frequencies of consumption of uncooked onions irrigated with wastewater that had been stored. Descriptive studies of the association between consumption of uncooked vegetables irrigated with untreated wastewater and Ascaris infection produced estimates of relative risks between 7.0 and 35 (Amoah, 2008).

The proportion of Ascaris infection in the study populations that was attributed to consumption of uncooked vegetables irrigated with wastewater (the attributable risk) varied between 34% and 60%. The attributable risk from consumption was 25% for children and 14% for adult men. There is some evidence in adult men that consumption of vegetables irrigated with wastewater had a greater effect when irrigation was with untreated wastewater. In children, irrigation of vegetables with untreated wastewater was associated with an increased risk of infection (Amoah, 2008).

2.8.2. Disease burden via drinking water

Disease causing organisms (pathogens) transmitted via drinking water are predominantly of faecal origin (and therefore known as enteric pathogens). Yet the drinking water associated outbreak of cholera in Germany during 1892 was the foundation point of our modern understanding of waterborne pathogens. The World Health Organization (WHO) estimates that about 1.1 billion people globally drink unsafe water and the vast majority of diarrheal disease in the world (88%) is attributable to unsafe water. a typical rural village water supply system, which is prone to faecal contamination from domestic animals and human excreta, due to the interactions between exposure to enteric pathogens via poor quality water, lack of sanitation and inadequate hygiene. Various estimates in North America suggest up to 15–30% of gastrointestinal disease may come via water, with a similar component via food (Nicholas, 2004).

2.8.3. Triggering of antibiotic resistance dissemination

A major driver for the dissemination of antibiotic resistance is supposed to be the use of antibiotics, which, between the years of 2000 and 2010, increased in 36%, with major contributions from Brazil, Russia, India, China, and South Africa (Celia *et al*., 2016). So the wastewater biological treatment process offers a suitable environment for the development of resistance due to the presence of sub-inhibitory concentrations of antibiotics. Consequently, the process makes it easy for bacteria to acquire resistance and enables the transference of antibiotic resistance genes (Luisa, 2016).

Several research studies have reported the occurrence of antibiotic resistant organisms in environmental samples and advocated a global public health concern due to these Bacteria. Ash *et al*., (2002) have studied the prevalence of antibiotic resistance of gram-negative bacteria in major rivers of United States. Studies have shown that highest ARB and ARGs were observed in hospital. biofilms, followed by activated sludge of municipal sewage, then surface water and then drinking water. Resistant bacteria can also be found in high numbers in lakes. In a study of two Spanish lakes, 71% of isolates were resistant to at least one antibiotic including erythromycin (31.1%), tetracycline (17.8%), Chloramphenicol (22.2%), and penicillin (68.9%). Populations` especially in rural areas, rely on untreated groundwater for their water supplies. Few studies have been done to determine the antibiotic resistance of isolates from ground water (Mariya, 2010).

2.9. Antibiotics in the environment

Antibiotic classes of compounds frequently used in agriculture include tetracycline, amino-glycosides, cephalosporin, macrolides, and fluoroquinolones, and sulfonamides (Christian *et al*., 2003). Antibiotic medicines have been shown to be released to soils and to persist in the environment. A study group indicated the potential for a range of veterinary medicines to be taken up from soil by plants used for human (Boxall *et al*., 2006). Different studies have been conducted to determine the presence of antibiotics in the soil, biosolids and manure samples. Indeed, tetracycline concentrations in the range of several hundred micrograms per kilogram have been detected in soil some months after manure application (Kummerer, 2004). Along with inappropriate use of antibiotics in human medicine, higher practice of growth promoters in the agricultural industry has given rise to bacterial resistant. Intensive animal production involves giving livestock animals large quantities of antibiotics to promote growth and prevent infection Wastewater Irrigated Vegetable Production (Kummerer, 2004).

2.10. Treatment strategies for microbial-contaminated water and wastewater

The two fundamental reasons for the treatment of water and wastewater are to safeguard public health and prevent the pollution of receiving water. The strategies for the treatment of microbial-contaminated water and wastewater are grouped into two: physical (solar radiation and ultraviolet radiation) and chemical disinfection (chlorination, chloramination, ozonation)

processes. Each of these processes involves the destruction or inactivation of microorganisms. Although, no disinfection process is known to provide 100% effectiveness, any good process should be characterized by the ability to penetrate and destroy pathogenic organisms during water treatment process and should be harmless to humans and the environment. It must be cheap to acquire and maintain, easy and safe to handle, store and transport. In addition, during the disinfection process, toxic residuals, mutagens and carcinogens should not be produced (Mark W*et al*., 2004)

2.10.1 Physical disinfection processes

The two main physical disinfection processes are solar and ultraviolet (UV) radiations. **Ultraviolet** disinfection involves the exposure of contaminated water to UV light that has been generated by an electronic discharge through mercury vapour. The radiations from the UV light penetrates cells of pathogenic organisms in water and destroys their cell genetic material and effective means of inactivating virus, spores and cysts, thereby preventing reproduction. Because UV radiation is not associated with the production of harmful by-products and known to improve the taste, color and odour of wastewater, it is considered a safe method of primary disinfection(Tomilola *et al*., 2014).

A **solar** disinfection method is a thermal inactivation and photo-oxidation used to inactivate pathogenic organisms in contaminated water of low turbidity. This method involves the use of the synergetic combination of optical and thermal processes for the treatment of contaminated water that is already contained in a transparent container for the inactivation of microorganisms such as bacteria, virus and protozoa. It is one of the simplest, inexpensive treatments available. It entails filling plastic containers with contaminated water and exposing to the sun for a whole day, thereby exposing microbial cells to ultraviolet radiation from sunlight, which leads to the destruction of their DNA. It is effective against bacteria, protozoa and viruses, its proven reduction of diarrheal disease incidence in users and acceptability to users due to its simplicity and low cost(Tomilola *et al*., 2014).

2.10.2 Chemical disinfection processes

There are three commonly used chemical disinfection processes: chloramination, chlorination and ozonation. **Chloramination**, refers to the use of chlorine and ammonia to disinfect water and is considered one of the best technologies for water disinfection. Chloramines are formed

from the combination of chlorine and ammonia. The disinfection method is by the frequent addition of ammonia to water that already contains free chlorine at pH 8.4. The method is effective and kills microbial cells by penetrating their cell walls, thereby obstructing their metabolism. Chloramination has the ability to improve the odour, taste, smell and flavor of water and it remains active for a very long period of time and less effective in killing some pathogenic microorganisms(Tomilola *et al*., 2014).

Chlorination, the method involves the use of chlorine to disinfect water. The chlorine could either be in solid (calcium hypochlorite) or liquid (sodium hypochlorite) and gas. When added to water, chlorine helps in the reduction of pathogens by affecting the reproduction and metabolism of microorganisms present in the water. This method involves introduction of chlorine, (about 0.2 – 0.4 mg/L) into contaminated water at pH of between 5.5–7.5.Chlorinationdisinfection method is a well-established technology and leaves a residual behind, which can prolong disinfection after the treatment process, thereby preventing microbial regrowth (Tomilola *et al*., 2014).

Ozonation, it involves the use of ozone for disinfection. Ozone is considered a very effective oxidizing agent for the treatment of water. The method is indicated to have good bactericidal and virucidal activities. It is considered the most effective disinfectant that can kill microorganisms including bacteria, viruses and protozoans. Ozone is considered as the most powerful oxidant which can inactivate microorganisms in contaminated waters and it is more effective than chlorine. When compared to chlorination and chloramination, it is considered more effective in killing microorganisms and fast in activity. It is also not associated with the production of harmful by-products and helps in the removal of taste and odour from water(Tomilola *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in Hasassa, westArsi zone in Oromia Regional State of southeastern Ethiopia. It is located at 7°10′N39°10′E / 7.167°N39.167°E (figure 2). According to CSA (2008), a survey of the land in this woreda shows that 76.9% is arable or cultivable, 17.3% pasture, 0.4% forest, and the remaining 5.4% is considered swampy, mountainous or otherwise unusable. Industry in the woreda includes two edible oil mills, four flour factories, one bottled water factory, four wheat mill factories and one brick factory. Hasassa River starts from the center of the city, used for range of activities such as recreation, bathing, washing clothes, and household utensils, small scale agricultural irrigation, car washing and other uses that enters Melka Wakena Dam

