Deanship of Graduate Studies

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"Prevalence of antibiotic resistant Enterobacteriaceae in Palestinian leaf vegetables"

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Dedication

My Dear Wife, Father, Mother, Brothers and Sisters

For their Patience, Encouragement and Support

With love and Respect

Al-Quds University

Deanship of Graduate Studies

Applied Industrial Technology

Science and Technology Department

Thesis Approval

Prevalence of antibiotic resistant Enterobacteriaceae in Palestinian leaf vegetables

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Declaration:

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis or any part of the same has not been submitted for a higher degree to any other university or institution.

Signed: 

Jasem Sobhi Yousef Naddaf

Date: 1/7/2015

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Abstract

Leaf vegetables are susceptible to contamination with different pathogenic agents due to soil, water and storage quality. Numerous food borne outbreaks were related to the consumption of contaminated leaf vegetables, lettuce types specifically. Contamination of Palestinian farming products with pathogenic agents had not yet been widely studied and scientific information in this regard is barely available. Here we present the first study addressing contamination of Palestinian lettuce, mint and watercress with antibiotic resistant *Enterobacteriaceae*. For this we collected lettuce, mint and watercress samples from different parts of the West Bank; Jericho, Jenin, Tulkarem, Bethany, Abu-Dies and Ramallah as well as few samples produced in Israel for comparison purposes. We purchased samples either directly from farmers or from greengrocers. Our results revealed that the majority of the samples tested positive for total *coliform*, *fecal coliform* and ampicillin resistant bacteria. Overall, *enterobacter* was detected in 64.91%, *E. coli* in 12.28%, *pseudomonas* spp. in 14.04% and *Yersinia enterocolitica* in 8.77% respectively. The most common type of resistance among *Enterobacteriaceae* causing contamination in Palestinian samples as tested per antibiotic disk diffusion was carbapenem resistant *Enterobacteriaceae* (CRE) followed by extended spectrum beta-lactamases (ESBLs). Finally, resistance to antibiotics was further analyzed using the molecular biology tool polymerase chain reaction (PCR) to amplify bacterial genes enabling bacterial resistance. This analysis was performed in all CRE and ESBLs positive samples and revealed that only 18.5% of the CRE resistance to carbapenem and 41.7% of ESBLs were genetically encoded. The high percentage of contamination illustrated in our results indicate that possible outbreaks could have went undetected in Palestine and urge for applying international standards and quality farming measurements to avoid possible future outbreaks.

انتشار المضادات الحيوية المقاومة Enterobacteriaceae في الخضروات الورقية الفلسطينية

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اشراف : د. ميساء العزة

الملخص

تعتبر الخضراوات الورقية من الخضراوات الاكثر عرضة للتلوث بالعوامل المسببة للامراض ويعود هذا التلوث لعدة عوامل منها: التربة والماء و جودة التخزين. كما ان تفشي الامراض الناتجة عن الغذاء تعود الى استهلاك هذا النوع من الخضراوات الورقية, خاصة الخس.

لا تتوفر دراسات واسعة ومعلومات علمية كافية حتى الان عن تلوث منتجات المزارع الفلسطينية بالعوامل المسببة للامراض. وهذه الدراسة تعتبر اول دراسة عنونة تلوث الخضراوات الورقية الفلسطينية مثل الخس والنعنع والجرجير بالبكتيريا المقاومة للمضادات الحيويه Enterobacteriaceae . لقد قمنا بجمع عينات الخس والنعنع والجرجير من مختلف مناطق الضفة الغربية مثل اريحا وجنين وطولكرم والعيزرية وابوديس ورام الله. بالاضافة الى عدد قليل من الخضراوات المنتجة في اسرائيل لاهداف المقارنة. لقد قمنا بشراء عينات اما مباشرة من المزارعين او من البقالين. اوضحت نتائجنا في هذه الدراسة ان غالبية العينات اعطت نتائج موجبة ل Total coliform, Fecal coliform and Ampicillin resistant bacteria وقد اظهرت نتائج الدراسة ان نسبة العينات المصابه بكل من (*Yersinia* , *pseudomonas* spp, *E. coli* , *enterobacter* *enterocolitica*) كانت (64.91% ,12.28 % ,14.04% ,8.77%) على التوالي .

اكثر الانواع المقاومة شيوعا بين Enterobacteriaceae *المسببة للتلوث في فلسطين التي تم فحصها عن طريق تكنيك يسمى انتشار اقراص المضادات الحيوية كانت (CRE)* carbapenem resistant Enterobacteriaceae يليها (ESBLs).

واخيرا المقاومة للمضادات الحيوية خضعت لمزيد من التحليل عن طريق استخدام تكنيك (PCR) لتكثير الجينات البكتيرية المسوؤلة عن مقاومة المضادات الحيوية, وقد اجري هذا التحليل على جميع العينات التي كانت موجبه ل CRE و ESBLs واوضحت نتائج هذا التحليل ان فقط 18.5% من CRE مقاومة ل carbapenem وان 41.7% من ESBL كانت معرفة وراثيا.

النسب العالية من التلوث كما تم الكشف عنها في نتائج الدراسة تكشف عن احتمالية تفشي العديد من المخاطر الغير مكتشفة في فلسطين وبذلك نحث على تطبيق المعاير الدولية والمقايس الزراعية عالية الجودة لتجنب تفشي المخاطر في المستقبل .

Abbreviations

CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem-resistant Enterobacteriaceae
ESBL	Extended-spectrum beta-lactamase
KPC	*Klebsiella pneumoniae* carbapenemase
SHV	Sulfhydryl variable
LB	Luria-Bertani
MAC	MacConkey Agar
MH	Muller Hinton
ONPG	O-NitroPhenyl--D-Galactopyranosidase
PCR	Polymerase Chain Reaction
MDR	Multidrug Resistance
OXA48	Oxacillin hydrolyzinfg capabilities
TEM	Temoneira
MFS	Facilitator superfamily
SMR	small multi-drug resistant
PBS	Phosphate buffer saline
MATE	multi antimicrobial extrusion
CTX-M	Cefotaxime hydrolyzing capabilities
ABC	resistance-nodulation-cell division
RND	resistance-nodulation-cell division
NACMCF	National Advisory Committee on Microbiological Criteria for Foods

ETEC	Enterotoxigenic E. coli
EPEC	Enteropathogenic E. coli
EHEC	Enterohemorrhagic E. coli
EIEC	Enteroinvasive E. coli
EAEC	Enteroaggregative E. coli
DAEC	Diffusely adherent E. coli
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
BMA	Bacteriological Analytical Manual
EDTA	Ethylene-diamine-tetra-acetic acid
bp	Base pair

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1. Introduction

1.1. Bacterial contamination of vegetables

One of the most common sources for human infections is the food we consume every day. Foodborne infections are mainly associated with consumption of fresh vegetables (Tyler and Triplett, 2008; lynch et al., 2009). It is widely accepted that fruits and vegetables are significant sources of pathogens and chemical contaminants (Uzeh et al., 2009). Outbreaks associated with fresh produce result in considerable economic losses to farmers, distributors and the food industry (Golberg et al., 2011).

Food contamination can be caused by Fungi, Protists, viruses and Prions (Murray et al., 2013). Despite the massive progress made internationally on the level of hygiene and food safeties, foodborne outbreaks are still a threat, worldwide. Gastrointestinal diseases had been strongly related to pathogenic contamination of food, particularly bacterial contamination with members of the Enterobacteriaceae family (Brand, 2006; Heaton and Jones, 2008). Beside the fact that fresh vegetables are colonized by natural nonpathogenic epiphytic microorganisms, animal and human can function as a new source of contamination with pathogenic bacteria during growth, harvest, transportation, and further handling of these products (Falomir et al.; 2013). Consumption of raw seasonal fresh vegetables specifically can be a major risk for foodborne infections (Beuchat, 1996, Brand, 2006; Heaton and Jones, 2008), especially among immunocompromised patients (Falomir et al., 2010a; Falomir et al.; 2010b). Khan et al. (1992) reported that bacterial contamination results from various unsanitary cultivation and marketing practices. In another study, Tambekar et al. (2006) reported that bacterial contamination of salad vegetables was linked to the fact that they are usually consumed without any heat treatment. Beside the major food safety concern related to the bacterial content in fresh vegetables, the presence of antibiotic resistances in both epiphytic and pathogenic microorganisms contributes to horizontal spreading of resistances among bacterial populations. Therefore, fresh products may play a role as a carrier and reservoir of antibiotic resistant bacteria (Walsh and Fanning, 2008; Falomir et al., 2010b; Schwaiger et al., 2011a).

Beside hospitals and commercial animal farming, which are the primary sources for emergence of antibiotic resistances, plant agriculture became another threatening source, due to the usage of large amounts of antibiotics, including misusage (lipsitch et al., 2002; McManus et al., 2002; Vidaver, 2002; Boehme et al., 2004; Sarmah et al., 2006; Todar, 2008a). A high level of antibiotic resistance is often related to the member of the Extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae (Johnson et al., 2005).

It is noteworthy here to mention that green house production is in some instances even favorable for the survival of foodborne pathogens (Nguyen-the and Carlin, 2000). Furthermore, using composted organic materials, as nutrients for plants may be critical depending on the origin of the compost and the treatment of the compost. Nevertheless, the recent development of organically grown fruits and vegetables will increase the use of organic fertilizers, which could contribute to reduction of pathogenic and antibiotic resistance pathogenic contamination of food. Doses of irradiation required to inactivate human pathogens may have an adverse effect on fruits and vegetables (European commission report, 2002).

Pathogenic contamination of vegetables and fruit can occur in the field by contaminated soil, by the use of contaminated water for irrigation or pesticide application or by deposition of feces by wild animals (Ingham et al., 2005; Johannessen et al., 2005, Andersen et al., 2015). Fecal bacteria (including enteric pathogens) are in particular in wet conditions and clouded weather (limited UV irradiation) able to survive for extended periods in soils (Islam et al., 2004a), manure (Nicholson et al., 2005) and water (Chalmers et al., 2000; Steele and Odumeru, 2004).

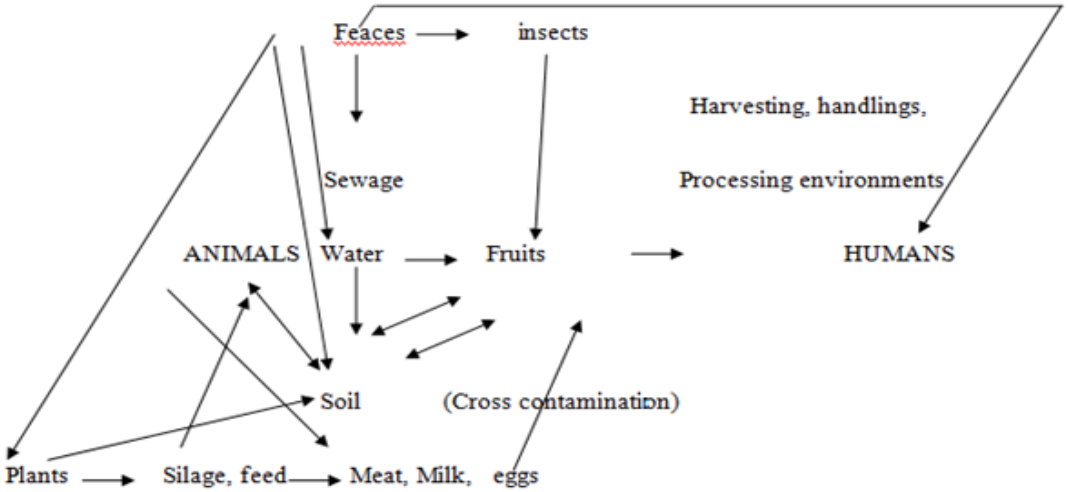


Figure 1.1. Mechanisms of food contamination with pathogenic microorganism (adapted from Beuchat, 1996).

1.1.1 Bacterial contamination by irrigation

Although water is considered as the main source of irrigation for plants, it is as well the major source of pathogenic contamination. Plants constantly absorb water and nutrients through a re-circulating process, therefore the quality of the water and the technologies used for irrigation are critical factors contributing to or protecting from microbiological hazards (NACMCF, 1999a). For example, the use of drop irrigation instead of flooding or spray irrigation reduces waterborne contamination and aerosols. Additionally, heavy rains and wind may provide further opportunities for the transfer of microorganisms from soil to plant surfaces.

1.1.2 Bacterial contamination of soil

Livestock agriculture is one of the primary causes of bacterial contamination of surface and ground waters (Jamieson et al., 2002). Using animal manures to tile drained land is a major pathway contributing to transport pathogens with subsurface drainage water to surface water systems (Jamieson et al., 2002). However this pathway is affected by soil type, moisture, temperature, pH, manure application rate and nutrient availability. Cool, moist environments are considered optimal for bacterial survival (Jamieson et al., 2002). There is an urgent need to apply farm management systems that minimize the risk of water contamination, especially with respect to human pathogens.

1.1.3 Food storage and bacterial contamination

Food is classified by storage potential in regard to their moisture content into three categories; perishable, semiperishable and stable or non-perishable. Typical stable foods are flour and sugar, which have no water activity. Semiperishable foods are nuts, which show a low water activity. Vegetables and fresh products including meat and fish belong to the perishable category as they have high water activity and therefore must be stored under specific condition to avoid water activity, which is a very good medium for bacterial growth, to spoil them. Bacteria found in the gut of animals such as Salmonella and Enteric bacteria are potential danger for meat whole slaughtering, lactic bacteria are potential danger for milk products, while bacteria found in soil and water are potential danger for fresh leaf vegetables.

Food preservation using cold, acidity, drying, dehydration, heating, chemical preservation, and irradiation works out with different foods, but does not allow fresh vegetables to be consumed as such. So far, there is no method to store leaf vegetables such as lettuce without causing damage to the leaves and affecting the shape and taste, therefore it belongs to one of the leaf vegetables, which needs serious precaution while the short handling process from harvesting until the minute of consumption.

1.2 Bacteria causing contamination

Enterobacteriaceae are the major contaminants of food and water, causing various intestinal and extra-intestinal infections such as urinary, central nervous system and respiratory tract infections (Murray et al., 2013). Enterobacteriaceae are coliforms, facultative anaerobic gram negative rods. The presence of Enterobacteriaceae such as E. coli, Enterobacter spp., Salmonella spp., Shigella spp. and Pseudomonas aeruginosa has been reported in salad vegetables (Khan et al., 1992; Tambekar, 2006). Mehmet and Aydin (2008) also reported the presence of *E. coli* in some green leafy vegetables. Fruits and vegetables comprise a diverse range of plant parts (leaves, roots, tubers, fruits, and flowers).

Bacteria such as *Clostridium botulinum*, *Bacillus cereus*, *Listeria monocytogenes* and *Pseudomonas* are also capable of causing illness as natural inhabitants of many soils, whereas *Salmonella*, *Shigella*, *Escherichia coli* and *Campylobacter* reside in the intestinal tracts of animals, including humans, and are more likely to contaminate raw fruits and vegetables through contact with feces, sewage, untreated irrigation water or surface water. On the other hand, contamination with other pathogens such as viruses, parasites and molds can also result from contact with feces, sewage and irrigation water (Andersen et al, 2015; Murray et al., 2013).

1.2.1 Enterobacteriaceae

The Enterobacteriaceae is a large family of gram-negative bacteria that includes *Escherichia coli*, *Salmonella* spp., *Yersinia* spp., *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Shigella* spp., and *Serratia* spp. (Madigan et al., 2009). Members of the Enterobacteriaceae are facultative anaerobes, fermenting sugars to produce lactic acid and various other end products. Many members of this family are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants. Enterobacteriaceae types commonly involved in food-borne infections are illuminated in the next paragraph.

Escherichia coli is a typical inhabitant of intestinal tract of human and warm-blooded animals. Although most strains are harmless, some *E. coli* strains are pathogenic and can cause diarrhea in infected patients, mainly due to consumption of contaminated food. Pathogenic strains of E. coli bacteria are typical food pathogens, which produce virulence factors and commonly cause diarrhea. There are six different E. coli pathotypes: enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), enterohemorrhagic E. coli (EHEC), enteroinvasive E. coli (EIEC), enteroaggregative E. coli (EAEC), and diffusely adherent E. coli (DAEC). In addition, each type has a different pathogenic scheme (Mange and Johnson, 2012). *E. coli* is a mesophile, however, it can survive at temperatures as low as 8°C and as high as 48°C with an optimum at 39°C.

