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ANTIBIOTIC RESISTANCE OF BACTERIA ISOLATED FROM LEAFY GREEN SALAD VEGETABLES

by
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
December 2011

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ABSTRACT

Bacterial isolates obtained from samples of five varieties of leafy green vegetables were tested for resistance to four different antibiotics: ampicillin, erythromycin, streptomycin, and tetracycline. Isolates were separated based on media type (TSA vs. R2A media), sterilization techniques (surface sterilized vs. unsterilized), cultivation methods (conventional vs. organic), and specific lettuce type origins. Resistances were tested using a microscale broth culture technique, which allowed for growth of isolates in the presence of different concentrations of antibiotic. Antibiotic resistance was observed in all groups of isolates, although levels of resistance varied depending on the particular isolate and the antibiotic tested. Isolates were generally the most resistant to ampicillin, with some isolates showing resistance to 5000 μg/mL, 60 times the amount of ampicillin which would be used to treat human infections. Isolates generally showed the least amount of resistance to tetracycline, although many isolates grew at tetracycline concentrations exceeding a typical human dose. Over two thirds of the isolates were resistant to multiple antibiotics, with four isolates showing high levels of resistance to all four antibiotics tested. These four isolates were all obtained from green leaf lettuce and all four are potential human pathogens. Overall, bacterial isolates from green leaf lettuce samples collectively were the most antibiotic resistant of any of the five lettuce types. These results show that leafy green vegetables contain bacteria which are antibiotic resistant and could serve as a mechanism for an increased transmission of antibiotic resistance to bacteria within the human body.

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INTRODUCTION

In recent years, there has been increased acknowledgement of the potential harm caused by pathogenic bacteria contaminating commercially produced food products throughout the growing and packaging stages of food production. Pathogen contamination of food can lead to the transmission of at least 200 known diseases (Bryan, 1982). Because of the potential presence of pathogenic bacteria in foods, stricter regulations regarding prevention have been applied to food safety standards, along with technology allowing for the better detection of contamination. Studies suggest that in the 1990's food borne pathogens caused 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year, with many cases of food borne illnesses never being detected (Mead et al., 1999). A more recent study suggests that 31 of the major food borne pathogens cause 9.4 million illnesses, 56,000 hospitalizations, and 1,400 deaths per year within the United States (Scallan et al., 2011). The latter estimates are based on known major pathogens and disregard cases associated with unidentified pathogens. If unidentified causes are included the number of current food borne illnesses, hospitalizations, and deaths significantly increases and approaches 1990's levels (Scallan et al., 2011). Because of growing demand for "ready-to-eat" nutritional and convenient food such as salad greens, the number of illnesses attributed to food borne pathogens has likely risen, as pathogens can survive the minimal processing that such foods undergo (Francis et al., 1999).

An increased interest in a nutritional diet, in conjunction with better transportation and preservation of food, has led to the availability of a wider variety of fresh produce (Glanz and Yaroch, 2004). Because of this, lettuce and other leafy vegetables have become more prevalent within Americans' diets over the last fifty years through the consumption of more salads, sandwiches, and wraps (Everis, 2004). Currently, the market encounters demands from the consumer to produce products that are minimally processed, prepackaged for convenience, and ready-to-consume (Everis, 2004). Increased market demands can lead to deteriorating quality of prepackaged items, such as the presence of cut surfaces on the leaf being left exposed, potentially increasing the exposure of pathogens to nutrients which can stimulate their growth (Heaton and Jones, 2007). Convenient food packaging has likely caused large outbreaks of pathogens such as *Escherichia coli* 0157:H7, *Salmonella typhimurium*, *Campylobacter jejuni*, and *Listeria monocytogenes*, and more localized outbreaks of *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella* sp., *Salmonella typhimurium*, *Salmonella* Newport, *Campylobacter jejuni*, and Norovirus have also been associated with leafy salad vegetables (Heaton and Jones, 2007). In response to these outbreaks, food microbiologists have taken a greater interest in studying leafy green vegetables as well as the specific microorganisms associated with them, and food microbiology as a discipline showed much growth over the second half of the twentieth century.

At the same time as interest in food microbiology increased, a second area of microbiology expanded greatly during the twentieth century - the study of antibiotics and their interactions with bacteria. Antibiotics are defined as chemicals that interfere with structures that are necessary for the targeted bacteria to continue to grow, without the

chemical causing harm to the eukaryotic host harboring the bacteria (Walsh, 2000). Although traces of substances resembling antibiotics had been found previously, antibiotics were not used to treat human diseases until 1928, following Alexander Fleming's discovery of antibacterial activity in *Penicillium*. Ten years after Fleming's discovery, Ernst Chain and Howard Florey purified *Penicillium*, which led to the production of penicillin, the so-called "miracle drug" (Bennett et al., 2001). The mass production of penicillin is considered to be one of the most significant achievements in science during the World War II era, saving thousands of soldiers from gas gangrene as well as from other bacterial diseases present within war zones (Neushul, 1993). Fleming, Chain, and Florey were awarded the Nobel Prize in Medicine in 1945 (Masic, 2008). The success of penicillin led to the development and production of several other antibiotics such as streptomycin, chloramphenicol, and tetracycline, and the subsequent decade was the "golden age of antibiotics" (Clardy et al., 2009). Some antibiotics such as penicillin and cephalosporin were discovered in fungi, whereas others, such as erythromycin, streptomycin, tetracycline, and vancomycin, were obtained from strains of bacteria such as *Actinobacterium* and *Streptomyces* (Walsh, 2000).