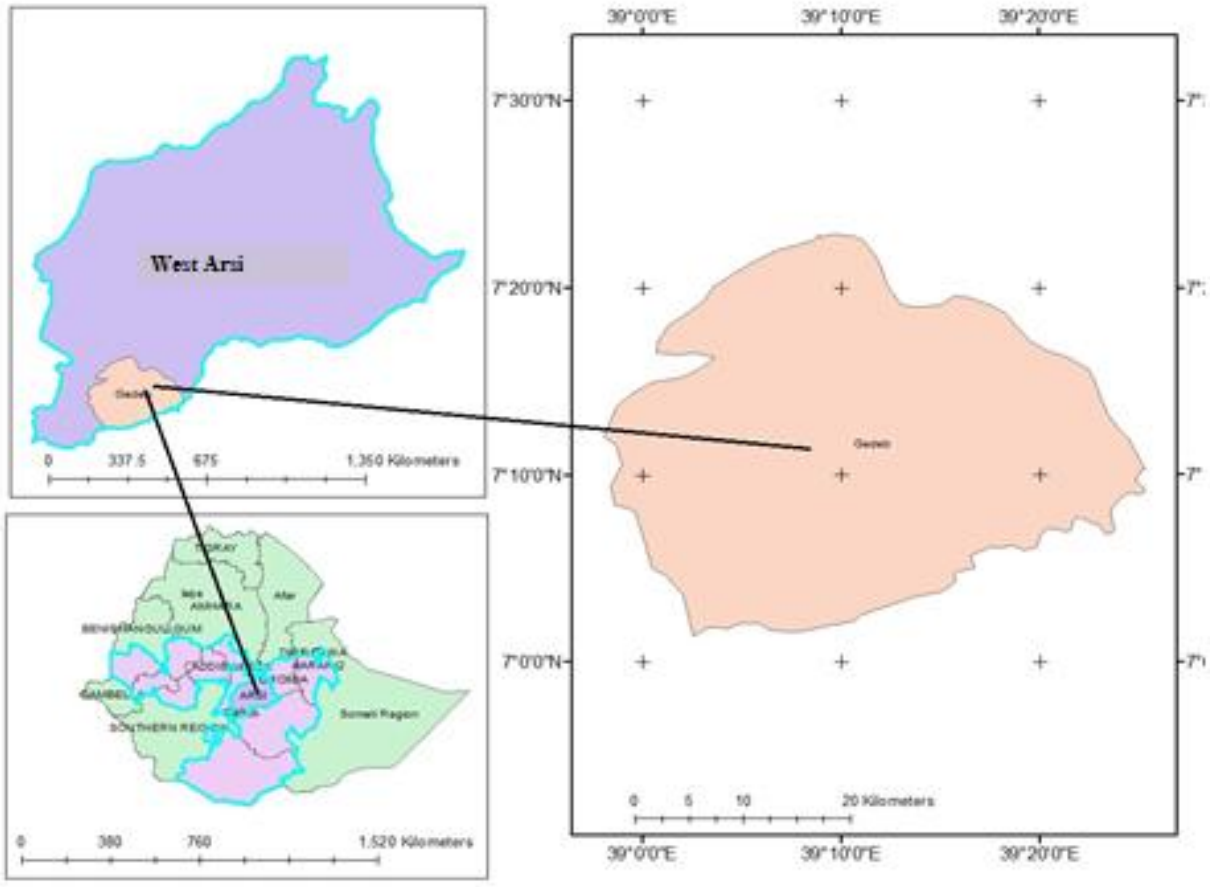


Figure 2.Map of study area with its longitude and latitude during April –July, 2018

3.2. Sample collection

Laboratory based cross sectional study design was used. A total number of 12 samples including three each vegetable(lettuce, garlic, and carrot) was randomly picked from both leaf and root aseptically cut in to pieces by using 90% ethanol sterilized scissors. All vegetable samples were collected aseptically in sterilized plastic bags and transported to Adama Science and Technology University. The samples were processed for bacteriological analysis within 1–8 hrs.

3.3. Samples from irrigation water

Three irrigation water samples were collected from Hasassa River at different sites. A total of 0.75L water samples were collected at the open surface flowing along the river course at the same time of the irrigation period. Samples were aseptically collected in sterile glass bottles maintained at 4°C in a cooler box and taken to the laboratory of Microbiology, school of Applied Natural Science, Adama Science and Technology University

3.4. Sample processing

Each vegetable samples (unprocessed and large sized) were aseptically chopped into smaller pieces. A 25 g of subsample of each vegetable were aseptically weighed and vigorously shaken in 225 ml of sterile 0.1% (w/v) peptone water for 3 min to homogenize the samples. They were prepared to appropriate serial dilutions from 10^{-4}, 10^{-5} and 10^{-6}. They spread-plated on a suitable solid media and incubated aerobically at 37°C for 24–48 hours. The same process was done for water samples.

3.5. Bacterial counts

A volume of 0.1 ml aliquot of appropriate dilution was spread-plated in duplicates on pre-solidified Plate Count Agar, MacConkey agar and Mannitol Salt Agar for counts of aerobic Mesophilic bacteria, Enterobacteriaceae and staphylococci, respectively. Homogenized samples were heated at 80°C for 10 minutes in a water bath to count aerobic spore formers. The inoculated plates were incubated at 32°C–37°C for 24–48 hrs. The mean colony counts on each given dilution were used to estimate the total viable count for the samples in colony forming units (CFU ml^{-1}). Liquid sample to be tested was diluted serially and inoculated in

lactose broth, The number of total Coliforms was determined by counting the number of tubes giving positive reaction (both color change and gas production) compared with standard statistical tables and the results were expressed as MPN 100 ml^{-1}.

3.6. Isolation and characterization of dominant Microflora

After enumeration of aerobic Mesophilic bacteria, 21 colonies with distinct morphological characteristics of colonies such as color, size, and shape were randomly picked from countable plates and aseptically transferred into a tube containing 5 ml nutrient broth and incubated at 30°C for 24–48 hr. The cultures were purified by repeated plating and pure cultures were temporarily preserved on nutrient agar slants at 4°C.An overnight activated culture of each isolates were further characterized by inoculating to the following standard tests such as cell morphology, gram staining, motility, catalase, Indole Test, Urease Test, Carbohydrate fermentation/Utilization test, Hydrogen Sulfide production (H_2S), Methyl Red (MR) test, Citrate Utilization test, Oxidation Fermentation (O/F), (Oluyege *et al*.,2015) to differentiate into genus and family levels. The characterization and identification of bacteria was done with the help of Bergey's Manual of Systematic Bacteriology.

3.7. Isolation and identification of some bacterial pathogens

For the identification of *Staphylococcus aureus*, yellow colonies on Mannitol Salt Agar plates were aseptically picked and transferred into 5 ml nutrient broth and incubated at 37°C for 24–36 hr for further purification. Then, a loop of culture from the nutrient broth was streaked on pre-solidified surface of nutrient agar supplemented with 0.75% sodium chloride and again incubated at 37°C for 24–36 hrs. Finally, the distinct colonies were characterized using the established microbiological methods such as gram staining and preliminary biochemical tests (the catalase).

For Isolation of *Salmonella* spp. 25g or 25ml of each sample was aseptically transferred into sterile flask, containing 225 ml buffered peptone water (BPW), and inoculated onto the Salmonella-Shigella (SS) agar and incubated at 35 °C for 24 h. The presumptive *Salmonella* colonies were then sub-cultured by streaking onto the fresh SS agar and incubated for 24 h at 37°C.

Since the selective media was used for the isolation, the presumptive *Salmonella* isolates were identified by two confirmatory biochemical tests, triple-sugar-iron (TSI) agar test and the urease test. The presumptive *Salmonella* colonies were directly stabbed into the TSI agar slant and incubated with loosened caps for 24 h at 37°C. For the urease test, two loopful of pure and well-isolated *Salmonella* colonies were inoculated into the urea broth. The inoculated tubes were incubated with loosened caps for 48 h at 35 °C in an incubator. The TSI agar was checked for the production of hydrogen sulphide (H_2S) gas, while the urease test was checked for the degradation of urea.