Salmonella species are facultative anaerobic Gram-negative rod-shaped bacteria and are part of the normal flora in the gut of vertebrates (Ibarra and Steele-Mortimer, 2009). Salmonella enterica serovar Typhimurium and S. enterica serovar Enteritidis cause prominent epidemics of gastroenteritis in human, worldwide (Rensch et al., 2013). Food-borne salmonellosis can be caused due to the consumption of contaminated meet, meet products, fresh cheese products, eggs and lettuce (Quinn et al., 2006).

Enterobacter spp. are found in various types of environments including water, sewage and intestinal tract of warm-blooded animals (Sanders and Sanders, 1997). Klebsiella spp. can often be confused with Enterobacter spp., but a distinguishing factor is the motility of Enterobacter species (Bouza and Cercenado, 2002). *E. cloacae* and *E. aerogenes* are the most common species causing infectious disease in humans due to consumption of raw vegetables, dairy products and raw shellfish types (Murry et al., 2013).

Beside Enterobacteriaceae, Pseudomonas spp. are widely spread in soil, water and plants. *Pseudomonas spp*. are also gram-negative bacteria and belongs to the family Pseudomonadaceae.

Antibiotic resistance in Enterobacteriaceae had become a very critical factor contributing to pathogenicity of these bacteria. Antibiotic resistance among these bacteria made them even more critical clinically, especially with these bacteria being major contributors to nosocomial infections (Sanders and Sanders, 1997; He et al., 2011; He et al., 2011).

1.2.2 Antibiotic targets in bacteria

Antibiotics are used to treat infectious disease caused by pathogenic microorganisms. The most common targets for prokaryotic cells are the bacterial cell wall, the bacterial ribosome, DNA replication, RNA synthesis, and specific enzyme pathways. Antibiotics targeting cell wall act against one of the several steps in the cell wall building process. Building blocks of cell wall; N-acetyl glucosamine and N-acetyl muramic acid made in the cytoplasm are transported across the cell membrane by a carrier molecule, a process called transglycosylation and are cross linked to another long chain in a process called transpeptidation. Bacteria make new cell wall material only when they are growing; therefore, antibiotics that disrupt this process are typically only effective on growing cells.

Luckily, bacterial ribosomes differ from human ribosomes, that antibiotics can effectively target them by binding with a ribosomal protein of the bacterial cells. Streptomycin is an example of an antibiotic that targets the ribosome and interferes with the movement of the ribosome along the mRNA. DNA replication is another target for antibiotics such as ciprofloxacin, which inhibits the DNA gyrase, which catalyzes the first step of replication and therefore it inhibits cell division (Guilfoile, 2007). Antibiotics like rifampin, which binds to bacterial RNA polymerase and prevents it from synthetizing RNA, leads to inhibition of protein synthesis required for cellular survival and therefore cause bacterial cell death.

1.3 Drug resistance in bacteria

Extensive treatment, prescription and misusage of antibiotics had led to a major public health concern due to the emergence, development and evolution of drug resistance bacterial strains (Levy, 2000; Alekshun and Levy, 2007). The fact that many patients do not complete their full course of prescribed antibiotics raise the chances of the bacteria to survive and initially adapt to the low concentrations of antibiotics, later even to higher doses (Pechere et al., 2007). In fact, antibacterial drug resistance is the ability of bacteria to grow in the presence of a substance that would normally kill it or limit its growth among the wide array of antibiotics; these antibiotics can be classified in a variety of ways.

Beta-lactams are considered the most variable and widely used class of antibiotics accounting for over 50% of all systematic antibiotics used (Bronsn and Barrett, 2001). Beta-lactams contain a highly active chemical compound, that can mimic a section of bacterial cell wall, and inactive enzymes normally involved in assembling cellular structure as well as the facts that these enzymes are localized to the outer leaflet of the bacterial cytoplasmic membrane and that they are specific to bacteria. There are four major groups in beta lactam antibiotics include: penicillin, cephalosporin, carbapenemes and monobactams, beside a variety of other antibiotics of different chemical classes (Guilfoile, 2007). In recent years bacterial resistance to beta-lactam antibiotics has clearly increased, which had been attributed to the spread of plasmid-mediated extended spectrum beta-lactamases (**ESBLs**), occurring predominantly in the family of Enterobacteriaceae. Whereas *Klebsiella pneumoniae* and *E. coli* are the main species in which ESBL enzymes have been the most commonly reported worldwide (Kohler et al., 1999). Beta-lactamase inhibitors include clavulanic acid, sulbactam and tazobactam, have little or no antimicrobial activity but they are potent inhibitors of many beta-lactamasees and thus they can protect the beta lactams drugs from inactivation by enzymes mentioned (Katzung, 1998). Both clavulanic acid and sulbactam are highly effective against staphylococcal penicillinase and TEM type beta-lactamases but less efficient in inhibiting cephalosporinase (Ryan, 1994). CTX-M type beta-lactamases, these enzymes are inhibited by the tazobactam (Bradford et al., 1998, Tzouvelekis et al., 2000).

1.3.1 Mechanisms of resistance to antibiotics

The mechanisms responsible for increased Drug and multidrug resistance among pathogens include biofilm formation, alteration of binding sites, enzymes that can inactivate antibiotics, decreased membrane permeability and active efflux of antimicrobials (Kumar and Varela, 2013; Alekshun and Levy, 2007) (illustrated in Figure 1.2).

1.3.1.1 Biofilms

Biofilms are highly condensed layers of bacteria, which cause weak diffusion of antibiotics through the biofilm polysaccharide matrix (Ander et al., 2000). On the other hand, biofilms may undergo physiological changes due to slow growth rates and starvation responses or phenotypic changes, which allows for resistance to antibiotics. Finally, the expression of efflux pumps is a crucial contribution to drug resistance in biofilms (Soto, 2013).

1.3.1.2 Modification of drug target

During the course of misusing antibiotics, bacteria developed this mechanism, by which mutations cause alteration of antimicrobial binding site, which inhibits its action and allows the bacteria to survive despite the presence of antibiotics (Kumar and Varela, 2013). An example is bacterial ribosomes. Changes in bacterial ribosomes inhibit the activity of antibiotics such as tetracycline (Poehlsgaard and Douthwaite, 2005; Schnappinger and Hillen, 1996).

1.3.1.3 Enzymatic deactivation of antimicrobial agents

Bacteria have evolved enzymes, which can deactivate or degrade antimicrobials chemically inhibiting their activities. ESBLs are the most common bacteria conferring such type of resistance mechanism. ESBLs Amidases cleave the beta lactam rings of the penicillin and cephalosporin classes of drugs (Gutkind et al., 2013) causing resistance to this class of antibiotics. Different bacterial enzymes encode for this resistance.

1.3.1.4 Reduced drug permeability cross the bacterial membrane

Using this mechanism, bacteria totally inhibit access of the antibiotics across bacterial membrane, which confer resistance to antibiotics targeting bacterial organisms (Kumar and Schweizer, 2005) by modifying the outer membrane lipid barrier and porin mediated

permeability. Phospholipids and lipopolysaccharide (LPS) of the outer membrane act as barrier for antibiotics like erythromycin and rifamycin (Nikaido, 2003). Furthermore, membrane channel proteins, such as porins may be altered by mutations, which inhibits antimicrobial access (Pages et al., 2008). Resistance to carbapenems is a typical example of this mechanism (Page, 2012).

1.3.1.5 Active efflux of drugs from bacterial pathogens

Hereby, bacteria acquire resistance to antimicrobials and even environmental toxic compounds by an active efflux. Integral membrane transporters known as drug efflux pumps inhibit the accumulation of drugs inside the bacterial cells by efficiently pumping them outside the bacterial So far, different classes of bacterial efflux transporters had been identifies and referred to using abbreviations. There are five known efflux transporter families causing this type of resistance: a. Facilitator superfamily (MFS); b. the small multi-drug resistant super family (SMR); d. the multi antimicrobial extrusion protein superfamily (MATE); f. the ATP-binding cassette super family (ABC); and g. the resistance-nodulation-cell division superfamily (RND) (Kumar and Varela, 2012; Kumar and Varela, 2013; Kumar et al., 2013).

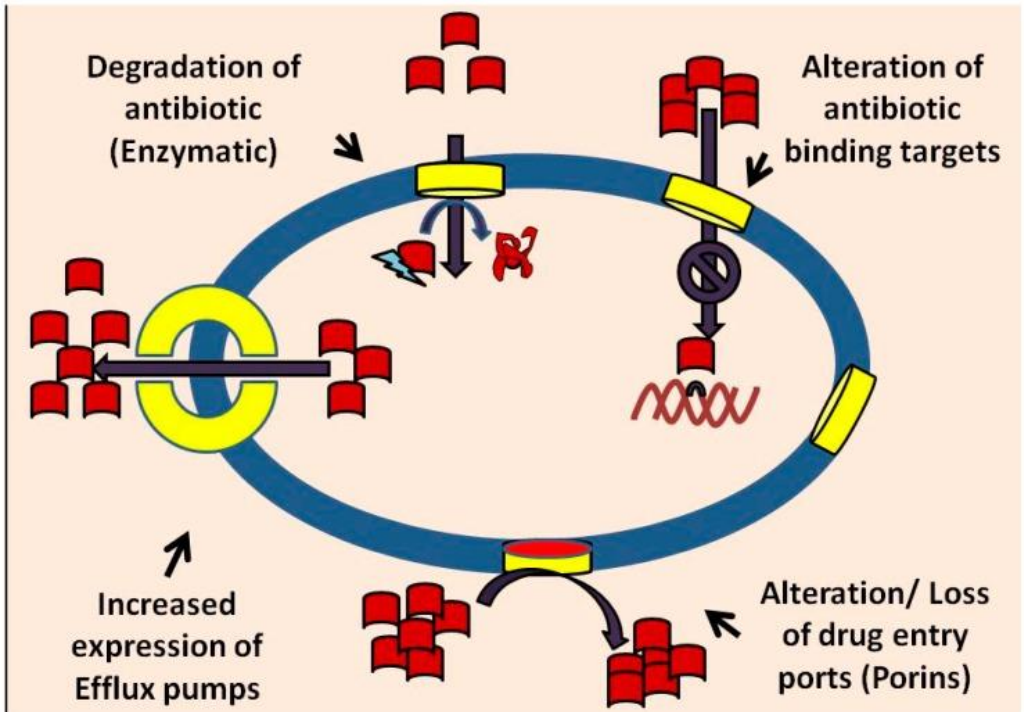


Figure 1.2. Bacterial antibiotic resistance mechanisms (from Andersen et al., 2015; Kumar and Varela, 2013). Antibiotics are illustrated as red blocks, while their entry sites are depicted in yellow rings. The mechanisms are: Degradation of antibiotics, alteration of antibiotic binding target, alteration/loss of drug entry, and increased expression of efflux pumps. All the mechanisms are genetically encoded. While the first two mechanisms do not allow entry of the antibiotics, the last two mechanisms lead to reduction of the intracellular concentration and permeability of the antibiotic into the cell.

1.3.2 ESBLs

Extended-spectrum beta-lactamases (ESBLs) are a rapidly evolving group of enzymes causing beta lactam resistant among bacteria. The ESBLs are usually plasmid encoded (are often located on plasmids). These ESBL plasmids may carry genes encoding resistance to other drug classes as well. Plasmids are transferable from strain to strain and between bacterial species via conjugation. Only limited antibiotic can be used in the treatment of infections caused by ESBL-

producing organisms (Paterson and Bonomo, 2005). ESBLs are enzymes capable of conferring bacterial resistance to the penicillins, 1st generation, 2nd generation, and 3rd generation cephalosporins, and monobactam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by beta lactamase inhibitors and generally derived from TEM and SHV-type enzymes. (Paterson and Bonomo, 2005). Although the prevalence of ESBLs is not known, it is clearly increasing, and in many parts of the world 10–40% of strains of *Escherichia coli* and *Klebsiella pneumoniae* express ESBLs.

1.4 Detection of bacterial drug resistance

Detection of bacterial drug resistance can occur by different ways after growing the bacteria to be tested. The most common technique used is antibiotic inhibition zone testing. However, this conventional method does not necessary reflects the existence of genes coding for that exact resistance. As described above in 1.3.1, resistance can be induced by different mechanisms; therefore, one can make use of molecular biology techniques to test for genes encoding resistance. Hereafter, one can delineate, whether drug resistance was evoked by genes encoding it or by other mechanisms illustrated in 1.3.1, figure 1.2.

1.4.1 Conventional detection of bacterial drug resistance

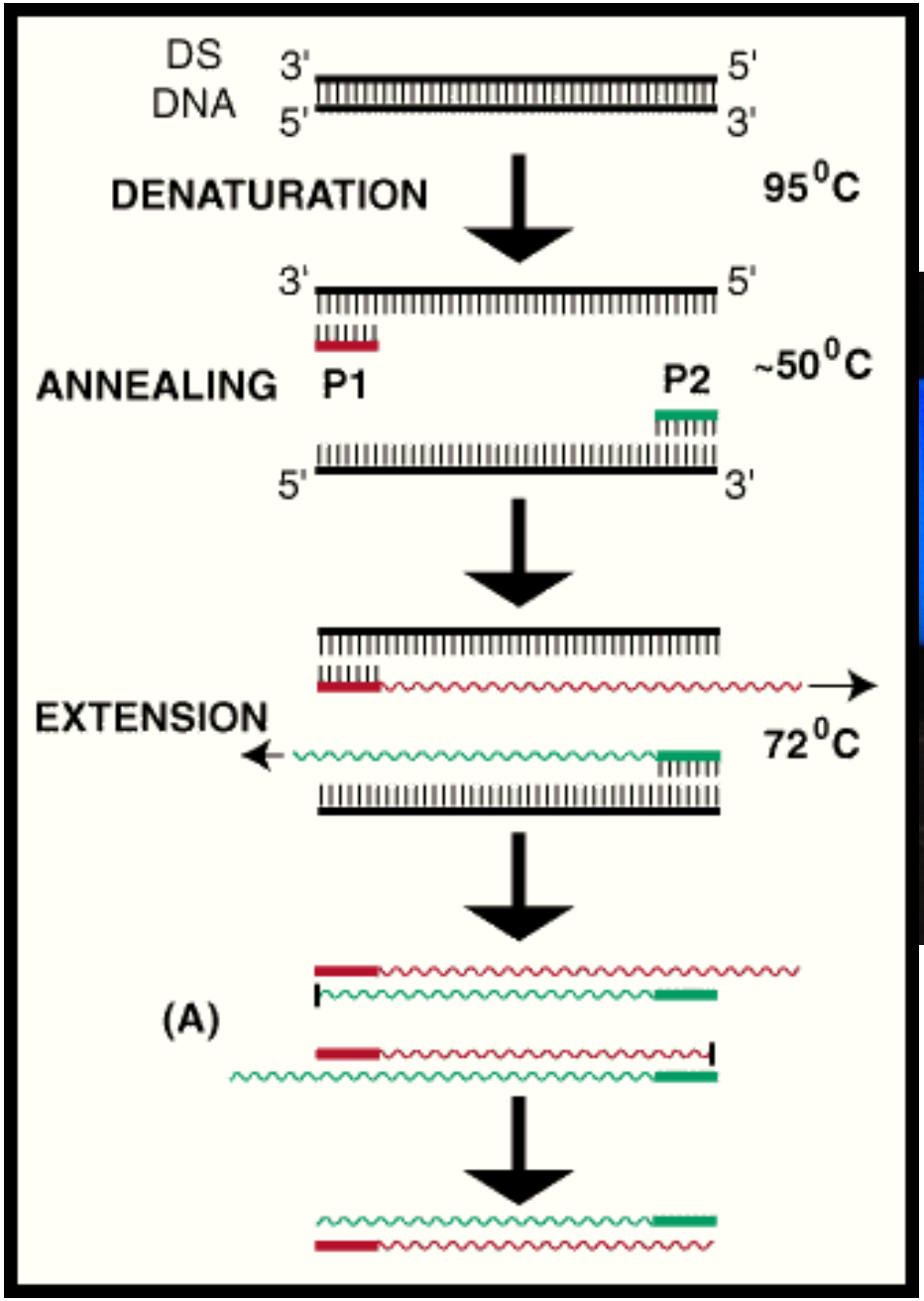
The most conventional method of detecting drug resistance in bacteria is using the antibiotic diffusion test. Hereby, a disk containing the antibiotic at the appropriate concentration is placed on a nutrient plate (Mueller Hinton) after spreading the bacteria to be tested. Plates are placed in an incubator to allow growth. After 12-24 hours, bacterial growth is detected and inhibition zones around the antibiotic disks are visual. Internationally recognized zones for specific antibiotics are measured to determine whether the bacteria is sensitive, resistant or intermediate sensitive to that antibiotic.