Antibiotics can be separated into different classes based on the chemical's mode of action, which can result in specific effects on different bacteria. Typically, antibiotics target bacteria by disrupting the process of cell wall biosynthesis, protein synthesis, or DNA replication and repair (Walsh, 2000). Commonly used classes of antibiotics include the beta-lactam antibiotics, macrolide antibiotics, aminoglycoside antibiotics, and polyketide antibiotics. Beta-lactam antibiotics such as ampicillin and penicillin target bacterial cell wall construction, killing bacteria by inhibiting the final stages of

peptidoglycan biosynthesis (Spratt, 1977). Since the discovery of penicillin, development of beta-lactam antibiotics has advanced with the finding of beta-lactam antibiotics that contain novel ring structures, which has led to a wide range of these potent antibiotics being available (Spratt, 1983). Macrolide antibiotics such as erythromycin block protein synthesis. Erythromycin works by binding to the 50S subunit of the bacterial 70S rRNA complex, blocking protein synthesis and even inhibiting bacterial cell division at higher concentrations (Katzung, 2007). Aminoglycosidic antibiotics such as streptomycin, neomycin, and gentamicin are also protein synthesis inhibitors. Antibiotics in this class act by binding to the 16S rRNA of the ribosomal 30S subunit, and interfere with the addition of formyl-methionyl-tRNA (Sharma et al., 2007). Polyketide antibiotics such as tetracycline also inhibit protein synthesis and bacterial growth by binding to the ribosomal subunits. Tetracycline binds to the 30S subunit of the bacterial ribosome, blocking the attachment of an aminoacyl-tRNA and preventing the addition of a new amino acid to the growing peptide chain (Connell et al., 2003).

Increased use of antibiotics has led to the emergence of antibiotic resistance within many bacteria. Antibiotic resistance occurs when bacteria are able to survive antibiotic treatment, and resistance may be acquired through genetic mutation or horizontal gene transfer. The phenomenon of resistance is typically witnessed within a period of months to years after a new antibiotic has been proven effective (Davies, 1996), and antibiotic resistance is certain to occur when antibiotics are used in treatment (Levy, 1998). Due to the ability of bacteria to multiply quickly, emergence of antibiotic resistance can also spread rapidly; so that the majority of bacteria within an environment can become antibiotic resistant. If a single mutation leads to antibiotic resistance, those

resistant bacteria may survive antibiotic treatment and grow to become the dominant strain of that bacterial species (Walsh, 2000).

Mechanisms that antibiotic resistant bacteria utilize to inhibit the effectiveness of antibiotics include antibiotic efflux pumps, antibiotic modification, and target site alteration (Weisblum, 1995). Antibiotic efflux pumps transport substrates to the outside of the cell, reducing the amount of antibiotic inside the cell to a point where it may not exceed the minimum concentration necessary to inhibit bacterial growth (Bambeke et al., 2000). Antibiotic modification can occur when a bacterium produces enzymes that can modify an antibiotic, reducing its toxicity. These enzymes are often coded by plasmids that can be transferred from cell to cell (Courvalin et al., 1977). Target site alteration functions by substituting several single amino acids in a protein that may be inhibited by an antibiotic, thereby decreasing the similarity to the antibiotics target. Since the antibiotic can no longer bind its specific site, the bacteria then grows tolerant of high levels of that antibiotic, increasing resistance (Spratt, 1994).

One of the major problems of antibiotic resistance is that it can be transferred from one bacterium to another through horizontal gene transfer in the form of conjugation, transformation, and transduction (Maiden, 1998). This can result in the rapid spread of resistance to different bacterial strains and species, and antibiotic resistance has become an important public health concern, threatening the effectiveness of antibiotics and resulting in the emergence of some that are resistant to multiple antibiotics (Li and Nikaido, 2009). While most antibiotic resistant bacteria were initially discovered in hospitals (Levy and Marshall, 2004), improper use of antibiotics in agriculture can cause influxes of antibiotics into the environment (Goni-Urriza et al.,