3.8. Antimicrobial susceptibility testing

Once the bacteria were isolated and identified from each sample, the standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profiles of the isolates. Bacterial inoculums were prepared by suspending the freshly grown bacteria in 4–5 ml sterile nutrient broth and the turbidity was adjusted to that of a 0.5 McFarland standard prepared from adding 0.5 mL of 1.0% $BaCl_2$ to 99.5 mL of 1% H_2SO_4 solution. The antimicrobial susceptibility testing was performed using Mueller-Hinton agar medium against the following antibiotics namely,Chloramphenicol30µg, penicillin g 10µg, Vancomycin30µg, erythromycin 15µg, Perfloxacin5µg, ampicillin (10 µg) obtained from pharmacy. The bacterial suspension was aseptically streaked uniformly on the entire agar surface using sterile cotton swab in three different directions by rotating the plates at 60° angles after each streaking. The Petri plates were left to dry for 20min. Afterwards, the antibiotics containing discs were placed on the agar surface with sterile forceps and pressed carefully down to ensure contact. The plates were incubated aerobically at 35°C for 18–24 hours. The plates were examined after incubation for diameters of zone of inhibition around the discs, which was measured using Ruler (mm). The data obtained was interpreted with reference to CLSI (2013). The criteria used to select the antimicrobial agents tested in this study were based on availability and frequency of prescription of the drugs for the management of bacterial infection in Ethiopia. *E. coli* (ACC25922) and *S. aureus* (ATCC6538) standard strains from Ethiopian Institute of Biodiversity were used as reference strains for quality control of the antibiotics used.

3.9. Data analysis

Bacterial counts were calculated as colony forming units per milliliter (CFU ml^{-1}) and converted into log10 values before computing means and standard deviations. total coliform was expressed by MPN100 ml^{-1}. Then Mean of Bacterial counts and standard deviation was calculated using Microsoft Excel. The statistical analysis was performed by single factor (ANOVA) comparisons to derive statistical differences ($p<0.05$) of microbial levels between all the studied samples

4. RESULTS

4.1. Bacterial counts

The mean bacterial counts (log CFU ml^{-1}) of aerobic Mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, and staphylococci of the three vegetable samples were, 7.62, 5.59, 6.58 and 6.38 log CFU ml^{-1} for carrot; 8.12, 6.58, 6.88, and 6.88 for lettuce; 6.76, 5.95, 5.68, and 6.12 for garlic; 7.09, 6.05, 6.77, and 6.40 log CFU $ml^{-1}$for water, respectively (Table 1).The average microbial counts of different vegetable samples of selected sites ranged from 5.59 to 8.21 log CFU ml^{-1}.

The minimal and maximal counts of aerobic Mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, and staphylococci obtained from three different vegetable samples and irrigation water was in the range of 6.2 and 8.32, 5.48 and 6.99, 5.43 and 7.45, and 5.72 and7.48 log CFU ml^{-1}, respectively. The highest mean count of AMB was (8.21 log CFU ml^{-1}) and the mean of staphylococci count was also relatively higher (6.88 log CFU ml^{-1}) in lettuce samples. In addition, MPN of total Coliforms count and their overall mean in vegetables ranged from 191 to >1100 and in irrigation water total coliform was >1100.

Mean counts of all bacterial groups differed significantly at ($p< 0.05$) between the deferent sample types with the highest mean count in lettuce samples (table 1).The Total Coliforms of the different samples differ significantly at ($p< 0.05$)

Table 1.Bacterial counts from irrigation water and vegetable samples irrigated with Hasassa River during April-July, 2018

samples type	Average of total viable bacteria in log of CFU mL^{-1}± S.D				
	AMB	Enterobacteriaceae	ASF	staphylococci	Total coliform by MPN$100ml^{-1}$
Carrot	7.62 ± 0.40	5.59± 0.11	6.58 ± 0.58	6.38 ± 0.7	>1100 ± 0
Lettuce	8.21± 0.12	6.58± 0.58	6.88 ± 0.49	6.88 ± 0.52	813 ± 496.5
Garlic	6.76± 0.55	5.95± 0.66	5.68 ± 0.45	6.12 ± 0.57	191 ± 84.9
Irrigation water	7.09± 0.16	6.05± 0.49	6.77 ± 0.53	6.40 ± 0.61	>1100±0

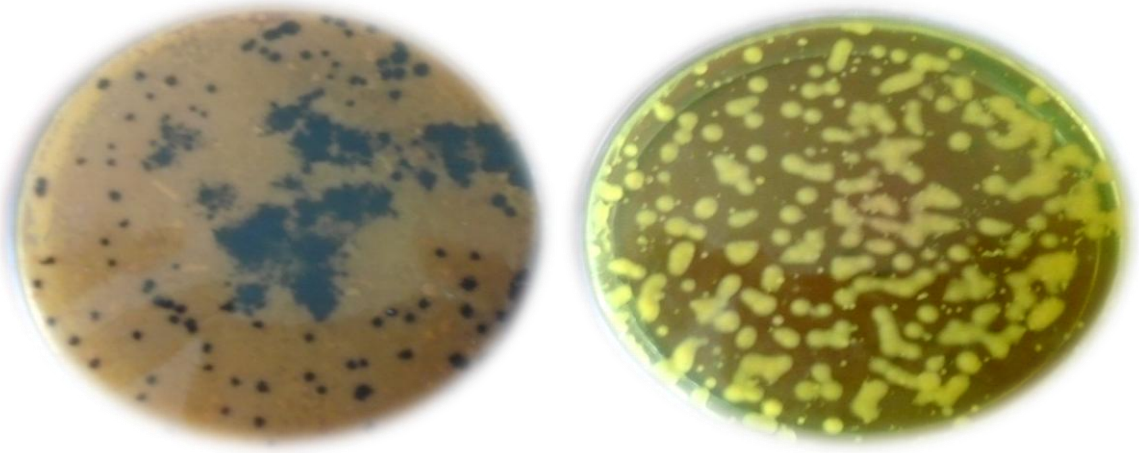
Note: AMB, Aerobic Mesophilic Bacteria; ASF, Aerobic Spore Formers

4.2. Analysis of Microflora and some pathogens

In this study of a total of eight genera of gram positive and gram-negative bacteria spp. were identified (Table 4).Aerobic Mesophilic bacterial flora identified from the water samples and different vegetables collected from the downstream Hasassa river was dominated by *Bacillus* spp. (21.88%), *Corynebacterium* spp. (12.5%), *Lactobacillus* spp. (12.5%), *Streptococcus* spp.(6.25%), *E. coli*. (6.25%), and *Neisseria* spp. (3.13%). Similarly, the most predominant genera in lettuce, carrot, garlic, and irrigation water samples were *Bacillus* spp. (57.1, 14.3, 31.7, and 0%), *Corynebacterium* spp.(0, 25, 25, and 50.0%), *Lactobacillus spp*. (50, 25, 25, and 0%), *Streptococcus* spp. (0, 50, 0, and 50%), *E. coli*. (0, 50, 0,and50.0%), and *Neisseria* spp. (100, 0, 0, and 0%), respectively. Lettuce were the vegetables most contaminated, followed by the samples of carrots, irrigation water and garlic (11, 8, 7, 6) respectively.

The Incidence of some pathogen such as *Staphylococcus aureus* ferments Mannitol and producing a yellow zone surrounding colony on Mannitol Salt Agar plates and isolated pure colonies confirmed by salt tolerance and catalase test. colonies displayed typical *Salmonella* morphological characteristics on Salmonella-Shigella agar, which were clearly with a black spot in the center due to H_2S gas production on Salmonella-Shigella agar (Figure 3).The presumptive colonies were scored to be *Salmonella* after the colonies showed positive reaction to triple sugar iron test and negative to urease test.

Staphylococcus aureus and *Salmonella* which isolated by special selective media was 12 bacteria with Percentage distribution of 21.88% and 15.6%, of total isolates respectively (Table 2). The distribution of these pathogens varied depending on the nature of vegetables. With regard to sample types, *Staphylococcus aureus* was equally distributed in lettuce (28.6%), garlic(28.6%), and irrigation water (28.6%), but relatively lower in carrot (14.2%). Higher Percentage of *Salmonella* spp. was encountered in lettuce (40.0%) but it was lower occurrence and equally distributed in irrigation water, carrot and garlic (20.0 %,) (Table 2). The Incidence of these all bacterial Microflora and pathogens was not significantly different at ($p< 0.05$) with respect to sample types.