1.4.2 Molecular biology detection of bacterial drug resistance

As describe in the section of mechanism of antibiotic resistance, either mutation or specific genes encode for different types of drug resistance. Although resistance is detectable using conventional antibiotic diffusion test, detecting genes responsible for resistance is essential, as different genes encode for the very same drug resistance. Genetic epidemiology and mutation causing resistance could be different in different countries and environmental circumstances. In order to perform genetic analysis of drug resistance genes, we applied the polymerase chain reaction method (PCR). This method is based on making copies (amplification) of specific sequences (in our case, the antibiotic encoding genes) if they exist. The PCR reaction is catalyzed using the enzyme DNA polymerase, which naturally amplifies DNA molecules in organisms. Beside the DNA polymerase, a PCR reaction contains the following:

1- Oligonucleotides (primer), which are complementary to the flanking target DNA, regularly a 20-25 nucleotides in length synthesized on oligonucleotide synthesizer.
2- Deoxy nucleic triphosphate (dNTPs), which are the building blocks necessary to build the DNA strand and deliver the DNA copies of the target sequence.
3- Stabilizing buffer: regularly $MgCl_2$.

A PCR reaction starts with a denaturation step at 95°C, which separates the double stranded DNA, followed by an annealing step, which allows the primer pair to hybridize to the target DNA. The Annealing step is dependent on the primer sequence. The last step for polymerase reaction is the DNA polymerizing extension at 72°C. These three steps are repeated normally 30-40 times allowing for building more and more copies of the target DNA sequence. When a PCR reaction is negative, it means that the target DNA does not exist and therefore could not be amplified. The PCR reaction is detailed in the methods section, please see figure 1.3 (page 14) describing the polymerase chain reaction steps.



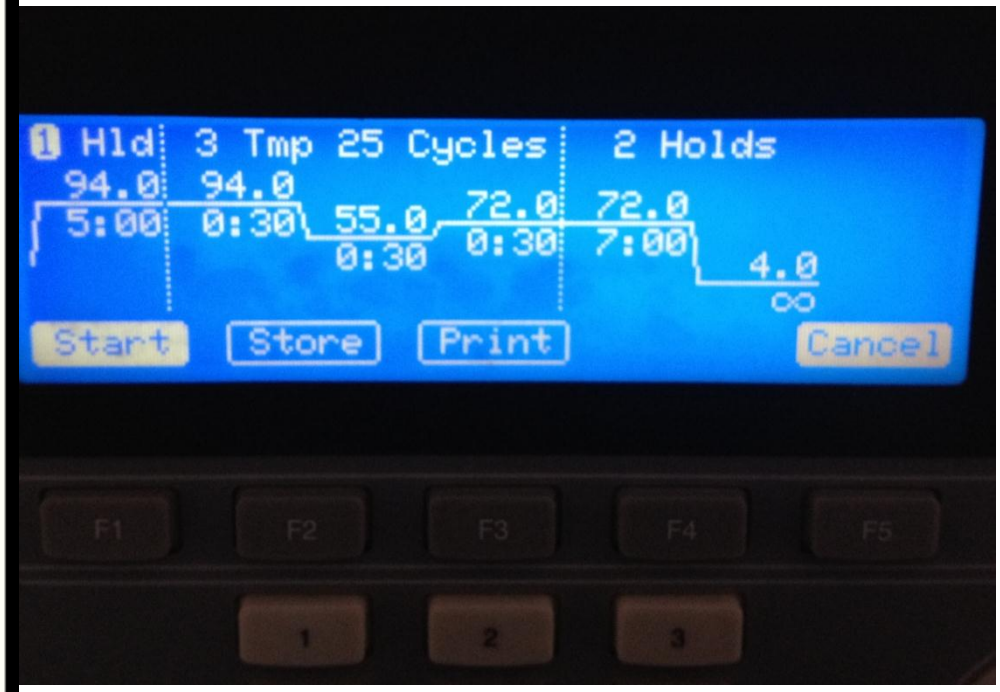


Figure 1.3. PCR reaction steps. On the left, PCR reaction steps are illustrated, copies of DNA generated are in green and red colors. On the right is a PCR reaction on the PCR machine showing the steps of the PCR reaction.

1.5 Aims of this study

The main aims of this study are summarized in the following points:

1- To detect the overall prevalence of bacterial contamination in some fresh Palestinian leafy vegetables
2- To detect the type of bacteria causing contamination in these vegetables
3- To test for resistance types in these bacteria
4- To test for resistance encoding genes in these bacteria
5- To compare the results retrieved from market samples versus farm sample

2. Materials and methods

2.1 Collecting samples

A total of 59 fresh samples of lettuce, mint, and watercress were collected between 24-2-2013 and 22-10-2014 from several regions in Palestine. 34 of the samples were lettuce, 18 were mint, while 7 were watercress respectively. The samples were collected from commercial regular markets or directly from distributing farm from Ramallah, Jericho, Tulkarem, Israel, Bethany, Jenin, and Abu-Dies as indicated in Table 3.1 (Result section).

2.2 Sample preparation

Purchased samples were placed in a plastic bag and either transported directly to the laboratory and subjected to examination or stored for max 12h in fridge until examination. On a disinfected bench, samples were cut into small pieces with a sterile knife, well milled in a stomacher (IUL Nr 1965/400) after the addition of phosphate buffer (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4) as following: For each 30 g of mixed cut sample, 270 ml of phosphate buffer was added for all samples in this research. Sample homogenate was then divided into three equal amounts before being subjected to the three different bacterial isolation methods as illustrated below

2.3 Bacterial isolation from sample homogenate

Homogenate was used for isolation of gram negative bacteria using either the total coliform test, the fecal coliform, or by direct streaking on ampicillin containing media. The reason for using the coliform and the fecal coliform tests was based on the fact that we were aiming to test the quality of water used for irrigation; therefore we took advantage of these well-known methods as indicators.

2.3.1 Total *coliform* and fecal coliform tests

m-Endo media (HIMEDIA, cat No.M1106) and m-FC media (HIMEDIA, cat No. M1122 prepared in 25 mm sterile Petri dishes were used to isolate coliform and fecal coliform respectively. 100 ml of homogenized sample was filtered onto the coliform test plate according to the instructions of Bacteriological Analytical Manual (BMA, 2015). m-Endo and m-FC Plates were incubated at 37 ºC for 24 h, and 44.5 ºC for 24 h respectively.

Growth and number of colonies were detected in each case separately. Colonies were then subjected to further analysis in accordance with the results obtained from the ampicillin plates (section 2.3.2).

2.3.2 Isolation of gram negative bacteria on ampicillin plates

Sorbitol MacConkey agar (OXOID, cat No. CMO813) was prepared with Ampicillin (Amp) at an end concentration of 100 µg/ml, added at 50 ºC. 1 ml of homogenate was spread on the Amp-MacConkey plate and incubated at 37 ºC for 24 -48 h. Growth and number of colonies was recorded. (BMA, 2015).

2.4 Verification of gram negative bacteria isolated in section 2.3.1 and 2.3.2

Colonies grown on total coliform, fecal coliform and the Amp-MacConkey plates were counted. Color, form, and phenotype of colonies were recorded. Based on this eye inspection a colony representing a group of colonies was sub-cultured on Amp-MacConkey. In most cases 2-4 colony types were dominant and these were sub-cultured to get clear single colonies for identification and antibiotic resistance testing. These single colonies representing one type of bacteria were frozen for back up and for future testing including identification, antibiotic resistance testing and polymerase chain reaction (PCR) analysis of resistance genes.

2.5 Storage of bacteria (bacterial stabs)

Storage of bacteria was necessary as mentioned above and was performed from one single colony as follows:

1. 3 ml of LB media (5 g NaCl, 5 g peptone water, 2.5 g yeast extract, 6 g agar) with Ampicillin (100 µg/ml) was prepared in sterile 10 ml glass tube.
2. A single colony of bacteria was taken by sterile disposable plastic loop from Agar plate and cultured over night (ON) in the Amp-LB media from point 1.
3. Overnight culture took place in shaker incubator at 37 ºC (100 rpm).
4. On the next day, 800 µl of cultured bacteria was transferred into a sterile well labeled 1.5 ml Eppendorf tube and 200 µl of glycerol was added
5. Bacterial culture and Glycerol were mixed well by vortexing.
6. Mixture was frozen immediately at -70 ºC

2.6 Bacterial identification

In case of frozen sample, eppendorf bacterial stab was placed on ice, a sterile disposable plastic loop was used to take an aliquot of the frozen sample and streaking it on Amp-MacConkey agar plates. After 24 h incubation by 37 ºC, a single colony was inoculated into an enterotest tube (Hy Labs) using a sterile disposable plastic loop and incubating for 24 h by 37 ºC. Bacterial identification was based on the following criteria/biochemical differentiation test:

1- Motility: This reflects the ability of the bacteria to move represented by diffuse growth in the lower layer of the enterotest tube.

2- H_2S production: This test determines whether the bacterium reduces sulfur-containing compounds to sulfide. If so, a black precipitate is produced from the Sulfide medium (in the butt upper layer of the enterotube test).

3- Indole production: This test determines the presence of tryptophanase, which hydrolyse Tryptophan into indole. The stopper of the Enterotest tube identifies this test. Hereby, the white stopper turns violet if the test is positive.

4- Urea test: This test determines the presence of Urease in the bacteria. Urease is an enzyme, which hydrolysis urea to ammonia and carbon dioxide. Urea test is presented by the lower layer of the enterotest tube and contains phenol red as a pH indicator, which turns pink if the test is positive due to the base activity of ammonia.

5- ONPG (Ortho-Nitrophenyl-b-Galactosidase) test: This tests the presence of b-Galactosidase, as this hydrolysis ONPG to Galactose and ortho-nitrophenol indicated by the yellow color.

6- Glucose test: This test is used to determine the ability of the bacteria to ferment glucose as well as to convert pyruvic acid into gaseous byproducts. Also here phenol red is used as an indicator in the butt upper layer of the enterotest tube. A yellow color reflects the production of acids. Gas production is measured by the appearance of bubbles.

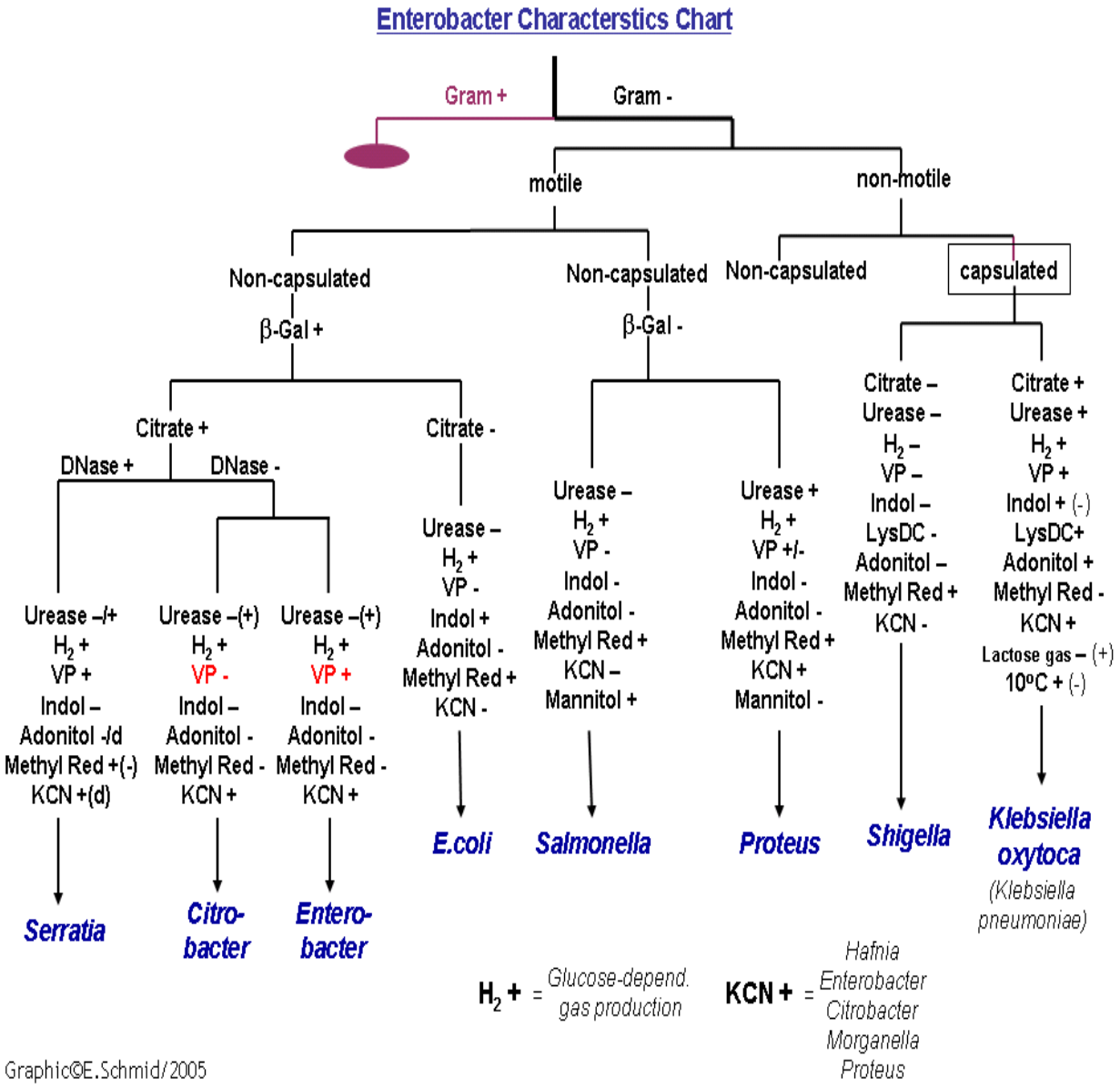


Figure 2.1. Biochemical differentiation tests of Enterobacteriaceae. This Diagram was used to identify the Enterobacteriaceae inoculated in the enterotest tube. The exact steps were followed based on the color reactions, motility and gas production as indicated in the text for each test (see above).

2.7 Antibiotic susceptibility testing

Conventional antibiotic sensitivity testing was performed using the disk diffusion method and the type of resistance was determined based on the guidelines described by Schreckenberger and Rekasius (2012). For this purpose 150 mm Mueller Hinton Agar plates (MH plates) were prepared a day before the indented susceptibility testing. The procedure was performed as the following:

1- MH agar plate and antibiotic disk dispenser were allowed to come to room temperature before use.

2- A 0.5 McFarland standard was prepared using sterile saline from freshly grown colonies as described in 2.4 or 2.6 above.

3- Bacterial suspension was distributed on MH Agar using a sterile cotton swab.

4- Using a sterile forceps, antibiotic disks (Oxoid) were distributed on the MH agar in positions as illustrated in Figure 2.2 according to Schreckenberger and Rekasius (2012).