Black colonies on Salmonella-Shigella agar yellow colonies on MSA agar

Figure 3.Presumptive salmonella on ss. agar and staphylococcus aureus on Mannitol Salt Agar from Hasassa Irrigation River and irrigated vegetables during April-July, 2018

Table 2.Percentage distribution of bacteria isolated from irrigation water and vegetables in Hasassa during April –July 2018

Isolates	Samples					
	Irrigation water	Carrot	Garlic	Lettuce	Total isolates	% Incidence
E. coli	1(50)	1(50)	-	-	2(100)	6.25
Bacillus spp.	-	2(31.7)	1(14.3)	4(57.1)	7(100)	21.88
Corynebacterium spp.	2(50)	1(25)	1(25)	-	4(100)	12.5
Streptococcus spp.	1(50)	1(50)	-	-	2(100)	6.25
Staphylococcus aureus	2(28.6)	1(14.2)	2(28.6)	2(28.6)	7(100)	21.88
Salmonella spp.	1(20)	1(20)	1(20)	2(40)	5(100)	15.6
Neisseria spp.	-	-	-	1(100)	1(100)	3.13
Lactobacillus spp.	-	1(25)	1(25)	2(50)	4(100)	12.5
Total	7(21.88)	8(25)	6(18.75)	11(34.37)	32(100)	100

4.3. Frequency of isolates in the samples

The frequency of occurrence of bacterial isolates on different selected fresh vegetables and irrigation water was showed that *Staphylococcus aureus and salmonella* occurred most frequently 4(4) 100% in all examined samples (irrigation water, carrot, garlic and lettuce).followed by *Bacillus* spp .,*Corynebacterium* spp .*And Lactobacillus* spp.3(4)75%), *Escherichia coli* and *Streptococcus* spp. 2(4)50% and *Neisseria* spp. Which have the least occurrence 1(4)25%).

Table 3. Frequency of occurrence of bacterial isolates from irrigation water and vegetables in Hasassa during April-July, 2018

Isolates	Irrigation water	Carrot	Garlic	Lettuce	IF%
Bacillus spp.	-	2	1	4	3(4) 75%
E. coli	1	1	-	-	2(4) 50%
Corynebacterium spp.	2	1	1	-	3(4) 75%
Streptococcus spp.	1	1	-	-	2(4) 50%
Lactobacillus spp.	-	1	1	2	3(4) 75%
Neisseria spp.	-	-	-	1	1(4) 25%
Staphylococcus aureus	2	1	2	2	4(4) 100%
Salmonella spp.	1	1	1	2	4(4)100%

Note: IF=Isolate Frequency

Table 4.Biochemical characterization of isolates from irrigation water, and vegetables in Hasassa during April-July, 2018

Isolates code	Gram stain	Catalase	Citrate	Indole	Methyl	Urease	Motility	Oxidative	Fermentative	Glucose	Lactose	Sucrose	Gas	H_2S	Identified bacterial Isolates
LETS	+ Rod	+	-	-	-	-	+	+	-	+	-	-	-	-	*Bacillus* spp.
ONIS	+ Rod	-	-	-	+	-	-	-	+	+	-	-	-	-	*Lactobacillus* spp.
WATS	+ Rod	+	-	-	+	-	-	-	+	+	-	-	-	+	*Corynebacterium* spp.
WATS	+ cluster	+	-	-	+	-	-	-	+	+	-	-	-	-	*Staphylococcus aureus*
CARS	- Rod	+	-	+	+	-	+	-	+	+	+	-	+	-	*E. coli*
LETS	+ Rod	-	-	-	-	+	-	-	+	+	-	-	-	-	*Lactobacillus* spp.
WATS	- Rod	+	-	+	+	-	+	-	+	+	-	-	+	-	*E. coli*
WATS	+ Cocci	-	+	-	+	-	-	-	+	+	-	-	-	-	*Streptococcus* spp.
LETS	- Cocci	+	+	-	-	-	-	-	+	+	-	-	+	-	*Neisseria* spp.
CARS	+ Cocci	-	+	-	-	-	-	-	+	+	-	-	-	-	*Streptococcus* spp.
LETS	+ Rod	+	+	-	-	-	+	+	-	+	-	-	-	-	*Bacillus* spp.
ONIS	+ Strep	+	-	-	+	+	-	-	+	+	-	-	-	+	*Corynebacterium* spp.
CARS	+ Rod	+	-	-	+	+	-	-	+	+	-	-	-	+	*Corynebacterium* spp.
CARS	+ Rod	+	+	-	-	-	+	+	-	+	-	-	-	-	*Bacillus* spp.
CARS	+ Rod	-	-	-	+	-	-	-	+	+	-	-	-	-	*Lactobacillus* spp.
CARS	+ Rod	+	+	-	+	-	+	-	+	+	-	-	-	-	*Bacillus* spp.
WATS	+ Rod	+	-	-	+	-	-	-	+	+	-	-	-	+	*Corynebacterium* spp.
ONIS	+ Rod	+	+	-	-	-	+	-	+	+	-	-	-	-	*Bacillus* spp.
LETS	+ Rod	-	-	-	+	-	-	-	+	+	-	-	-	-	*Lactobacillus* spp.
LETS	+ Strep	+	+	-	-	-	+	+	-	+	-	-	-	-	*Bacillus* spp.
LETS	+ Rod	+	-	-	-	-	+	+	-	+	-	-	-	-	*Bacillus* spp.

Note: += positive, - = negative

4.4. Antimicrobial resistance patterns of bacterial isolates

Table 5 presents the number of isolates that were resist to different antibiotic of the thirty-two isolates. the highest 32(100%) isolates were resistant to penicillin g10µg, 26(81.25%) to vancomycin30µg, 23(71.9%) toampicillin10µg, 15(46.9%) to chloramphenicol 30µg, 15(46.9%) to erythromycin 15µg, and the least 3 (9.4%) to perfloxacin 5µg.

Table 5.Distribution and proportion of antibiotic resistance among bacterial isolates from vegetable samples and irrigation water in Hasassa during April-July, 2018

Identified Isolates	Total (%)	Vancomycin 30µg	Chloramphenicol 30 µg	Perfloxacin 5 µg	Penicillin g 10 µg	Ampicillin 10 µg	Erythromycin 15µg
Bacillus spp.	7(100)	5(71.4)	2(28.6)	-	7(100)	4(57.1)	1(14.3)
E. coli	2(100)	2(100)	2(100)	1(50)	2(100)	2(100)	1(50)
Corynebacterium spp.	4(100)	3(75)	1(25)	1(25)	4(100)	3(75)	-
Streptococcus spp.	2(100)	1(50)	2(100)	-	2(100)	2(100)	-
Lactobacillus spp.	4(100)	3(75)	3(75)	1(25)	4(100)	1(25)	3(75)
Neisseria spp.	1(100)	1(100)	1(100)	-	1(100)	1(100)	1(100)
Staphylococcus aureus	7(100)	6(85.71)	3(42.86)	-	7(100)	7(100)	6(85.71)
Salmonella spp.	5(100)	5(100)	1(20)	-	5(100)	3(60)	3(60)
Total	32(100)	26(81.25)	15(46.9)	3(9.4)	32(100)	23(71.9)	15(46.9)

4.5. Multiple drug resistance patterns of bacterial isolates

Patterns of multiple drug resistance (MDR) among bacterial isolates varied from two to six antibiotics(Table 6). The maximum MDR registered was resistant to 6antibioticsVancomycin/ Chloramphenicol/ Penicillin/ Erythromycin/ Ampicillin/ Perfloxacilin, followed by 5 with the combination of (Vancomycin, Chloramphenicol, Penicillin, Erythromycin, and Ampicillin). The highest MDR resistance patterns were observed in *bacillus spp, staphylococcus aureus*

and *salmonella spp* respectively, with combination of the following antibiotics (Van, ampic), (Penic, ampic), (Van, peni).

Table 6.Multiple drug resistance patterns in isolates identified from irrigation water and vegetables in Hasassa during April-July, 2018

Isolates	No of isolates that demonstrated resistance	Antibiotic resistance pattern observed	No of Antibiotics
Bacillus spp.	1(7)	Van, chlora, peni, eryth, ampic	5
	2(7)	Van, chloram, Penic,ampic	4
	4(7)	Van, peni,ampic	3
	5(7)	Van,ampic	2
E. coli	1(2)	Van,chlora,peni,eryth,ampic,perf	6
	2(2)	Van, chlora,penic,ampic	4
	2(2)	Van,Penic	2
Corynebacterium spp.	1(4)	Van, perf,peni,chlora,ampic	5
	3(4)	Ampi,peni,van	3
Streptococcus spp.	1(3)	Van,peni,ampic	3
	2(3)	Penic,ampic	2
Lactobacillus spp.	1(4)	Van,chlora,peni,eryth,ampic,perf	6
	3(4)	Van,chlora,peni,eryth	4
Neisseria spp.	1(1)	Van,chlora,peni,eryth,ampic	5
Staphylococcus aureus	2(7)	Van,chlora,peni,eryth,ampic	5
	4(7)	Eryth,peni,van,ampic	4
	5(7)	Penic,ampic	2
Salmonella spp.	1(5)	Van,chlora,peni,eryth,ampic	5
	3(5)	Eryth, ampic, Van,peni	4
	5(5)	Van, peni	2

Van = Vancomycin, peni = penicillin, ampic = ampicillin, chlora= Chloramphenicol, Perf = Perfloxacilin, eryth = erythromycin

5. DISCUSSION

The highest bacterial load in lettuce samples probably due to the larger surface area exposed to irrigation water. This in line with the work of Halablab, (2011) who reported that lettuce carried higher incidence of *E. coli* and *S. aureus* organisms (42.30 and 50%) than parsley samples (13.80 and 37.93%), and the higher microbial loads on lettuce samples than parsley counterpart may be due to the large surface area of the former leaves. Other study also showed that high bacteria counts could likely be associated with the morphology of leaves which have a broad and rather rough surface, indeed, in both vegetables their large surface made easily coming in contact with the ground and the irrigation water (Cinzia *et al*., 2015).

The overall mean aerobic Mesophilic count observed in the present study ranged from 6.76 to 8.21 log CFU ml^{-1}, relatively higher than previous reports (6.94 to 8.06 log CFU ml^{-1}) from Ethiopia, by Desta and Diriba,(2016). but, lower bacterial counts that ranged from 6 to 7 log CFU ml^{-1}were reported by Daniele *et al*. (2013). According to Hazard Analysis and Critical Control Points-Total Quality Management (HACCPTQM) Technical Guidelines, (1998) the microbial quality for raw foods, belong to the category of poor and spoiled food.

The overall mean count of Enterobacteriaceae in the present study ranged from 5.56 to 6.88 log CFU ml^{-1}. This was lower than other study conducted in Ethiopia by Desta and Diriba (2016) on lettuce, carrot and tomato which ranged from 6.09 to 7.10 log CFU g^{-1},but higher than the microbial load of lettuce and green pepper 5.08 and 4.84 log CFU g^{-1}, respectively reported by Biniam and Mogessie, (2010) in Ethiopia. According to (Gilbert *et al*.,2000) guideline recommended for fresh fruit and vegetables in London, overall mean counts log CFU $g^{-1)}$ of Enterobacteriaceae in carrot (7.10), cabbage (6.70), tomato (6.24), and lettuce (6.09) revealed unsatisfactory level (≥4 log CFU g^{-1}). Biniam and Mogessie, (2010) suggested that the high level of Enterobacteriaceae in vegetables might indicate that the water used for irrigation could be heavily contaminated with fecal matter from sewerage effluent.

In case of aerobic spore formers, the overall mean counts ranged from 5.68 to 6.88 log CFU ml^{-1}. In all vegetables the counts were higher compared to reports by Biniam and Mogessie,(2010) where the counts ranged between 3.47 and 3.50 log CFU ml^{-1} in green pepper and lettuce, respectively, from Addis Ababa,

In the present study, the overall mean count of staphylococci from vegetable samples ranged from 6.12 to 6.88 log CFU ml^{-1}. This is higher than the microbiological studies made on lettuce and green pepper from super market in Addis Ababa, Ethiopia, by Biniam and Mogessie, (2010), they reported bacterial count of 4.55 and 4.97 log CFU ml^{-1}, respectively. Higher counts of staphylococci from this study may be due to skin contact and environmental contamination. Halablab (2011) concluded that *Staphylococcus aureus* is a dangerous pathogen that surface of vegetables may be contaminated through human handling and other environmental factors and is able to survive for several weeks. Human skin and nasal cavity are the main reservoir of staphylococci.

The overall mean counts of total Coliforms from vegetable samples in the present study were relatively lower except for carrot >1100 MPN 100 ml^{-1} than the report of Nipa *et al*.,(2011), who observed >1100 MPN 100 $ml^{-1}$from salad vegetables. The observed difference in the counts could be attributed in part to the degree of original contamination, storage conditions, and the hygienic conditions of utensils and vegetables handlers. The total coliform counts from water samples in the present study were >1100 MPN 100 mL^{-1} which was higher than the WHO recommended standard. According to the standard, the fecal coliform level must not exceed 1000 counts 100 $ml^{-1}$for the safe use of wastewater for irrigation of vegetables. The presence of Coliforms might be attributed to cattle faeces and excretion by farmers and others who use the farm environment as toilet.

Microbial groups that belong to eight genera were isolated from the examined samples at varying percentages with gram positive and gram-negative flora accounting for 75% and 25%, respectively. The gram-positive cells were represented by bacteria from the genera: *Bacillus, lactobacillus* spp., *Corynebacterium* spp., *Staphylococcus aureus,* and *Streptococcus* spp. while the gram-negative microflora constituted members of *Escherichia coli, Neisseria* spp., and Salmonella. This is partly similar to the previous reports by Ankita *et al*. (2014) in raw fruits and vegetables from India.

The predominant microflora of fresh vegetables in the present study was *Bacillus* spp. That occurred in all samples except in irrigation water, followed by *Corynebacterium* spp. and *Lactobacillus* spp. The predominance of Bacillus isolates in this study among the gram-positive bacteria was in agreement with Desta and Diriba, (2016). *Salmonella* spp. isolates

were the dominant bacteria among gram negative isolates contrary to the previous reports by Biniam and Mogessie, (2010) on lettuce and green pepper from Ethiopia which showed the dominance of *Pseudomonas* isolates.

Gram-positive bacteria could be higher effect in the spoilage of vegetables than Gram-negative bacteria. This observation agrees with the findings of Yaji, (2016) who indicated that mainly Gram-positive organisms were responsible for initiating and causing spoilage in cucumber, garden egg and pawpaw fruits. From wastewater-irrigated and manure-treated farmland of Nigeria samples, Oluyege, *et al*. (2015) reported the predominant genera that include *Bacillus* spp. (33%), *Staphylococcus aureus* and *Pseudomonas* spp. (15%). The presence of Endospores that could made more resistant than the vegetative cells to harsh weather conditions and even to antimicrobial treatments may resulted high percentage of *Bacillus* spp.

Corynebacterium spp. is the inhabitants of the soil. Predominance of Corynebacterium in the present study may come from the soil or from feacally-contaminated; water used for irrigation and received sewage from diverse sources. Ankita *et al*., (2014) also reported dominant bacteria such as *Corynebacterium* spp., *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Pseudomonas* spp. respectively, from raw fruits and vegetables. *Lactobacillus spp* are nutritionally fastidious and associated with a variety of plants and animals. Oladele and Olakunle,(2011) reported the presence of *Bacillus subtilis*, *Serratia marcescens*, *Lactobacillus spp* and *Proteus mirabilis* on the deteriorated spinach samples may be linked to the fact that these microbes are widely distributed in air, dusts and soils

The incidence of *Staphylococcus aureus* in the current study was (21.88%) lower than what was mentioned in the study of Halablab *et al*., (2011) who reported higher incidence of *Staphylococcus aureus* (51.5%) from Lebanon. In addition, Ijabadeniyi, (2010) also reported 67.0% in broccoli and 33.0% in cauliflower in South Africa. The presence of *Staphylococcus aureus* (28.6%) in the irrigation water in this study was lower than those obtained by Ikpeme *et al*.,(2011) (25–33%) from two rivers that are used for irrigation of vegetables in South Africa.

In the present study, the incidence of *Salmonella* spp. In all vegetable samples was higher (15.6%) than in other reports by Biniam and Mogessie, (2010) who reported 10% in lettuce and green peppers. This difference might be attributed to the variation of the test techniques

employed (pre-enrichment steps), the origin of sample or geographical differences and difference in management practice. The presence of *E. coli* can represent the existence of fecal pathogens like Salmonella and Shigella. Thus, it can be good indicator of poor sanitary conditions of sources of water used and the use of water with fecal contamination.

In present study another important point was antimicrobial resistance rate was high and this may be causes a serious challenge to the management of common infections. The overall resistance rates among the bacteria isolates remarkably, demonstrated 100% resistance to Penicillin g,81.25% were resistant to Vancomycin, 71.9% to Ampicillin, 46.9% were resistant to Chloramphenicol, 46.9%were also resistant to Erythromycin and 9.4% were resistant to Perfloxacilin.