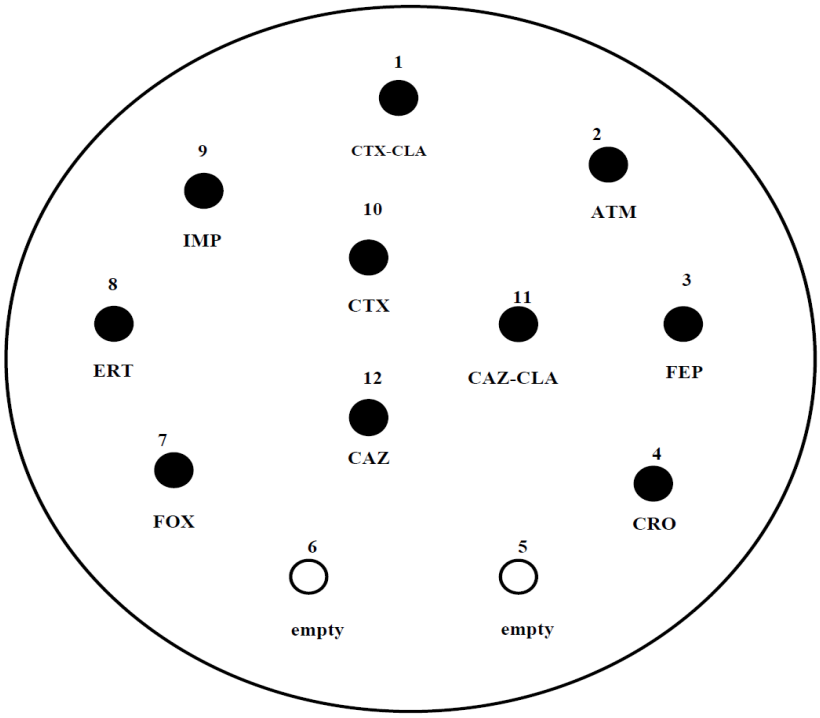


Figure 2.2. Template for antibiotic disk positions for Detecting ESBL and ampC beta-lactamases. The antibiotics included first, second (here Cefoxitin; FOX), third (here ceftriaxone; CRO, cefotaxim; CTX, and ceftazidime; CAZ), and fourth (here cefepime; FEP) generation cephalosporins. Antibiotic concentration was as following: Aztreonam (ATM, 30 µg), Ceftazidime (CAZ, 30 µg), Ceftazidime + Clavulante (CAZ-CLA, 30/10 µg), Cefotaxime (CTX, 30 µg), Cefotaxime + clavulante (CTX-CLA, 30/10 µg), Cefoxitin (FOX, 30 µg), Ceftriaxone (CRO, 30 µg), Cefepime (FEP, 30 µg), Ertapenem (ETP, 10 µg), Imipenem (IMP, 10 µg). All antibiotic disks were purchased from Oxoid, UK.

1- Detection of ESBLs:

Detection of ESBLs was based on the sensitivity of ceftazidime and cefotaxime disks with and without clavulanic acid. Thereafter sizes of inhibition zones for these antibiotics were measured; if an increase of 5 mm or more was detected in the combination with clavulanate for either antibiotics, the isolate was considered an ESBL.

Furthermore, if the inhibition zones of any of the cephalosporin antibiotics and the clavulanate combination disks open towards each other causing a phenomenon called "keyhole" effect, ESBL isolate is predicted (Figure 2.3).

Figure 2.3: Keyhole effect phenomena between ceftazidime (CAZ) in combination with clavulanic acid (CAZ-CLA) and aztreonam (ATM)/cefepim (FEP). The figure clarifies the keyhole effect, which shows the inhibition zones of CAZ-CLA and ATM, CAZ-CLA and FEP extending into each other's.

2- Detection of AmpC beta lactamases:

Detection of AmpC beta lactamases was based on cefepime and cefoxitin disks. Hereby, AmpC isolates are resistant to the cephamycins, here cefoxitin (FOX), susceptible to cefepime (FEP). Furthermore, high-level AmpC producers are resistant to 1^{st}, 2^{nd}, and 3^{rd} generation cephalosporins and monobactams, here Aztreonam (ATM).

3- Detection of K1 beta lactamases:

The detection of K1 beta lactamases was based on the antibiotics aztreonam, ceftazidime, cefotaxime and ceftriaxone. An isolate is K1 beta lactamase positive, when monobactams antibiotic aztreonam (ATM) is resistant, while third generation antibiotics CAZ, CTX and CRO are sensitive and no CLA effect is detectable.

4- Detection of Carbapenemase:

Carbapenemases Enterobacteriaceae isolates, also known as carbapenem resistant Enterobacteriaceae (CRE), are resistant to ertapenem and sensitive to imipenem. Furthermore such isolates can also be Klebsiella pneumoniae Carbapenemase (KPC) producers, which can only be confirmed by PCR test.

If the isolates were none of the mentioned above, sensitivity and resistance to the antibiotics was recorded. Record all disk diffusion mm zone size readings in the culture work up.

2.8 Molecular biology detection of antibiotic resistance

Resistance to antibiotic is regularly encoded by plasmid or on chromosome, for detection polymerase chain reaction (PCR) of the suspected gene encoding resistance is performed.

2.8.1 Preparing sample for PCR; "colony PCR"

Isolates were not subjected to DNA extraction by the means of isolating the bacterial chromosomal or plasmid DNA, rather than lysed by heating, which causes the release of plasmid DNA. We used the fast boil method (Holmes and Quigley, 1981) to lyse the bacterial cell wall and membrane and set the plasmid DNA free, a method also known as colony PCR. One bacterial colony, grown as described in 2.6 above, was picked using sterile disposable plastic loop and transferred into 50 µl sterile distilled water in 1.5 ml safe lock eppendorf tube. Thereafter the tube was placed in dry heat block at 95 ºC for 10 minutes and centrifuged at 5000 rpm for 2 minutes. Finally the supernatant was transferred to a new sterile eppendorf tube and was used as sample DNA mixture for the following PCR step.

2.8.2 Detection of resistant genes using PCR

Specific primer pairs were used to amplify the resistance encoding genes *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, OXA-48, NDM, and KPC (table 2.1).

Table 2.1. Primers used to detect resistance in the bacterial isolates. Once an isolate was detected as ESBL, it was subjected to PCR analysis for TEM, CTX and SHV. CRE isolates were subjected to PCR analysis of KPC, NDM and OXA48.

Gene	Primer Sequence	PCR product (bp)	Reference
bla$_{SHV}$ F	5'-TCGGGCCGCGTAGGCATGAT-3'	625	Melano et al., 2003
bla$_{SHV}$ R	5'-AGCAGGGCGACAATCCCGCG-3'		Melano et al., 2003
bla$_{CTX-M}$ F	5'-TGATGACTCAGAGCATTCG-3'	864	Melano et al., 2003
bla$_{CTX-M}$ R	5'-TATTGCATCAGAAACCGTG-3'		Melano et al., 2003
OXA-48F	5'-GAGCACTTCTTTTGTGATGGC-3'	718	Poirel et al., 2004
OXA-48R	5'-TTGGTGGCATCGATTATCGG-3'		Poirel et al., 2004
KPC- F	5'-CATTCAAGGGCTTTCTTGCTG-3'	686	Our design
KPC- R	5'-GATTTTCAGAGCCTTACTGCC-3'		Our design
*bla*TEM-F	5'-TTGGGTGCACGAGTGGGTTA-3'	972	Melano et al., 2003
*bla*TEM-R	5'-TAATTGTTGCCGGGAAGCTA-3'		Melano et al., 2003

The total PCR reaction of 25 µl included 5.25 µl DNA mixture from step 2.8.1, 1 µl (10pmol/ µl) of each primer (forward and reverse) and 12.5 µl PCR master mix (ready mix PCR master mix, cat # AB-0575/Dc/LD, Thermo Scientific). The PCR reaction started with a single hot start step for 4 minutes at 95 ºC followed by 30 cycles in the following order, 40 seconds at 95 ºC (denaturation step), 40 second at 55 ºC (annealing step) and 1 min at 72 ºC (extension step) respectively. To assure the complete extension step, additional extension step was performed for

7 minutes at 72 ºC, finally the reaction was cooled down to 4 ºC and the product either frozen at -20 ºC or directly subjected to gel electrophoresis for result analysis.

2.8.3 Detection of PCR product

To determine the amplified gene product of the PCR reactions, agarose gel electrophoresis was used to separate the PCR product. 1% agarose (Ameresco) gel was prepared in 1x TAE (50x TAE= 2M Tris-Acetate, 0.05 M EDTA, pH 8). The agarose was boiled until it was fully dissolved; Ethidium bromide was added when agarose suspension cooled down to 40 ºC, carefully mixed, poured into agarose gel casting system (Bio Rad, UK or cleaver, USA) then a desired comb was inserted and 10 µl of the PCR product was added directly into the gel well. Along with the samples, 2 µl of 100 bp marker (gene ruler express DNA ladder, Fermentas, CAT# SM1558) was added and gel electrophoresis was run for 30 min at 100mV. PCR and marker bands were visualized under UV light. A digital image of the gel was taken using digital camera of the gel documentation system (Pharmacia, biotech).

3. Results

3.1 Regional distribution of samples

Vegetable samples were collected from different regions in the West Bank, however over half of the sample were from Jericho being a major Palestinian vegetable farming site. A total of 59 samples of lettuce, mint and watercress were collected. Some of the samples were purchased from farms, other from local markets. The number of samples, whether they were purchased directly from farm or market are illustrated in Table 2.1.

Table 3.1. Sample types and their regional distribution. The region from which samples were collected is indicated under region. The number, type (lettuce, mint, or watercress) and purchase source (market or farm) are also indicated for each type.

Region	# of samples	Lettuce Sample	Source	# of samples	Mint Sample	Source	# of samples	Watercress Sample	Source	# of samples
Ramallah	3	2	Market	1	1	Market	1	0	Market	0
			Farm	1		Farm	0		Farm	0
Jericho	31	13	Market	3	11	Market	10	7	Market	7
			Farm	10		Farm	1		Farm	0
Tulkarem	9	9	Market	2	0	Market	0	0	Market	0
			Farm	7		Farm	0		Farm	0
Israel	7	4	Market	4	3	Market	3	0	Market	0
			Farm	0		Farm	0		Farm	0
Bethany	3	1	Market	1	2	Market	2	0	Market	0
			Farm	0		Farm	0		Farm	0
Jenin	1	1	Market	1	0	Market	0	0	Market	0
			Farm	0		Farm	0		Farm	0
Abu-Dies	5	4	Market	4	1	Market	1	0	Market	0
			Farm	0		Farm	0		Farm	0
Total	**59**	**34**			**18**			**7**		

3.2 Microbiological methods

All lettuce, mint and watercress samples were subjected to three test methods for bacterial growth and resistant bacterial growth upon preparing the homogenate as described in 2.2. Detection of bacterial growth using the Coliform and fecal Coliform was essential to test whether bacterial growth is occurring on first place. Nevertheless the third methods using ampicillin MacConkey plates occurred parallel to have an overall evaluation of enterobacterial growth and resistant bacterial growth simultaneously.

3.2.1 Detection of bacteria and resistant isolates from samples

All 59 samples were subjected to the three culture methods mentioned above. Lettuce samples from different regions in Palestine were subjected to three microbial tests as MacConkey +Ampicillin, Coliform and fecal Coliform (see supplementary data for example of growth). As shown in Fig 3.1 a lettuce samples from Jericho, Ramallah, Tulkarem, Israel, Abu-Dies, Bethany and Jenin tested positive for bacterial growth on MacConkey +Ampicillin and total Coliform.

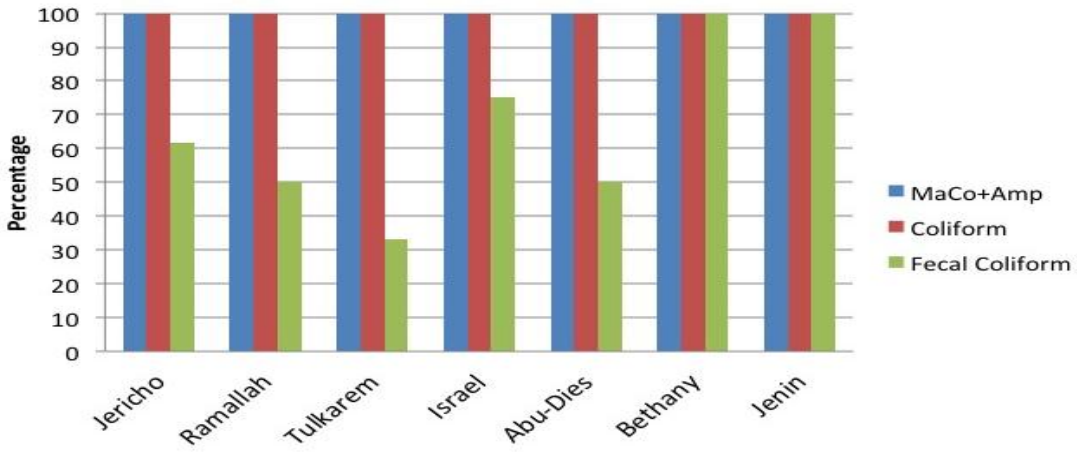


Figure 3.1. Detection of bacterial growth in different lettuce samples purchased from different regions. The total sample size of lettuce was 34 lettuce samples, 13 from Jericho, 2 from Ramallah, 9 from Tulkarem, 4 from Israel, 4 from Abu-Dies, 1 from each Bethany and Jenin respectively.

All tested lettuce samples from Bethany and Jenin tested positive for fecal coliform, while 25-60% of the samples from the others areas tested positive for bacterial growth with this method (Figures 3.1).

Testing mint samples for bacterial growth with the three methods revealed that with the exception of the samples from Jericho all others tested positive for ampicillin resistant isolates (Figure 3.2). All samples from Ramallah, Israel and Bethany tested positive for total Coliform, all those from Ramallah and Israel tested also positive for fecal Coliform (Figure 3.2).

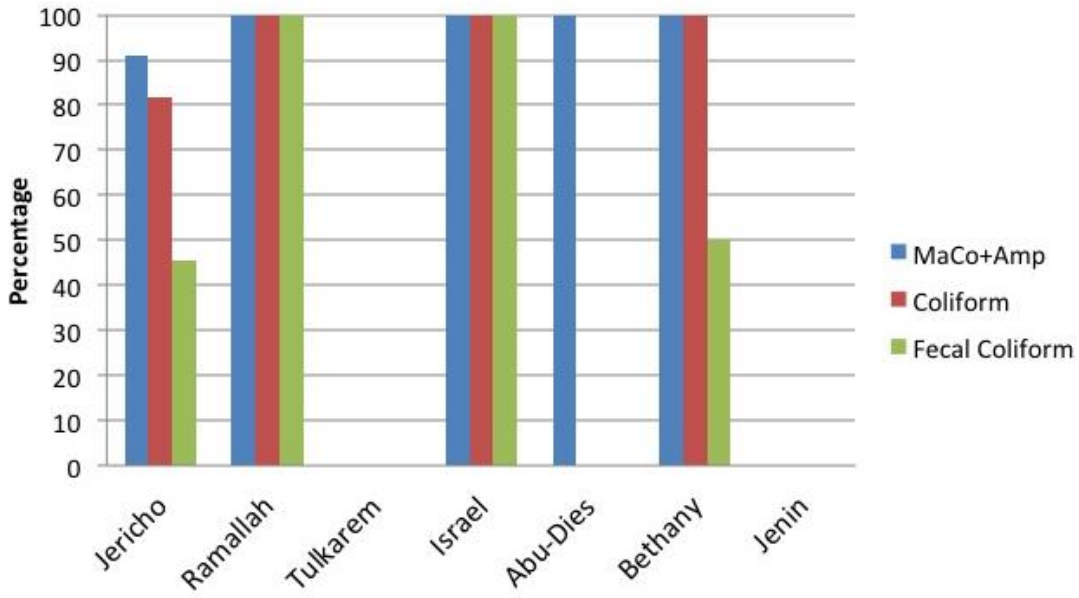


Figure 3.2: Detection of bacterial growth in different mint samples purchased from different regions. The total sample size of Mint was 18 Mint samples, 11 from Jericho, 1 from Ramallah, 3 from Israel, 1 from Abu-Dies and 2 from Bethany respectively.

All seven watercress samples purchased from Jericho tested positive for bacterial growth on ampicillin plates, for Coliform and fecal Coliform respectively (Figure 3.3).