In other study of two Spanish lakes, 71% of isolates were resistant to at least one antibiotic including Erythromycin (31.1%), Tetracycline (17.8%), Chloramphenicol (22.2%), and penicillin (68.9%) (Mariya, 2010).The antibiotic resistance pattern demonstrated by the bacterial isolates in this study revealed that resistance was highest in all tested antibiotic except Perfloxacilin (9.4%). Golly *et al*., (2016) reported that All the Gram-positive bacteria isolated showed 100% overall total resistance to penicillin, ampicillin and Flucloxacillin. The antibiotic resistance pattern obtained in this study is a serious challenge to public health because of the higher demanding for raw vegetables in different homes, societies and functions.

Twenty different multiple resistance patterns were observed when analyzed with the number of antibiotics (Table 6). Only two isolates (*E. coli* and *Lactobacillus* spp.) were resistant to all six antibiotics tested and six isolates were resistant to only five antibiotics. Highest 55.9% (19) of isolates showed resistance to any of two antibiotics and 41.1% (14) of them were resistant to four antibiotics, 23.53% (8) of them were resistant to Vancomycin, penicillin, ampicillin respectively. Multi antibiotic resistance (MAR) bacteria were isolated and also showed some level of resistance to almost all antibiotics tested. Such multi antimicrobial resistance pattern clearly indicates vegetables were potential vehicle for microbial food poisoning as well as a source of infectious diseases that cannot be treated with commonly used antibiotics.

The resistance ability of the isolates can be transferred from on to another, through the antibiotic resistance plasmids (Olsen *et al.,* 2003).Long ago, bacteria resistant to multiple

drugs were found mostly in hospitals, where antimicrobial agents were used most extensively, however, resistance is currently found anywhere almost as frequently in the community. This is therefore to emphasize the need to be given to good hygienic practices, proper handling, storage and retail of fresh vegetables in a sanitized environment.

6. CONCLUSION

In conclusion, the present study revealed the potential hazard of raw vegetables (lettuce, carrot and garlic) collected from Gedeb Asassa areas, which were irrigated, by Hasassa River. This study was the first to evaluate the microbiological quality of vegetables grown by Hasassa river, where these vegetables harbored high microbial loads including aerobic Mesophilic bacteria, Coliforms, Enterobacteriaceae, aerobic spore formers, and staphylococci might be due to irrigation by untreated contaminated water in that area. The data obtained provide a first-hand indication of which microorganisms was present in fresh vegetables and the potential microbiological risks of these products. The large number of Aerobic Mesophilic bacteria, indicator organisms (Coliforms and *E. coli*) and pathogens (*S. aureus*),*salmonella* spp. detected in the vegetable samples revealed that the contamination of these foods by pathogenic microorganism might present a potential health hazard to consumers in this area. The *in vitro* assay result of the present study showed that irrigation water and samples (lettuce, carrot and garlic) contained bacteria with multiple antibiotic resistance patterns. this may be caused by high concentrations of microorganisms, nutrients, and antibiotics found in contaminated water makes it a favorable environment for bacterial growth and horizontal transfer of resistance genes.

7. RECOMMENDATIONS

- Based on the findings of the present study, the following recommendations were given:
- The traditional identification of bacteria based on phenotypic characteristics is not as reliable as recent identification, which is based on genotypic methods. Therefore, molecular characterization of the isolates is needed.
- Special concern has to be taken to the risk with droppings from wild and domestic animals entering the production area and from fresh manure used as fertilizer.
- Main source of contamination of fresh products whether it was from wind, soil, water, animals and humans have to be further investigated.
- Further studies are required on the microbiological status and survival of various pathogens in the agricultural environment, in/on raw fruits and vegetables
- Further study should be conducted to isolate and characterize different bacterial and fungal species by increasing the sample size because the current study was carried out only on small number of bacteria and small sample size

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APPENDICES

Appendix 1.Analysis of variance of mean counts of four types bacterial groups such as Aerobic Mesophilic bacteria, Staphylococcus, Aerobic spore former and Enterobacteriaceae from vegetables and Hasassa irrigation river, during April –July, 2018

ANOVA:
Single factor
Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	4	29.68	7.42	0.402867
Column 2	4	24.17	6.0425	0.167425
Column 3	4	25.91	6.4775	0.298025
Column 4	4	25.78	6.445	0.100367

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.088725	3	1.362908	5.62788	0.012092	3.490295
Within Groups	2.90605	12	0.242171			
Total	6.994775	15				

Appendix 2.Analysis of variance of the prevalence of all bacterial Microflora and pathogens from vegetables and Hasassa irrigation river, during April –July, 2018

ANOVA:
Single Factor
Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	8	7	0.875	0.696428571
Column 2	8	8	1	0.285714286
Column 3	8	6	0.75	0.5
Column 4	8	11	1.375	1.982142857

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.75	3	0.583333	0.673539519	0.575491	2.946685266
Within Groups	24.25	28	0.866071			
Total	26	31				

Appendix 3.Analysis of variance of Each three sample counts of Total Coliforms of all four samples from vegetables and Hasassa irrigation river, during April –July, 2018

ANOVA:
Single Factor
Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	3300	1100	0
Column 2	3	2440	813.3333	246533.3
Column 3	3	573	191	7203
Column 4	3	3300	1100	0

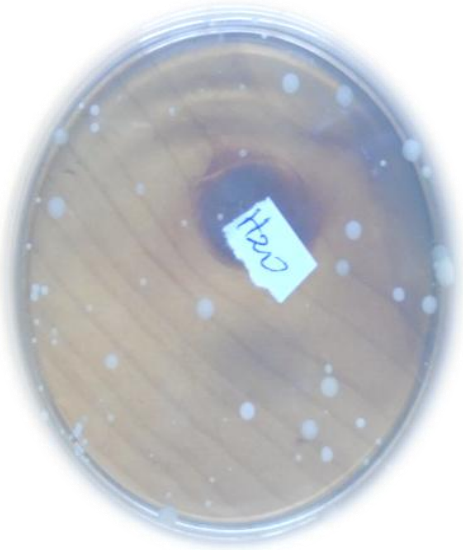
ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1653162.25	3	551054.1	8.687035	0.006747	4.066180551
Within Groups	507472.6667	8	63434.08			
Total	2160634.917	11				

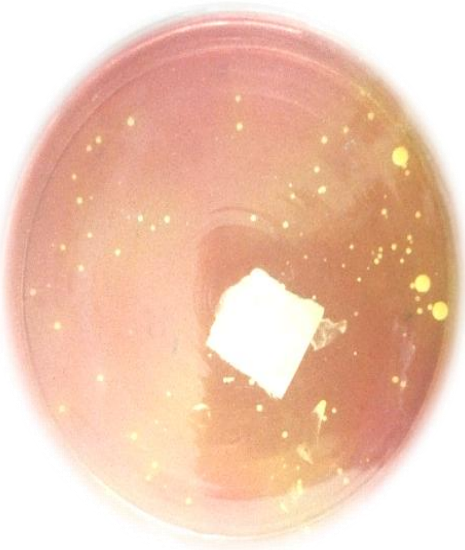
Appendix 4. Farm land around irrigated by the Hasassa river, during April –July, 2018

Appendix 5. Contaminated Hasassa river water used for irrigation purpose, during April – July,

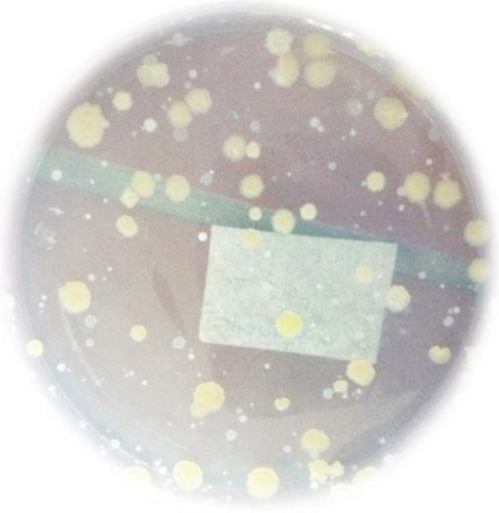
2018



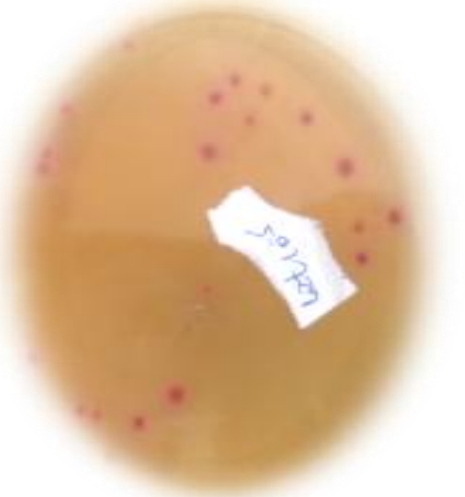
AMB counting



Staphylococcus counting

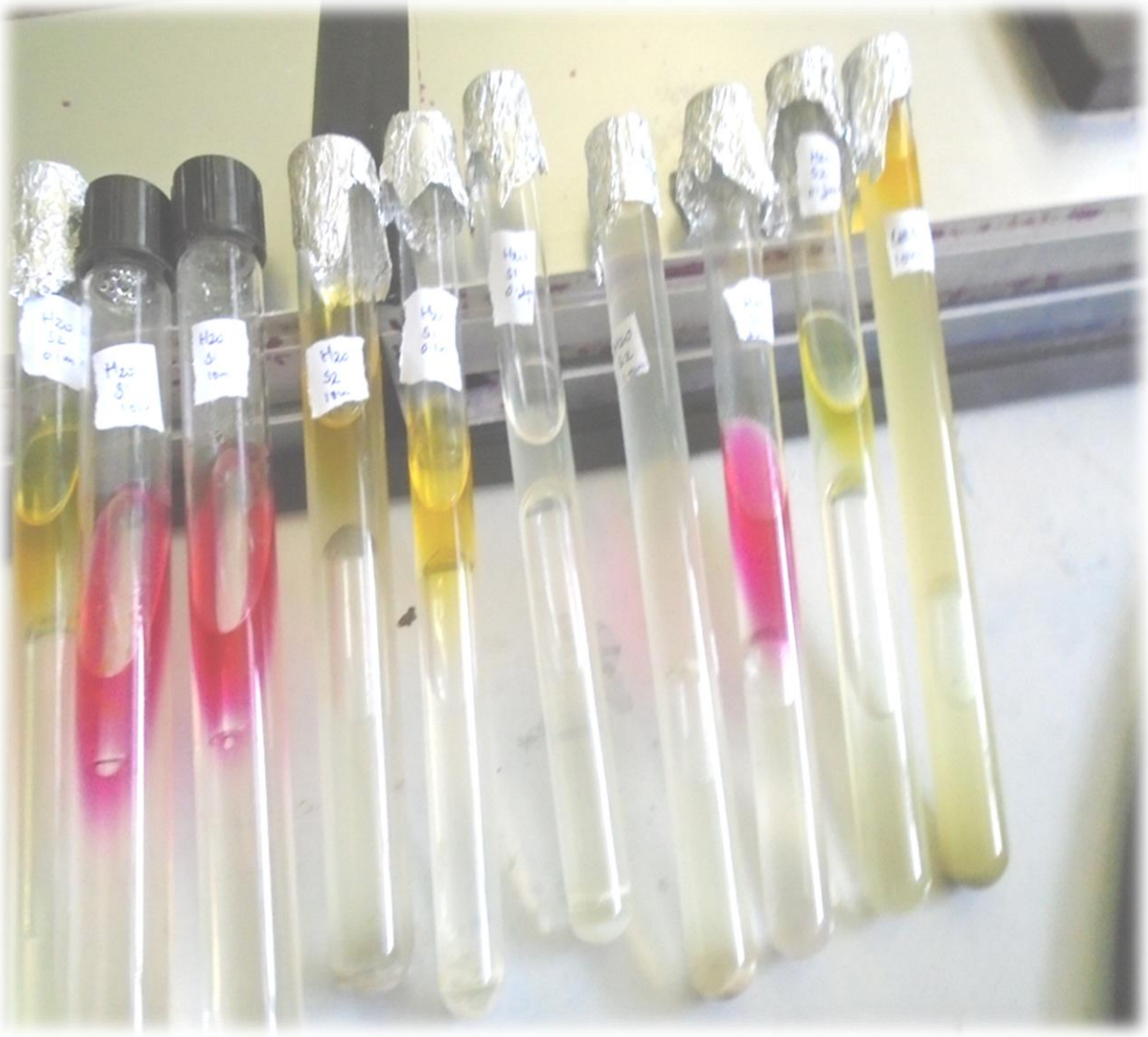


Aerobic spore former counting

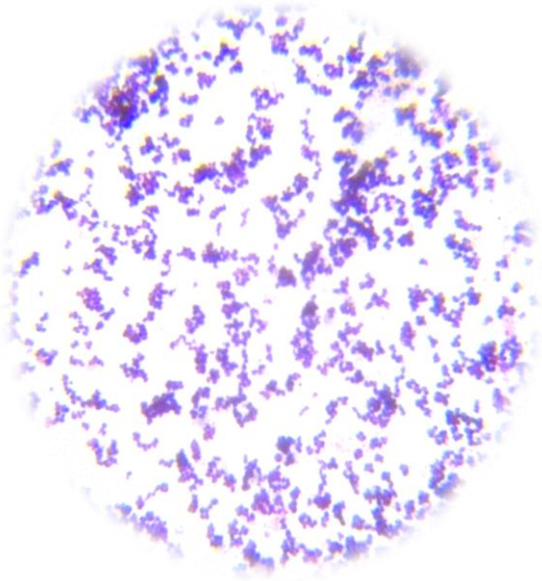


Enterobacteriaceae counting

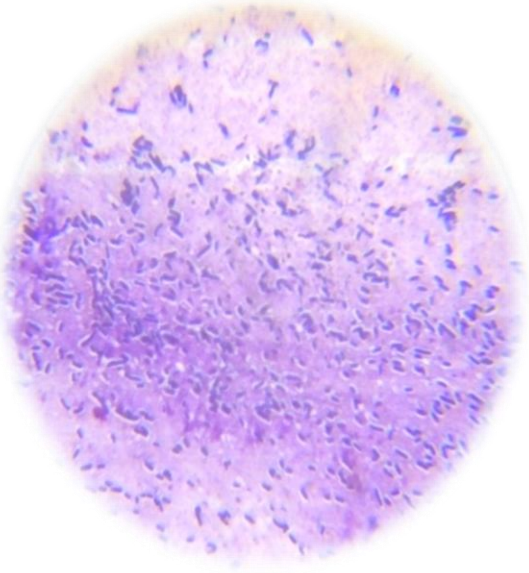
Appendix 6. Colony color of four types (AMB, staphylococcus, Aerobic Spore Former and Enterobacteriaceae) of counted bacteria from irrigated vegetables and Hasassa river, during April –July, 2018



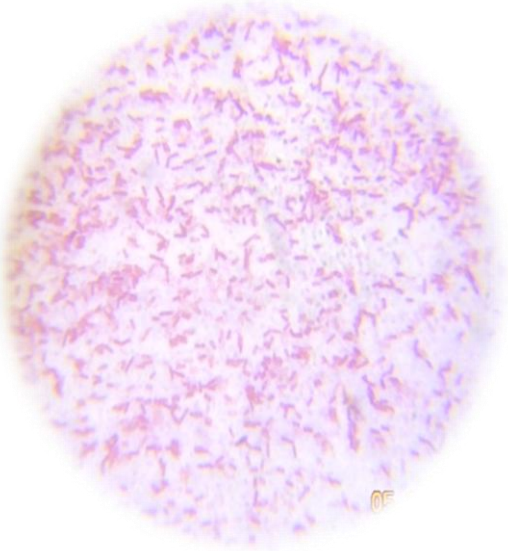
Appendix 7. Gas formation color change of coliform in the durem tube from liquid form of vegetables and Hasassa irrigation river, during April –July, 2018