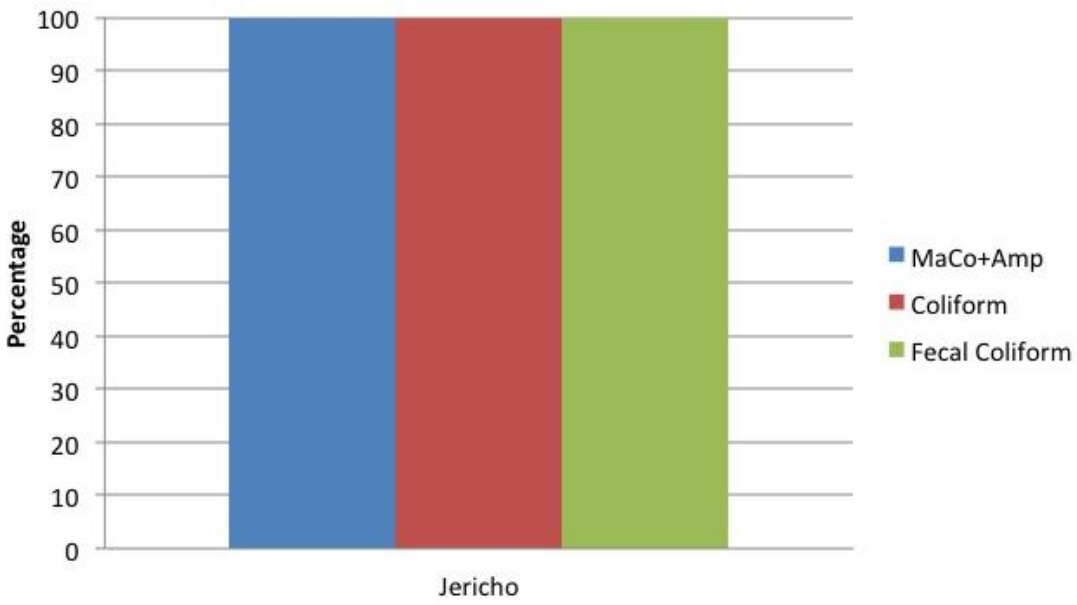


Figure 3.3. Detection of bacterial growth in different watercress samples purchased from Jericho. The total sample size of watercress samples was 7.

3.3 Differentiation of bacteria isolated from raw vegetables

Enterotest tube system is widely used for differentiation of Enterobacteriaceae based on variable simultaneous biochemical reactions as detailed in 2.6. The most dominant Enterobacteriaceae detected overall was Enterobacter, followed by *Pseudomonas spp*., *E. coli* and *Yersinia enterocolitica* (Figure 3.4). Beside Enterobacteriaceae, *Pseudomonas spp.* was present in many samples with variation in prevalence based on the vegetable tested. *Enterobacter spp.* was the most predominant bacteria (67.65%) in lettuce samples, followed by *Pseudomonas spp.* (14.7%), then by *E. coli*, and *Yersinia enterocolitica* (8.82% each) (Figure 3.5). *Enterobacter spp.* was also the most predominant (68.75%) bacteria isolated from mint samples, followed by *E. coli*

(18.75%), *Yersinia enterocolitica* and *Pseudomonas spp.* (6.25% each) (Figure 3.6). In contrary to lettuce and mint, *Pseudomonas spp.* (28.57%) was the second predominant isolate from watercress samples after *Enterobacter spp.* (42.86%), followed by *E. coli* and *Yersinia enterocolitica* (14.29% each) as clearly demonstrated in Figure 3.7.

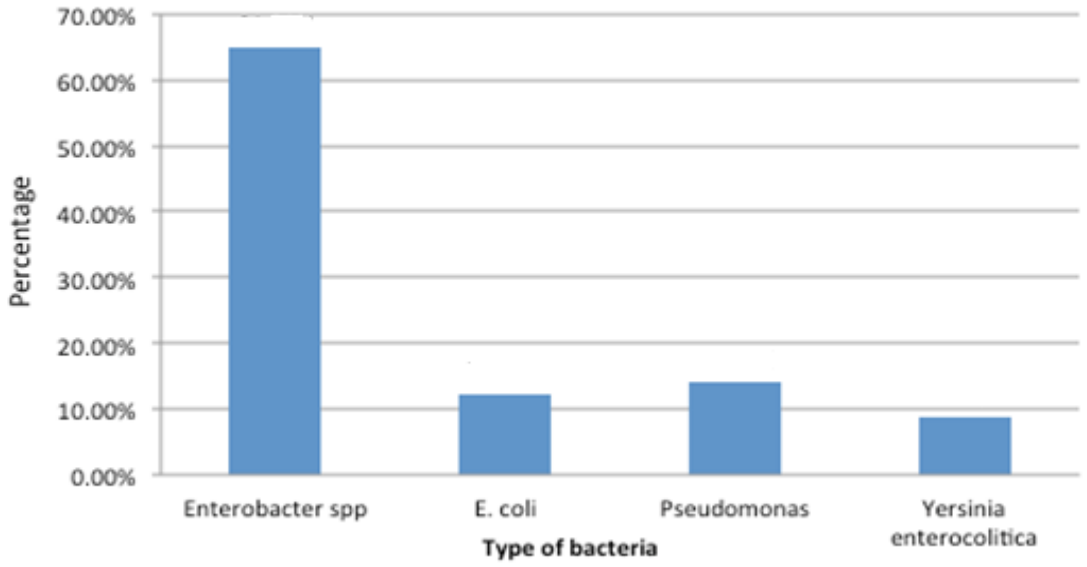


Figure 3.4. Types of bacteria isolated from the raw vegetable samples. Enterotest tube was used for differentiation of Enterobacteriaceae, while smell, color and antibiotic resistance was used for confirmation of Pseudomonas isolates. Growth was detected in 52 out of the 59 total samples subjected for analysis.

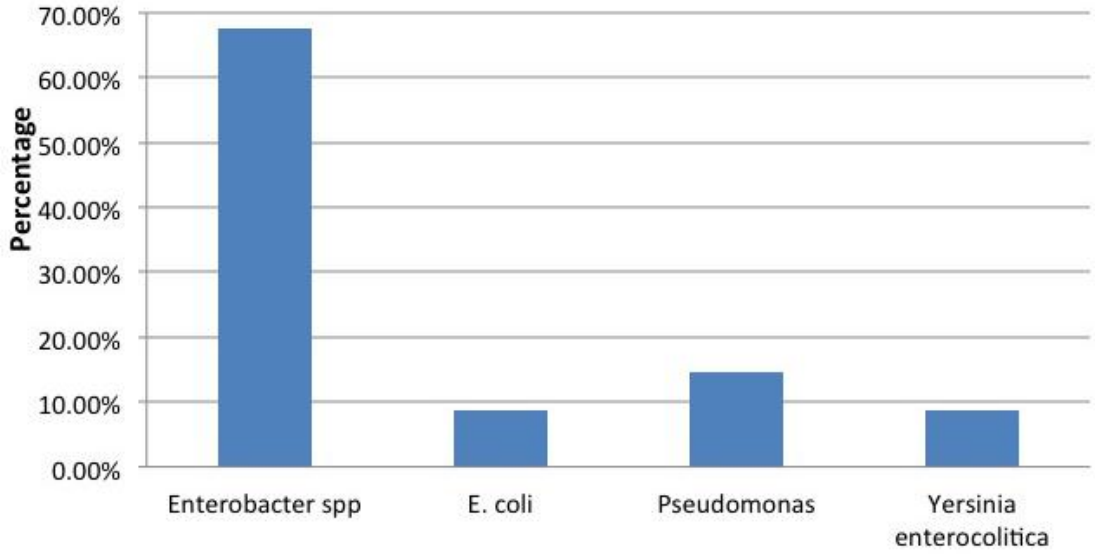


Figure 3.5. Types of bacteria isolated from lettuce samples. Growth was detected in 30 out of the 34 total samples subjected for analysis, 4 of which resulted in isolation of two different types of bacteria.

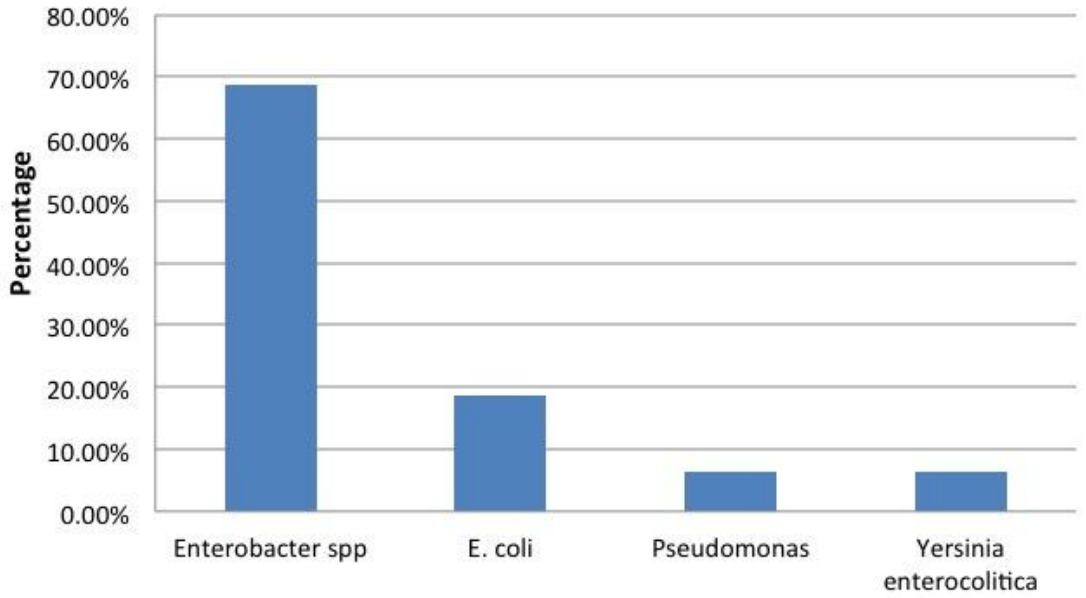


Figure 3.6. Types of bacteria isolated from the mint samples. Growth was detected in 15 out of the 18 total samples subjected for analysis, one of which resulted in isolation of two different types of bacteria.

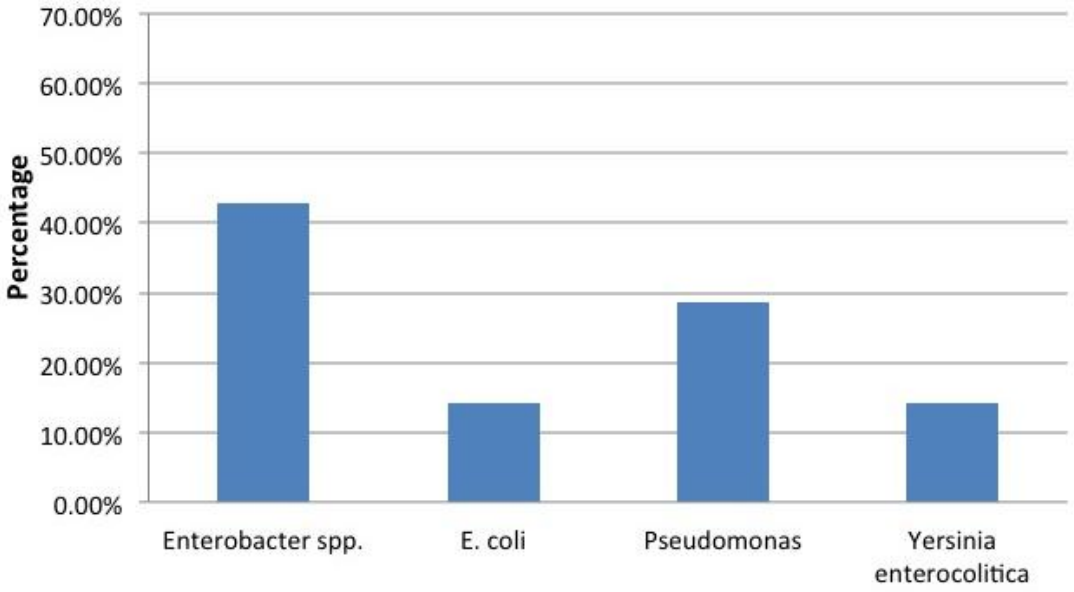


Figure 3.7. Types of bacteria isolated from watercress samples. Growth was detected in all 7 samples subjected for analysis.

3.4 Antibiotic Susceptibility testing

All 49 *Enterobacteriaceae* isolates were subjected to antibiotic susceptibility testing using the guidelines described by Schreckenberger, PhD and Violeta Rekasius (2012) as detailed in section 2.7. An example of CRE isolate is illustrated in Figure 3.8 from one of the samples tested ion this work, which shows a 100% resistance to ertapenem.

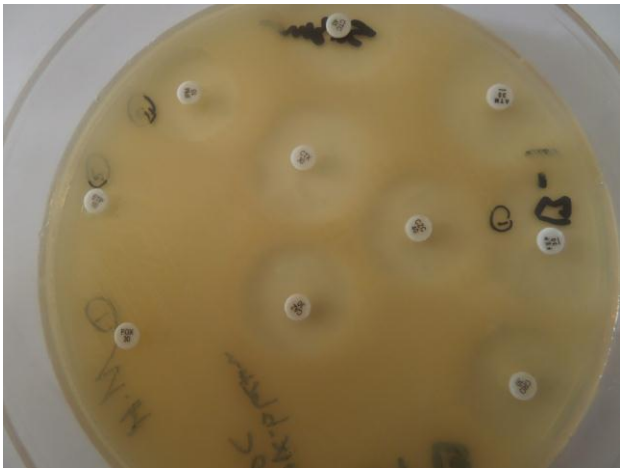


Figure 3.8. A CRE isolate detected using the conventional disk diffusion test (photo from this current research). Resistance to antibiotic disk ertapenem is crucial test for detection of CRE resistance as clearly visible on the left side of this example plate by 9 O'clock.

Interestingly, CRE was most common resistant Enterobacteriaceae (55.01%) detected in all raw vegetables samples, followed by ESBLs (24.49%). There was one case of multidrug resistance (MDR) and few cases of AmpC beta lactamases (12.25%) and K1 beta lactamases (6.12%) (Figures 3.9). Please also see supplementary data on antibiotic inhibition zone for the samples.

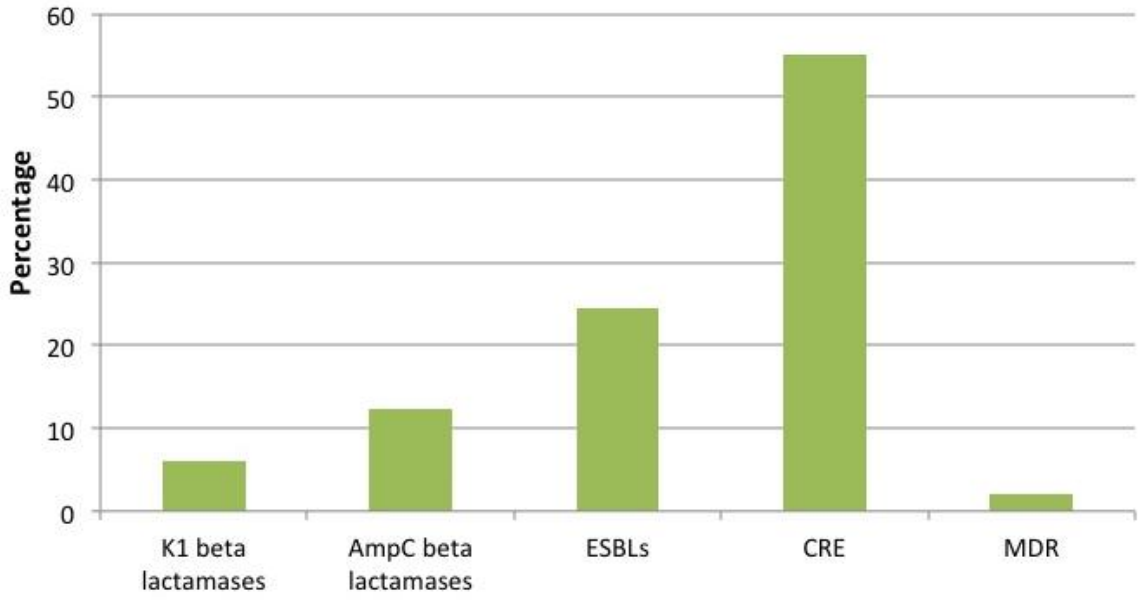


Figure 3.9. Type of resistance in *Enterobacteriaceae* isolates. Resistance was determined using antibiotic susceptibility testing based on guidelines described by Schreckenberger and Rekasius (2012). The percentages represent isolates in all samples tested. Total number of Enterobacteriaceae was 49.