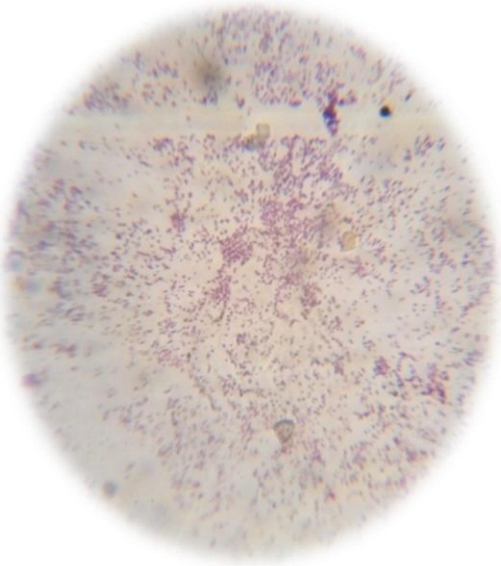
Gram-positive cocci in cluster



gram-positive rod



Gram-negative rod



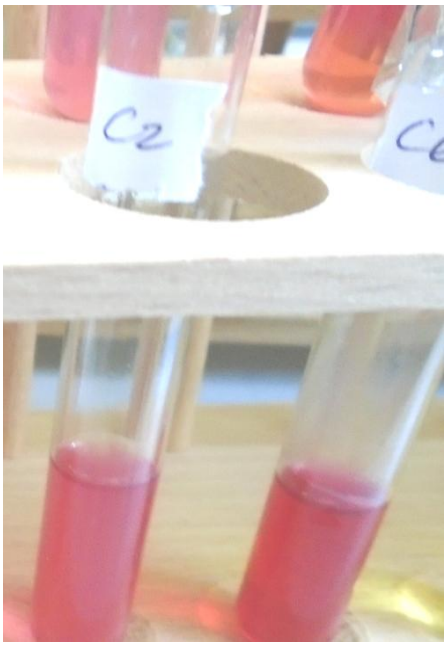
Gram-negative cocci

Appendix 8. Both Gram-positive and Gram-negative cocci and rod picture of bacteria from vegetables and Hasassa river, during April –July, 2018

Hydrogen sulfide producer

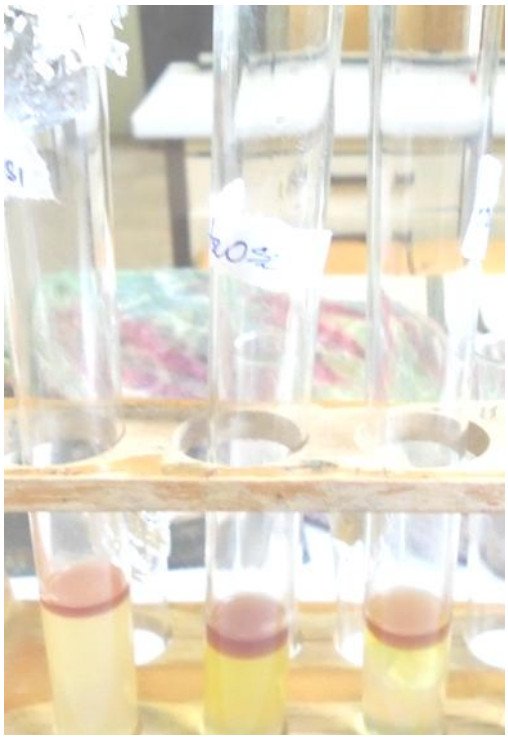
urease negative

Urease positive



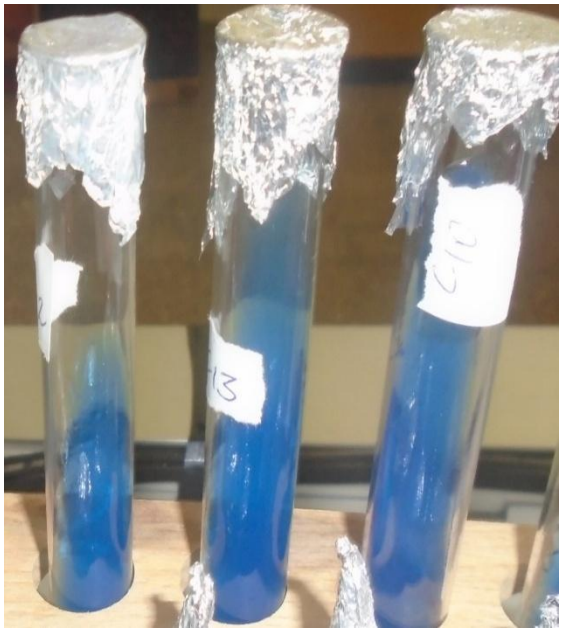
methyl red positive

Methyl red negative



Indole positive

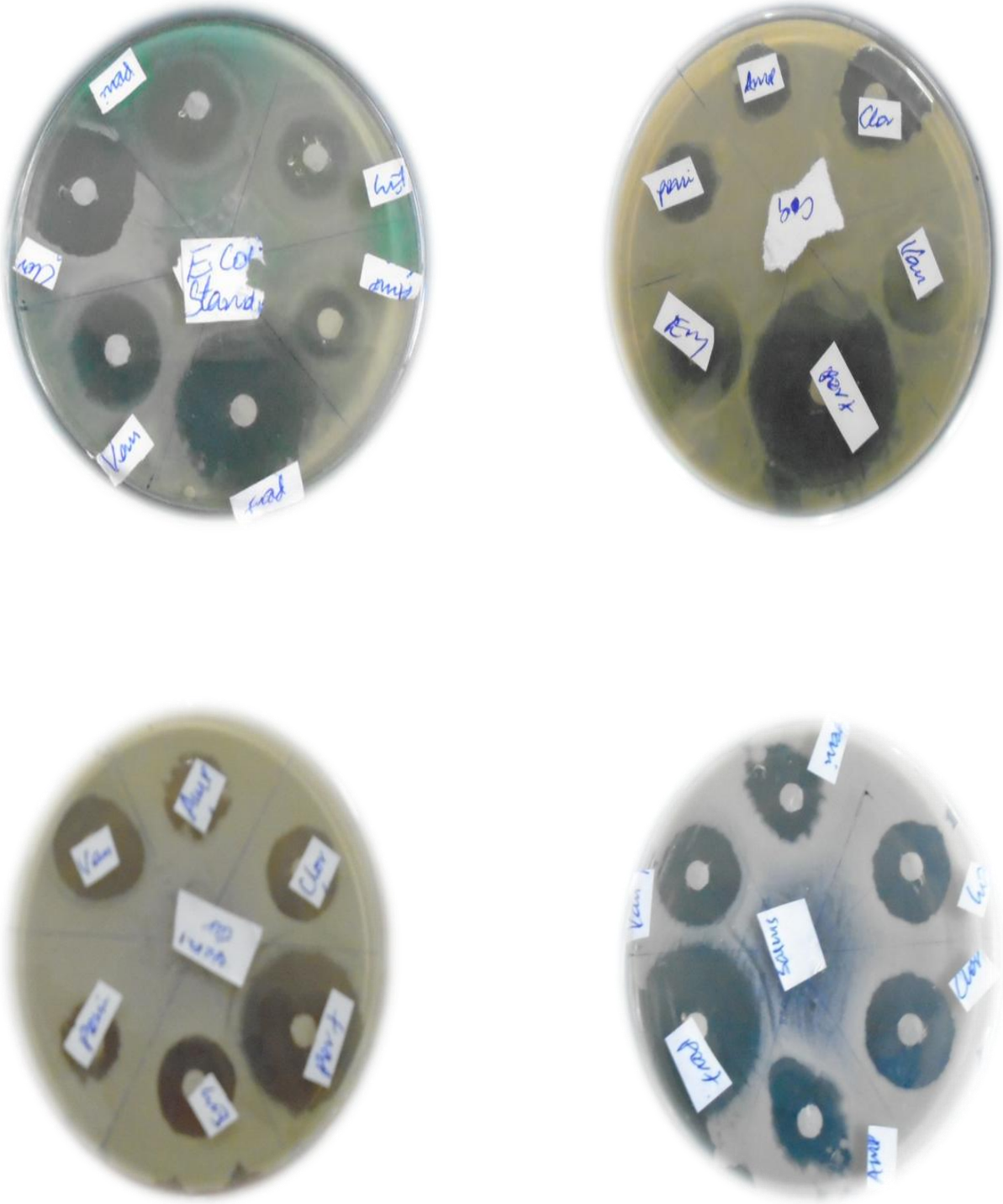
Indole negative



Citrate positive

citrate negative

Appendix 9. Different types of biochemical characterization picture of bacteria from vegetables and Hasassa river, during April –July, 2018



Appendix 10. Inhibition zone of control strain and isolated bacteria from vegetables and Hasassa irrigation river, during April –July, 2018