Figure 3.9 summarizes the result for each sample type, CRE, ESBLs, and K1 beta lactamases were present in all samples types; lettuce, mint, and watercress. AmpC beta lactamases and MDR were present in lettuce only.

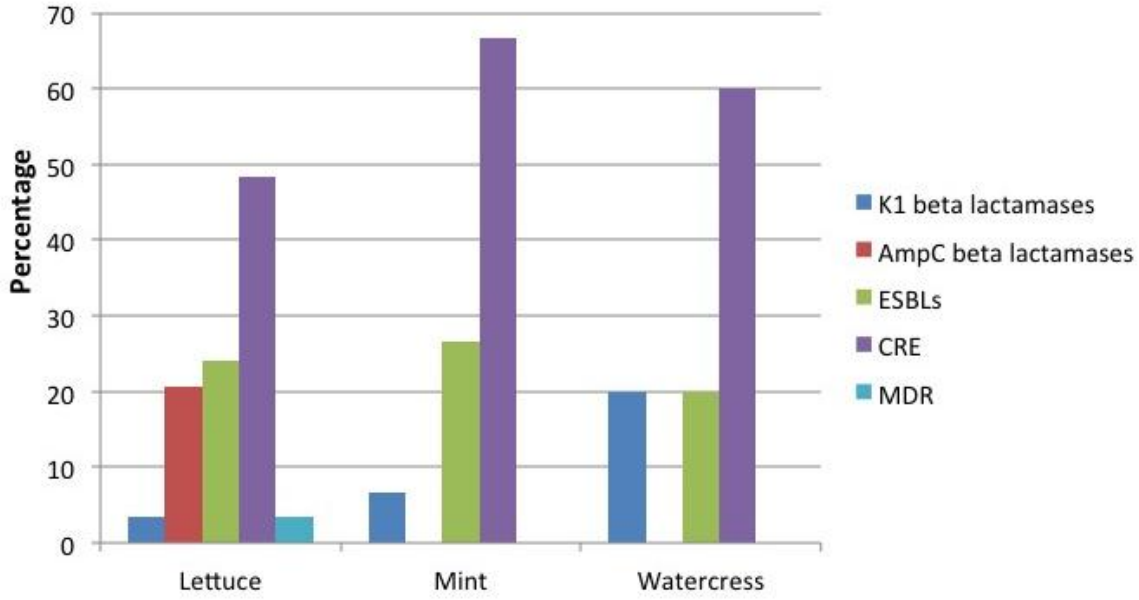


Figure 3.10. Distribution of resistance types in the different samples. Total number of Enterobacteriaceae detected in lettuce was 29, in mint 15 and in watercress 5 respectively.

Furthermore we investigated the resistance in each type of the Enterobacteriaceae detected. The highest rate of CRE resistance was detected in *Enterobacter* spp. and *Yersinia enterocolitica*, while ESBLs were highest in *E. coli* isolates (Figure 3.11). AmpC was detected only in *Enterobacter* spp., K1 in *Enterobacter* spp. and *E. coli* (Figure 3.11). The only case of MDR was detected in *Yersinia enterocolitica* (Figure 3.11).

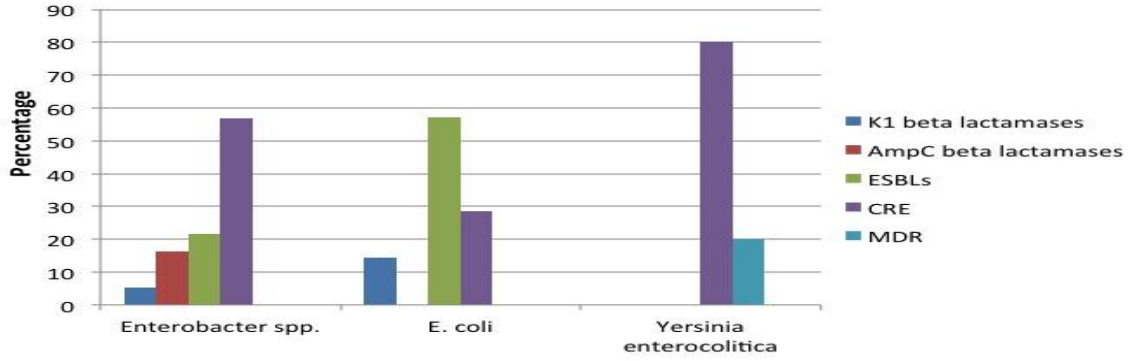


Figure 3.11. Distribution of resistance types in isolated Enterobacteriaceae types. The total number of *Enterobacter* spp. isolates was 37, of *E. coli* 7 and of *Yersinia enterocolitica* 5 respectfully.

3.5 Detection of gene encoded resistance

Enterobacteriaceae isolates, which were identified with drug resistance using the antibiotic susceptibility testing, were also subjected to PCR analysis for resistance-encoding genes. All CRE isolates were subjected to PCR testing using KPC and OXA48 primer, while those identified as ESBLs were subjected to TEM, CTX and SHV PCR testing. An example of PCR testing is illuminated in Figure 3.12.

Out of the 27 CRE positive isolates, five tested positive for KPC resistance-encoding gene, but none tested positive for OXA48 (Figure 3.12). Three and two out of the 12 ESBL isolates tested positive for TEM and SHV resistance-encoding genes respectively (Figure 3.13).

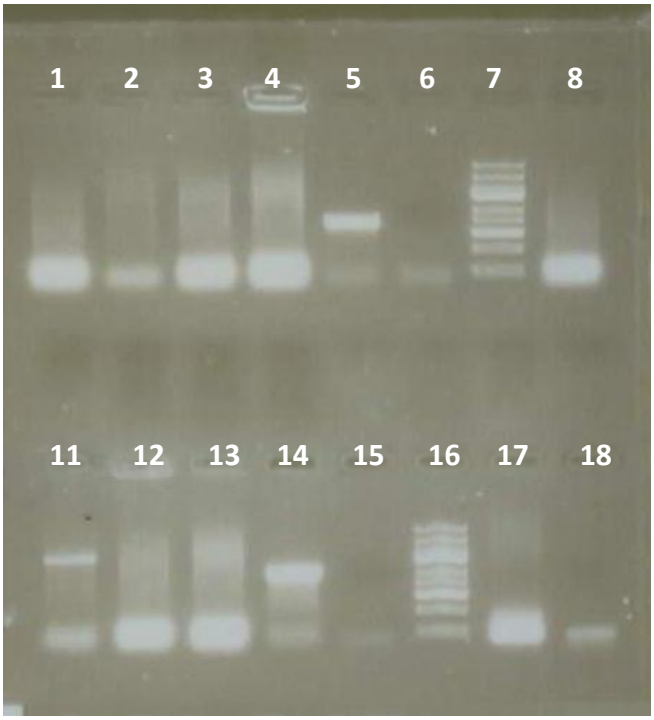


Figure 3.12: Gel electrophoresis of different PCR products. Upper gel: KPC PCR. Lane 1-4= samples 2, 7, 9 and 10; lane 5=positive control (686bp); lane 6=negative control. Lower gel and lane 8: TEM PCR. Lane 11-13=samples 7, 9 and 10; lane 14=positive control (~900bp); lane 15=negative control; lane 16=bp size marker; lane 17=sample 7, lane 18=sample 8.

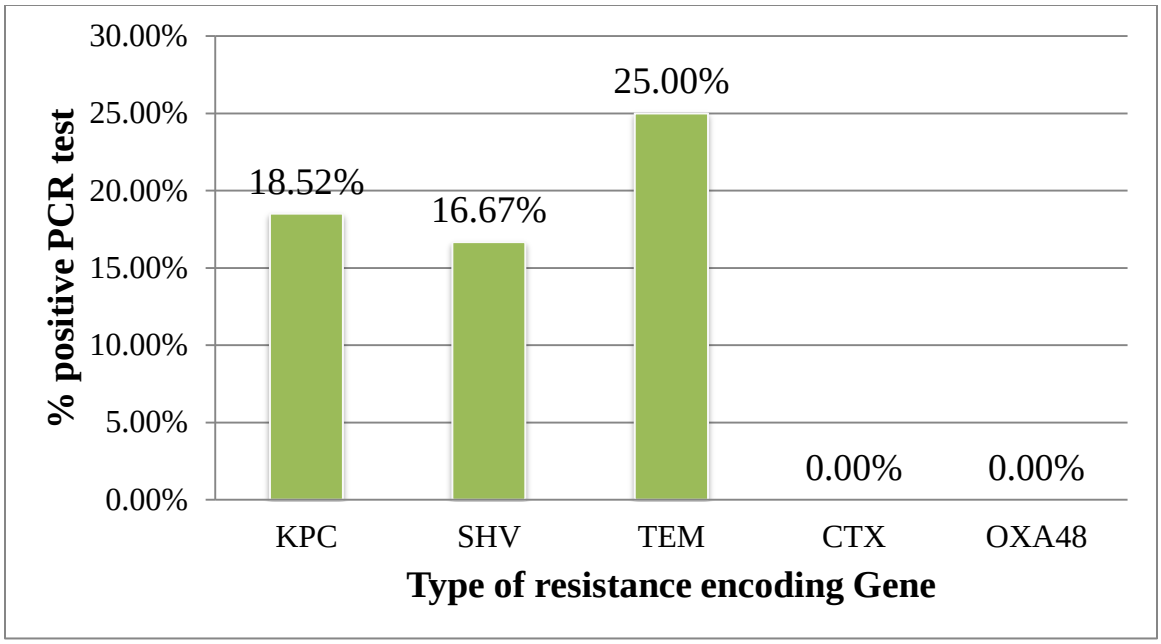


Figure 3.13: PCR Detection of resistance-encoding genes. All CRE isolates (27) were subjected to KPCR and OXA48 PCR testing, while all those ESBL isolates (12) were subjected to TEM, SHV, and CTX PCR testing.

3.6 Market samples versus farm samples

We compared the results for the samples obtained from the market with those obtained directly from farms. For this we excluded watercress and peppermint samples, as most samples were lettuce and a comparable number of samples from market versus farms were present. A total of 18 lettuce samples came from farms, while 16 came from markets.

Comparing the initial testing for coliform and growth on ampicillin containing MacConkey plates revealed no difference, but 25% less fecal coliform growth among farm samples (Figure 3.14) respectively. On the other hand, types of bacteria isolated from farm samples were not significantly different than those isolated from the market samples; while rather more *Enterobacter spp*. were isolated from farm samples, they did not contain *E. coli*, which was present in Market samples only (Figure 3.15). Finally the incidence of resistant bacterial isolates in farm samples was as high as in market samples (figure 3.16).

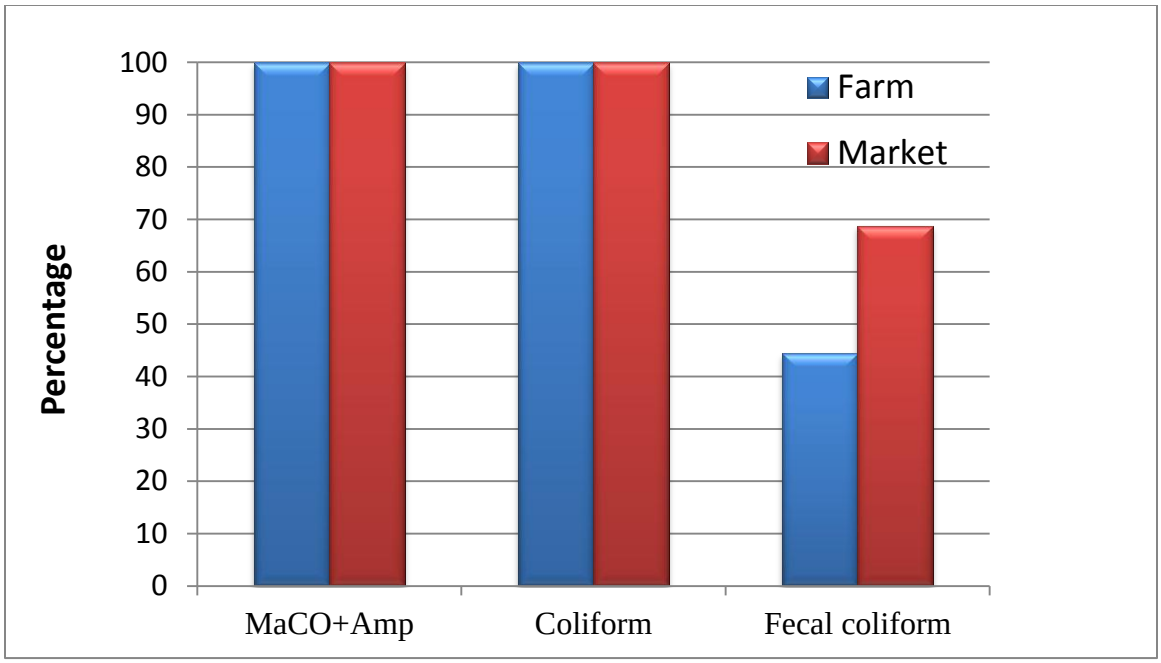


Figure 3.14: *Coliform, fecal coliform* and growth on ampicillin containing MacConkey in market and farm lettuce samples. A total of 18 lettuce samples from farms and 16 from markets were compared for the presence of bacterial growth using the three methods of detection.

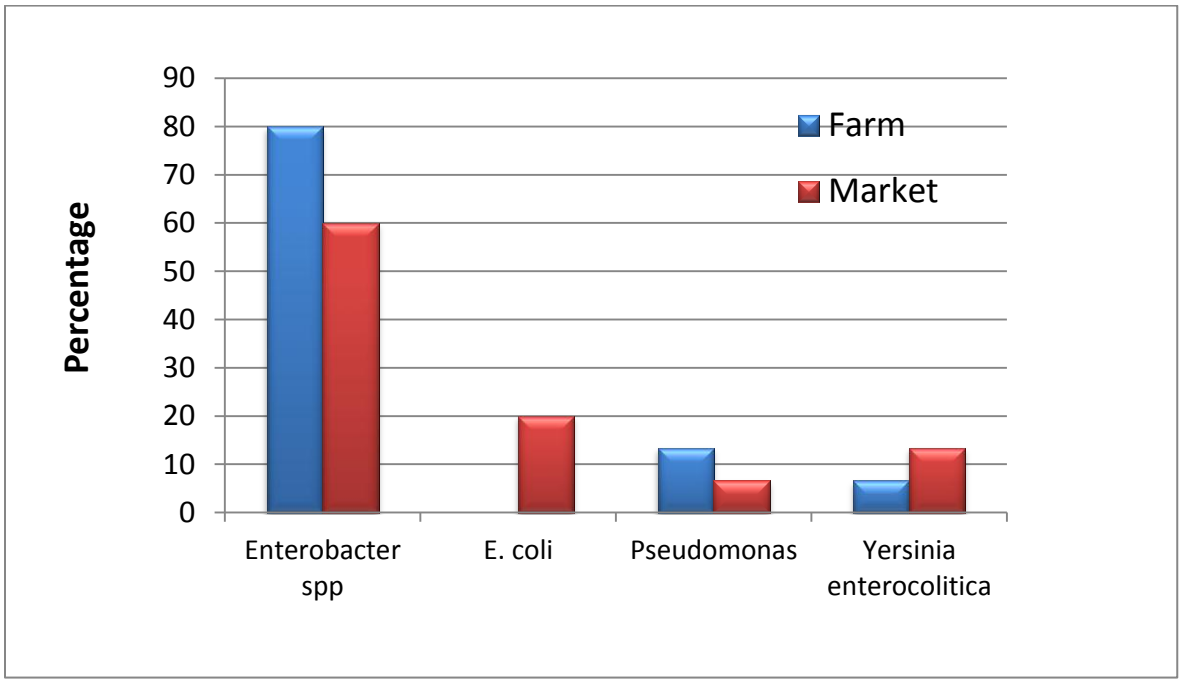


Figure 3.15: Types of bacteria isolated from farm lettuce sample versus market lettuce samples. Types of bacteria isolated from 18 farm lettuce samples were compared with those isolated from 16 market samples.

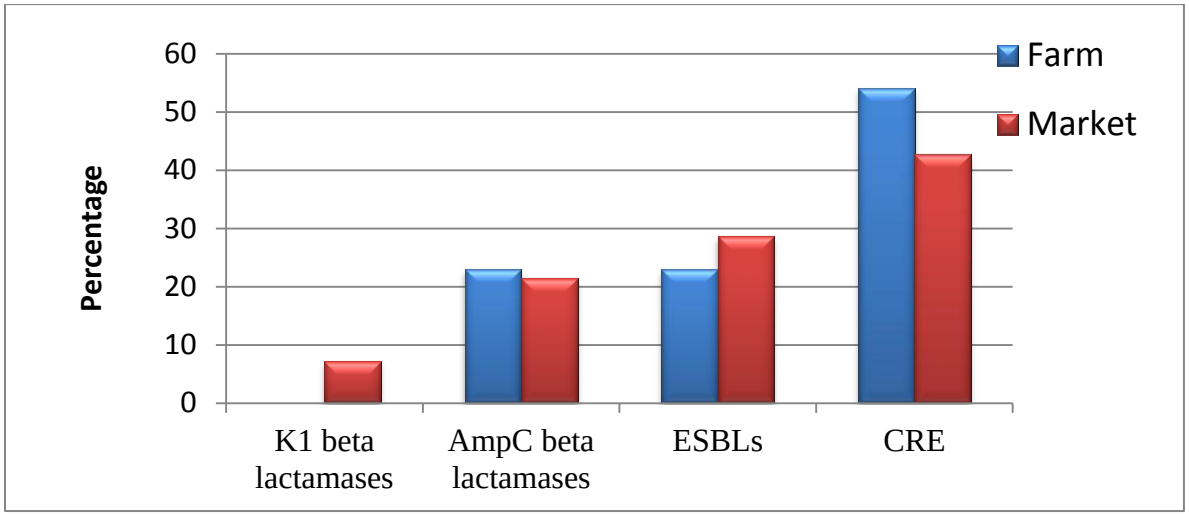


Figure 3.16: Type of resistance detect in farm lettuce sample versus market lettuce samples. A total of 18 lettuce samples from farms and 16 from markets were compared for the presence of the different resistance types in isolated strains.

4. Discussion

The level of safety eating raw vegetables is pretty unpredictable. While manufacturer of different edibles guarantee the safety of their conserved canned or packed food, while farmers and markets cannot give an exact data on the safety of raw vegetables. Raw vegetables are not subjected to any treatment, which may guarantee the lack of contamination, was it chemical or biological. Generally people tend to buy vegetables based on their knowledge and basic information given by the market dealer or farmer. In Palestine, markets do not show any etiquette on their vegetables, people depend on the information provided by that market dealer/merchant/greengrocer. All these problematic aspects were the driving force to perform this research to identify the possible contaminants in Palestinian fresh leaf vegetable products.

4.1 Overall contamination of leaf vegetables

Our study reveals that most leaf vegetables are highly contaminated with coliform and fecal coliform. Contamination was highest in lettuce and watercress. Indeed, all lettuce samples were contaminated with ampicillin resistant bacteria as well as coliform, while fecal coliform were less prevalent overall. The seven watercress samples tested positive for all three initial tests, ampicillin resistant bacteria, coliform and fecal coliform respectively. Coinciding with our results, salads were shown to possess levels of coliforms contamination up to 100% (Falomir et al., 2010a).

One can argue that the high level of contamination indicated in this research could be referred to the fact that the leafy vegetables were not washed, however different studies showed that standard washing procedure of vegetables with water did not remarkably affect the level of contamination (Falomir et al., 2010a). The main reason for this lies in the ability of coliform bacteria to attach to vegetables surfaces, a mechanism, which is not affected by water washing (Brandl, 2006; Tyler and Triplett, 2008; Heaton and Jones, 2008).

Most mint samples came from Jericho and they were 10% less contaminated than the lettuce samples. It had been demonstrated earlier by several studies, that the essential oils contained in mint have antibacterial activities (Hyldgaard et al., 2012). In fact,

these oils had been considered for usage in food preservatives due to this activity (Hyldgaard et al., 2012). An earlier study performed in Japan documented that essential oils of mint inhibit different types of gram-positive and gram-negative bacteria, including drug resistant strains (Imaitt et a., 2001). A very recent study illuminated the role of peppermint essential oil in inhibiting coliform bacteria using a very delicate experiment (Witkowski and Sowinska, 2013).

Taken together, despite the unequal number compared with lettuce, it was expected to detect less contamination in mint samples.

4.2 Bacteria causing contamination

The overall most prevalent bacteria causing contamination was *Enterobacter* spp., followed by *Pseudomonas spp*., *E. coli*, and *Yersinia enterocolitica*. *Pseudomonas* was not the target of this study; however, it is worth mentioning that it plays a major cause of bacterial contamination of raw vegetables including strains with multiple drug resistance (reviewed in Allydice-Francis and Brown, 2012).

This profile of bacterial types causing contamination in leaf vegetables was similar for lettuce and watercress samples, with the exception that both *E. coli* and *Yersinia enterocolitica* presented equally in the samples. In the case of mint samples *E. coli* was the second type of bacteria causing contamination, while *Pseudomonas spp.* and *Yersinia enterocolitica* presented equally in the samples after were the third prevalent type.

While *Enterobacter spp.*, *Pseudomonas spp*. and *E. coli* are typically found in contaminated fresh vegetables, *Yersinia enterocolitica* is more likely to be prevalent in those packaged refrigerated vegetables (Szabo et al., 2000). With the exception of one samples, all five *Yersinia enterocolitica* positive samples were purchased from markets, two from Israel, two from Tulkarem, and one from Jericho. Only the two samples from Israel were refrigerated, the other 3 samples were refrigerated at some point before sale. Another source of contamination with these specific bacteria could be due to refrigeration step before performing this research at our laboratory.

Enterobacteriaceae belong to the most common bacteria contributing to vegetable contamination causing outbreaks (Hamilton et al., 2006; DuPont, 2007; Tyler, 2008; Falomir et al., 2010a). The major factors contributing to contamination with these bacteria are treating organic fertilizers and irrigation water (Hamilton et al., 2006; Heaton and Jones, 2008).

4.3 The prevalence of the types of resistance causing contamination

Our research revealed surprising findings regarding the resistance profile in bacteria contaminating leaf vegetables. Hereby, carbapenem *resistance Enterobacteriaceae* (CRE) was the most common type of resistance, followed by ESBLs, AmpC beta lactamases, K1 beta lactamases, and one single multi-resistant type, which was resistant to all antibiotics used in this study.

The percentage of drug resistant bacteria detected in this study is extremely high and unexpected. ESBLs had been regularly tested in different studies on raw vegetables including salad (Rasheed et al., 2014, Blaak et al., 2014; Ben Said et al., 2015; Van Hoek et al., 2015; Reuland et al., 2014; Niage and Buys, 2014). Although a high prevalence of ESBLs had been described earlier in study on generally fresh produce in Netherlands (Blaak et al., 2014), another two Dutch study on different types of vegetables, which were purchased from stores including a type of lettuce, revealed a low prevalence of bacterial isolates ESBLs and AmpC beta lactamases (Reuland et al., 2014; Van Hoek et al., 2015). A high incidence of ESBL, in lettuce specifically had been reported in South Africa (Niage and Buys, 2014), which is in accordance with our findings introduced in this research.

Studies on CRE isolates from vegetables were generally rare. A US study reveals that gram-negative isolates resistant to imipenem were detected in 38.3% of triple washed ready-to-eat baby spinach (Walia et al., 2013).

4.4 The prevalence of genes encoding resistance in bacteria causing contamination

There are different mechanisms causing resistance of bacteria (see introduction). One of these mechanisms is caused by enzymes, which degrade the antibiotic. Such enzymes are genes' encoded and we tested for the most common genes causing CRE and ESBL, which were the most common types of resistance detected in this research.

All enteric bacteria isolated from the leafy vegetables, which tested positive for CRE using conventional methods were examined for the existence of genetic markers encoding enzymes causing this resistance; i.e. KPC and OXA48 genes. Only 5 (18.52%) out of 27 CRE samples tested positive for the most common gene causing CRE; KPC. None of the samples tested positive for OXA48. The 12 enteric bacteria tested positive for ESBLs using conventional methods were subjected to PCR analysis of three genes encoding for ESBL resistance; only three carried the TEM gene and another 2 carried the SHV gene respectively. None tested positive for CTX, another common gene encoding for ESBL resistance.

As enzymes encoding resistance is only one of the major mechanisms encoding resistance in bacteria, our data strongly suggest that the 22 CRE and 7 ESBL enteric bacteria are resistant due to mechanisms other than degrading enzymes. One of these other mechanisms is "efflux pumps", which force antibiotics out of the cell causing resistance to that antibiotic.

There are different types of efflux pumps regulated by different genes and cause resistance to different antibiotics. The most common efflux pumps found in *Enterobacter spp.* cause resistance to different antibiotics and drugs are EmmdR Multidrug Efflux Pump, the AcrAB-TolC Efflux Pump System, outer Membrane Proteins OmpD and OmpF, and SugE Multidrug Efflux Pump (Andersen et al., 2015). The outer Membrane Proteins OmpD and OmpF, which associates with the AcrAB-TolC Efflux Pump System is the one responsible for carbapenem resistance (Andersen et al., 2015; Bornet et al., 2003).

E. coli encodes for different efflux pumps, which are involved in pumping out

different drugs, including those found in the environment as a survival strategy, however the AcrA, AcrB, and TolC Multidrug Efflux Pump is the one responsible for resistance to a wide a range of antibiotics (Anderson et al., 2015; Sikora and Turner, 2005).

Furthermore, carbapenem resistance in Enterobacteriaceae, *E. coli* and *Enterobacter* spp. specifically can be granted via reduced drug permeability. Hereby, a loss or functional changes in porins, which normally allows drug entry into the cell, results in resistance to carbapenems and other classes of antibiotics (Page, 2012).

4.5 Effect of water quality on vegetable safety

It is generally accepted that water quality used for irrigation affects the microbiological quality of vegetables being irrigated. Farms, which sell the products tested in this study, depend on rainwater or/and open canals' distributions for irrigation; none of which is subjected to controlled inspections. Farms in Jericho depend mainly on canal distribution, which receive water from natural springs, while those from Tulkarem depend mainly on rainwater collection wells. In both cases, water quality for irrigation is not controlled in all stages. This means that even if spring water is inspected regularly, running through the canal system may be very risky for the water quality, as animals as well as human are using this water system, even for unhealthy purposes. Rainwater collection is dependent on very primitive methods, which cannot guarantee this water quality on the long term or even before being accumulated in the designated wells. There is a lack of inspection on water as well as the field used for that exact type of vegetables. An earlier research project at Al-Quds University showed that most drinking and irrigation wells in Palestine are infected with Enterobacteriaceae, ESBLs specifically (Atteyeh, 2007, MSc thesis). Indeed the data provided by Atteyeh (2007) coincides with the results retrieved in this research.

4.6 Effect of the soil quality on vegetable safety

Major soil contamination with pathogens occurs due to the employment of organic fertilizers and as a consequence of water contamination. Organic fertilizers are generally accepted, however, animal manure contain animal feaces, known to be highly contaminated with pathogens, drug resistance pathogens included, which can enter the food chain and cause foodborne diseases (Witte, 2000; Lau and Ingham, 2001; Locking et al., 2001; Wachtel et al., 2002; Islam et al., 2004b; Mootian et al., 2009). We do not have any official records on farming soil quality in Palestine, however we were informed that animal manure is regularly used as organic fertilizers across Palestine. This may not be a major problem if manure is tested for microbiological quality or subjected to treatments before application to the soil. Furthermore, the farms growing the leafy vegetables tested in this work we simple and are generally accessible for animals, who can urinate of dispose Feaces on soil and vegetables raising the risk for contamination. The current data presented here does not indicate any difference in pathogenic growth between the samples retrieved from different areas in Palestine, indicating that the contamination is not restricted to some areas, but is rather a general problem in Palestine.

4.7 Effect of harvesting and handling on vegetables quality

Harvesting of leaf vegetables is mostly primitive in Palestine, which subjects the crops for contamination during harvesting. Such contamination can come from the way the vegetables are cut; stacked and stored until being sold. We cannot refer the contamination found in our samples to the primitive harvesting method, as we have no proof, but it could be a contributing factor.

4.8 Effect of transport and storage on vegetable safety

Transport of the leafy vegetables in Palestine is generally primitive and in most cases storage is not needed as these items are subjected to market sale very effectively, mostly on the same day of the harvest. During market sale, most of these items are crowded together, allowing cross contamination. As described in the results, point

3.6, we were unable to record any considerable difference between farm and market sample in contamination, type of bacteria or antibiotic resistance.

None of the Palestinian samples purchased and tested in this research came from a supermarket shelve, but rather a greengrocer shop or farm as detailed in the results. Nevertheless, we purchased seven samples from supermarkets, produced in Israel. Also here we were unable to detect any remarkable difference in contamination (see figure 3.1), although earlier studies had shown that supermarket samples were less contaminated with coliform (Falomir et al., 2010a). It is known that storage of vegetables for longer periods may subject them to other types of bacterial contamination, which can commonly survive the refrigerating temperature such as *Listeria monocytogenes*, which we did not test in this study.

4.9 Recommendation

The data presented here reveals a high percentage of contamination of lettuce, mint and watercress across Palestine. Yet, this study is a random one and did not cover a wide range of farms and market samples or equal number of samples from all regions of Palestine. Samples were purchased randomly according to a specific plane. However, overall, we believe that primitive farming in the case of these types of leaf vegetables specifically is the major contributor for such contamination in Palestine. The data presented here are not pleasant and raise high demands on the farming industry and ministry of agriculture to pay an adequate attention and apply standard measures for farming leaf vegetables. It is generally accepted that controlling soil, water and storage of such vegetables is the first step in improving the crops and avoiding contamination.

Farming of leafy vegetables in Palestine does not follow specific standards or measurement and therefore, it may be possible, that this could have been a major contributing factor for these results. We hope that we were able to shed light on the urgent need for such control measures in Palestine.

5. Literature

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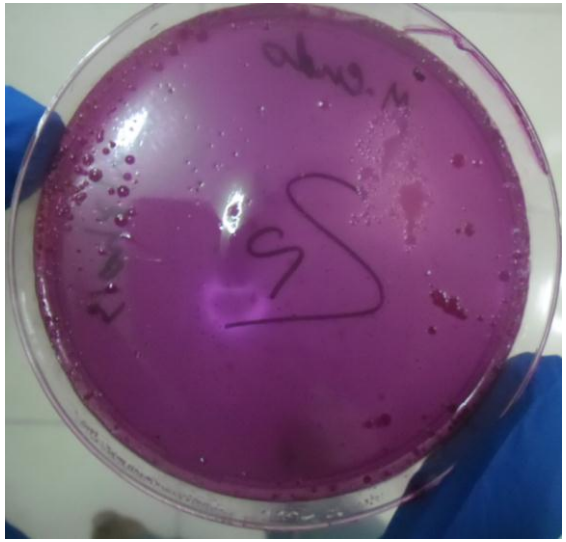
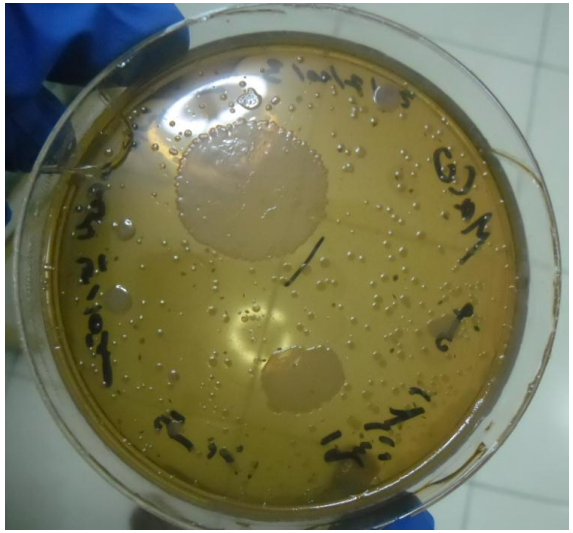
Coliform



Fecal coliform

MacConkey+Ampicillin



#	Sample #	CTC		ATM		FEP		CRO		FOX		ETP		IPM		CTX		CZC		CAZ		Antibiotic Resistance in Enterobacteriacae				Name Of Samp	Type of bactera
		RorS	d/cm	RorS	d/cm	RorS	d/cm	RorS	d/cm	RorS	d/cm	RorS	d/cm	RorS	d/cm	RorS	d/cm	RorS	d/cm	RorS	d/cm	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	**Lettuce**	
1	**1**																					not growth	not growth	not growth	not growth	Lettuce	
2	**2**	Sensitive	1.04	Resistance	0	Sensitive	3.31	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.14	Sensitive	1.24	Sensitive	2.19	Sensitive	2.16	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
3	**3**	Sensitive	2.63	Sensitive	2.53	Sensitive	2.96	Sensitive	2.45	Resistance	0	Sensitive	1.59	Sensitive	2.3	Sensitive	2.78	Sensitive	2.31	Sensitive	2.13			AmpC beta lactamases		Lettuce	Enterobacter species
4	**4**																					not growth	not growth	not growth	not growth	Lettuce	
5	**5**	Sensitive	2.65	Sensitive	2.87	Sensitive	3.17	Sensitive	2.79	Resistance	0	Sensitive	1.96	Sensitive	3.37	Sensitive	2.62	Sensitive	2.39	Sensitive	1.9			AmpC beta lactamases		Lettuce	Enterobacter species
6	**6**	Sensitive	2.8	Sensitive	3.07	Sensitive	3.17	Sensitive	2.81	Resistance	0	Sensitive	1.7	Sensitive	2.39	Sensitive	2.87	Sensitive	2.47	Sensitive	2.46			AmpC beta lactamases		Lettuce	Enterobacter species
7	**7**	Sensitive	3.32	Sensitive	2.66	Sensitive	2.59	Sensitive	2.94	Sensitive	3.35	Resistance	0	Sensitive	3.48	Sensitive	3.48	Sensitive	3.09	Sensitive	2.6			AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
8	**8**																					not growth	not growth	not growth	not growth	Lettuce	
9	**9**	Sensitive	2.85	Sensitive	3.2	Sensitive	3.49	Sensitive	3.3	Resistance	0	Sensitive	2.32	Sensitive	2.12	Sensitive	2.99	Sensitive	2.76	Sensitive	2.4			AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
10	**10**	Sensitive	2.94	Sensitive	3.18	Sensitive	3.31	Sensitive	2.68	Resistance	0	Sensitive	1.97	Sensitive	6.64	Sensitive	2.93	Sensitive	2.52	Sensitive	2.37			AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
11	**11a**	Resistance	0	Resistance	0	Sensitive	2.64	Resistance	0	Resistance	0	Resistance	0	Sensitive	3.12	Resistance	0	Sensitive	2.29	Sensitive	1.34	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	Mint	Enterobacter species
13	**12**	Resistance	0	Resistance	0	Sensitive	2.85	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.89	Resistance	0	Sensitive	2.35	Sensitive	1.67	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
14	**13**																					not growth	not growth	not growth	not growth	Lettuce	
15	**14**																					not growth	not growth	not growth	not growth	Mint	
16	**15**																					not growth	not growth	not growth	not growth	Mint	
17	**16**	Sensitive	3.25	Sensitive	3.4	Sensitive	3.27	Sensitive	3.09	Sensitive	2.32	Sensitive	2.46	Sensitive	2.68	Sensitive	3.18	Sensitive	2.8	Sensitive	2.81				ESBL E.Coli	Lettuce	E. coli
18	**17a**	Resistance	0	Resistance	0	Resistance	0	Resistance	0	Resistance	0	Resistance	0	Resistance	0	Resistance	0	Resistance	0	Resistance	0		KPC beta lactamase	AmpC beta lactamases		Lettuce	Yersinia enterocolitica
19	**18a**	Resistance	0	Sensitive	2.55	Sensitive	3.01	Sensitive	1.93	Resistance	0	Resistance	0	Sensitive	2.59	Sensitive	1.71	Sensitive	2.57	Sensitive	2.32		KPC beta lactamase	AmpC beta lactamases		Lettuce	Yersinia enterocolitica
20	**19**	Sensitive	1.58	Sensitive	2.59	Sensitive	2.84	Sensitive	1.81	Resistance	0	Resistance	0	Sensitive	2.53	Sensitive	1.69	Sensitive	2.57	Sensitive	2.47		KPC beta lactamase	AmpC beta lactamases		Lettuce	Yersinia enterocolitica
21	**20**	Sensitive	1.61	Sensitive	2.58	Sensitive	2.81	Sensitive	1.81	Resistance	0	Resistance	0	Sensitive	2.61	Sensitive	1.9	Sensitive	2.61	Sensitive	2.31		KPC beta lactamase	AmpC beta lactamases		Mint	Yersinia enterocolitica
22	**21a**	Resistance	0	Sensitive	1.73	Sensitive	2.93	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.41	Resistance	0	Resistance	0	Resistance	0		KPC beta lactamase	AmpC beta lactamases		Lettuce	Enterobacter species
24	**22**	Resistance	0	Resistance	0	Sensitive	3.01	Resistance	0	Resistance	0	Resistance	0	Sensitive	3.09	Sensitive	1.08	Sensitive	1.7	Sensitive	2.26		KPC beta lactamase	AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
25	**23a**	Resistance	0	Sensitive	1.54	Sensitive	2.84	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.62	Sensitive	1.19	Sensitive	0.8	Sensitive	0.8		KPC beta lactamase	AmpC beta lactamases		Lettuce	Enterobacter species
28	**24a**	Sensitive	2.76	Sensitive	3.05	Sensitive	3.24	Sensitive	2.68	Resistance	0	Sensitive	1.4	Sensitive	2.54	Sensitive	2.99	Sensitive	2.76	Sensitive	2.4			AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
30	**25**	Sensitive	3.51	Sensitive	3.61	Sensitive	3.17	Sensitive	3.66	Sensitive	3.24	Resistance	0	Sensitive	2.01	Sensitive	3.72	Sensitive	3.09	Sensitive	2.84		KPC beta lactamase	AmpC beta lactamases		Lettuce	E. coli
31	**26**	Sensitive	1.2	Sensitive	2.55	Sensitive	3.1	Sensitive	1.76	Resistance	0	Resistance	0	Sensitive	2.64	Sensitive	1.7	Sensitive	2.74	Sensitive	2.45					Lettuce	Pseudomonas
32	**27**	Sensitive	0.9	Sensitive	1.68	Sensitive	2.61	Sensitive	1.76	Resistance	0	Resistance	0	Sensitive	3.54	Sensitive	1	Sensitive	1.54	Sensitive	1.01		KPC beta lactamase	AmpC beta lactamases	ESBLs	Mint	Enterobacter species
33	**28**	Sensitive	0.5	Resistance	0	Sensitive	2.31	Sensitive	1.66	Resistance	0	Resistance	0	Sensitive	3.1	Sensitive	0.8	Sensitive	2.12	Sensitive	2.51	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
34	**29**	Sensitive	1.3	Sensitive	2.57	Sensitive	3.09	Sensitive	0.4	Resistance	0	Resistance	0	Sensitive	2.91	Sensitive	1.6	Sensitive	2.73	Sensitive	2.49					Lettuce	Pseudomonas
35	**30**	Resistance	0	Resistance	0	Sensitive	3.01	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.9	Sensitive	0.8	Sensitive	2.7	Sensitive	1.7	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
36	**31**	Sensitive	2.43	Sensitive	2.92	Sensitive	3.04	Sensitive	2.48	Resistance	0	Sensitive	1.94	Sensitive	2.55	Sensitive	2.04	Sensitive	2.41	Sensitive	2.28			AmpC beta lactamases		Lettuce	Enterobacter species
37	**32**	Sensitive	2.89	Sensitive	3.33	Sensitive	3.13	Sensitive	2.77	Resistance	0	Sensitive	2.52	Sensitive	2.76	Sensitive	2.9	Sensitive	2.75	Sensitive	2.41			AmpC beta lactamases	ESBLs	Mint	Enterobacter species
38	**33**	Sensitive	1.3	Sensitive	2.98	Sensitive	2.86	Sensitive	2.49	Resistance	0	Sensitive	1.99	Sensitive	2.58	Sensitive	2.77	Sensitive	2.45	Sensitive	2.41			AmpC beta lactamases	ESBLs	Mint	Enterobacter species
39	**34**	Sensitive	2.59	Sensitive	3.02	Sensitive	2.89	Sensitive	2.54	Resistance	0	Sensitive	2.06	Sensitive	2.59	Sensitive	2.75	Sensitive	2.49	Sensitive	2.3				ESBLs	Lettuce	Enterobacter species
40	**35**	Sensitive	3.13	Sensitive	3.27	Sensitive	3.02	Sensitive	2.73	Sensitive	2.35	Sensitive	2.56	Sensitive	2.96	Sensitive	3.22	Sensitive	2.58	Sensitive	2.65				ESBLs	Mint	E. coli
41	**36**	Sensitive	2.94	Sensitive	3.28	Sensitive	3.23	Sensitive	2.96	Sensitive	2.73	Sensitive	2.48	Sensitive	3.07	Sensitive	3.15	Sensitive	2.83	Sensitive	2.62			AmpC beta lactamases	ESBLs	Lettuce	E. coli
42	**37**	Sensitive	2.65	Sensitive	3.1	Sensitive	2.84	Sensitive	2.58	Sensitive	2.48	Sensitive	2.22	Sensitive	2.63	Sensitive	2.97	Sensitive	2.62	Sensitive	2.5		KPC beta lactamase	AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
43	**38**	Sensitive	1.69	Sensitive	2.81	Sensitive	3.01	Sensitive	1.95	Resistance	0	Resistance	0	Sensitive	3.37	Sensitive	1.83	Sensitive	2.69	Sensitive	3.01		KPC beta lactamase	AmpC beta lactamases		Mint	Enterobacter species
44	**39**	Sensitive	2.82	Sensitive	3.05	Sensitive	3.04	Sensitive	2.7	Sensitive	2.48	Sensitive	2.2	Sensitive	2.81	Sensitive	3.07	Sensitive	2.64	Sensitive	2.41			AmpC beta lactamases		Lettuce	Enterobacter species
45	**40**	Sensitive	2.9	Sensitive	3.1	Sensitive	3.15	Sensitive	2.8	Resistance	0	Sensitive	2.3	Sensitive	2.5	Sensitive	2.9	Sensitive	2.5	Sensitive	2.4	K1 beta lactamases				Lettuce	Enterobacter species
46	**41**	Resistance	0	Sensitive	1.9	Sensitive	2.9	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.9	Resistance	0	Sensitive	1.4	Resistance	0		KPC beta lactamase	AmpC beta lactamases	ESBLs	Mint	Enterobacter species
47	**42**	Resistance	0	Sensitive	2	Sensitive	3.1	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.9	Resistance	0	Sensitive	1.3	Resistance	0		KPC beta lactamase	AmpC beta lactamases	ESBLs	Mint	E. coli
48	**43**	Sensitive	3.01	Sensitive	3.3	Sensitive	3.1	Sensitive	2.98	Sensitive	2.41	Sensitive	2.48	Sensitive	2.81	Sensitive	3.09	Sensitive	2.76	Sensitive	2.74					Lettuce	Pseudomonas
49	**44**	Resistance	0	Resistance	0	Sensitive	2.3	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.64	Resistance	0	Resistance	0	Resistance	0		KPC beta lactamase	AmpC beta lactamases		Mint	Enterobacter species
50	**45**	Sensitive	1.23	Sensitive	2.61	Sensitive	3.3	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.61	Sensitive	1.39	Sensitive	2.99	Sensitive	2.26	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases		Mint	Enterobacter species
51	**46**	Resistance	0	Resistance	0	Sensitive	2.42	Sensitive	1.54	Resistance	0	Resistance	0	Sensitive	3.1	Sensitive	0.8	Sensitive	2.23	Sensitive	1.5	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	Lettuce	Enterobacter species
52	**47**	Sensitive	3	Sensitive	2.6	Sensitive	2.3	Sensitive	2.5	Resistance	0	Sensitive	2.5	Sensitive	2.6	Sensitive	2.7	Sensitive	2.6	Sensitive	2.2			AmpC beta lactamases		Lettuce	Enterobacter species
53	**48**	Resistance	0	Sensitive	1.2	Sensitive	2.42	Resistance	0	Resistance	0	Resistance	0	Sensitive	2.41	Resistance	0	Sensitive	0	Resistance	0	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases		Mint	Enterobacter species
54	**49**	Sensitive	2.79	Sensitive	3.14	Sensitive	2.97	Sensitive	2.53	Resistance	0	Sensitive	1.92	Sensitive	2.67	Sensitive	2.99	Sensitive	2.55	Sensitive	2.6	K1 beta lactamases				Mint	E. coli
55	**50**	Sensitive	1.7	Sensitive	3	Sensitive	3.2	Sensitive	2	Resistance	0	Resistance	0	Sensitive	3.1	Sensitive	2.5	Sensitive	3.1	Sensitive	2.6					Mint	Pseudomonas
56	**51**	Sensitive	2.7	Sensitive	3.2	Sensitive	3.2	Sensitive	2.7	Resistance	0	Sensitive	2.1	Sensitive	2.4	Sensitive	2.7	Sensitive	2.8	Sensitive	2.3			AmpC beta lactamases	ESBLs	Mint	Enterobacter species
57	**52**	Sensitive	3.2	Sensitive	3.5	Sensitive	2.6	Sensitive	3.1	Sensitive	2.5	Sensitive	2.5	Sensitive	3.3	Sensitive	3.5	Sensitive	3	Sensitive	2.5				ESBLs	Watercress	E. coli
58	**53**	Sensitive	1.71	Sensitive	2.81	Sensitive	2.91	Sensitive	1.94	Resistance	0	Resistance	0	Sensitive	2.53	Sensitive	1.8	Sensitive	2.9	Sensitive	2.69					Watercress	Pseudomonas
59	**54**	Sensitive	3.52	Sensitive	3.68	Sensitive	3.26	Sensitive	3.34	Sensitive	2.36	Sensitive	2.51	Sensitive	2.51	Sensitive	3.2	Sensitive	3.16	Sensitive	3.15	K1 beta lactamases				Watercress	Enterobacter species
60	**55**	Sensitive	1.6	Sensitive	2.69	Sensitive	3.06	Sensitive	2.07	Resistance	0	Resistance	0	Sensitive	2.91	Sensitive	2.05	Sensitive	2.97	Sensitive	2.54					Watercress	Pseudomonas
61	**56**	Resistance	0	Sensitive	1.1	Sensitive	3	Sensitive	1.6	Resistance	0	Resistance	0	Sensitive	3.9	Sensitive	1.8	Sensitive	0.6	Sensitive	2	K1 beta lactamases	KPC beta lactamase	AmpC beta lactamases	ESBLs	Watercress	Enterobacter species
62	**57**	Sensitive	2.5	Sensitive	3.2	Sensitive	3.3	Sensitive	2.2	Resistance	0	Resistance	0	Sensitive	2.07	Sensitive	2.6	Sensitive	2.7	Sensitive	2.2		KPC beta lactamase		ESBLs	Watercress	Enterobacter species
63	**58**	Sensitive	0.8	Sensitive	1.1	Sensitive	2.5	Sensitive	2.6	Resistance	0	Resistance	0	Sensitive	3.8	Sensitive	1.2	Sensitive	2.1	Sensitive	1.5		KPC beta lactamase	AmpC beta lactamases		Watercress	Yersinia enterocolitica
64	**59**																					not growth	not growth	not growth	not growth	Mint	