2000) and because of the expanding spread of antibiotic resistance, it is likely that antibiotic resistant bacteria can be found in many natural environments. Annually, the United States is estimated to use 23 million kilograms of antibiotics, only half of which is used for human medicinal purposes; the remainder is largely used in agriculture (Levy, 2002). Agricultural use of antibiotics results in an increase in antibiotic resistance genes in that setting, and agricultural animals can serve as a harboring ground for resistant bacterial populations to become established and multiply (Mellon et al., 2001). Agricultural use of antibiotics is not confined to their use in animals, as antibiotics such as tetracycline and streptomycin have been used to treat diseases in a variety of produce plants since 1950 (Levy, 1992; McManus, 2002). The U.S. Environmental Protection Agency reports that approximately 136,000 kg of antibiotics are used on fruit trees in the southern United States alone, with many other Central and South American countries following the same example (Harrison and Lederberg, 1998). Because of the overwhelming amounts of antibiotics used in the production of these and other plant conditions to promote antibiotic resistance are likely present in many agricultural settings (Levy, 2001). Antibiotic resistant strains of *Pseudomonas syringae* have been found in pear orchards in Oregon and Washington, with multiple strains showing resistance to more than one antibiotic (Spotts and Cervantes, 1995), and outbreaks of streptomycin resistant strains of *Pseudomonas* sp. and *Xanthomonas campestris* have been reported in other agricultural systems (McManus et al., 2002).

Recently, more attention has been paid to the use of food as a medium for the spread of antibiotic resistant bacteria (Perreten et al., 1997). The transmission of antibiotic resistance from bacteria associated with agricultural products to bacteria found

within humans can occur through food consumption (Teuber et al., 1999). In particular, fermented dairy products that are not heat treated before consumption have been found to provide a direct pathway for antibiotic resistant bacteria to embody the human gastrointestinal tract (Bates et al., 1994). Commensal bacteria, such as lactic acid bacteria, can act as a reservoir for antibiotic resistance genes, and have the ability to transfer these resistance genes to pathogens (Mathur and Singh, 2005). Genes that code for resistance to tetracycline, erythromycin, and vancomycin have been discovered in strains of *Lactococcus lactis*, *Enterococcus* sp. and *Lactobacillus* sp. isolated from fermented milk products and meat (Mathur and Singh, 2005). In recent years, probiotic supplements in foods such as yogurt have been adapted from lactic acid bacteria strains such as *Enterococcus faecium*, *Lactobacillus plantarum*, and several species of *Bifidobacterium* and *Propionibacterium*, any of which could show antibiotic resistance (Tannock, 1998).

Other potential food products that could harbor antibiotic resistant bacteria are raw vegetables such as those consumed in salads (Levy, 2001), and lactose-fermenting bacteria showing multidrug resistance have been found in carrots, celery, lettuce, cucumbers, peppers, and tomatoes (Levy, 1984). In the mid-1990's, several European countries experienced an outbreak of *Shigella sonnei*, which infected over 100 individuals within Norway, Sweden, and the United Kingdom (Kapperud et al., 1995). The outbreak was traced to iceberg lettuce, and the strain of *S. sonnei* identified showed resistance to antibiotics such as ampicillin (Kapperud et al., 1995). Also within the mid-1990's, an outbreak of antibiotic resistant *Escherichia coli* O157:H7 that infected 40 people in Montana was traced to leaf lettuce from numerous farms in Montana and

Washington (Ackers et al., 1998). These examples suggest that illnesses caused by antibiotic resistant bacteria residing in produce are potentially a growing problem, and could be a significant cause of death around the world (Baird-Parker, 1994).

A previous study (Randolph, 2011) isolated a number of bacterial strains from commercially available fresh produce, including five specific types of conventionally and organically grown green leafy vegetables. The objective of the study described here was to determine if these bacterial strains showed resistance to various antibiotics, and if so, to determine the level of antibiotic resistance. Specifically, I sought to determine the upper limit of antibiotic resistance displayed by these bacterial isolates to four different classes of antibiotics as represented by ampicillin, erythromycin, streptomycin, and tetracycline.

METHODS

Source of bacterial isolates

Bacterial isolates were obtained from a previous study (Randolph 2011) which sought to determine the numbers and types of bacteria associated with different types of lettuce and salad greens. That study obtained several types of fresh salad greens (romaine lettuce, baby spinach, green leaf lettuce, iceberg lettuce, and red leaf lettuce) from the Kroger supermarket in Oxford, MS in the fall of 2010. For each salad type, conventional and organic varieties were purchased (A summary of the salad greens used in that study is presented in Table 1).

Each of the ten samples was analyzed in two separate conditions: surface sterilized and unsterilized. Washing of the lettuce occurred in such a way that specimens from unsterilized samples should consist of interior and exterior bacteria, whereas surface sterilized samples should only yield bacteria found inside the leaves. Therefore, for each type of leaf vegetable both a conventional and organic cultivation method was tested, each in a sterile and unsterile state, and each plated onto both R2A agar and TSA agar media. Bacterial isolates from each sample type were obtained on R2A agar (R2A) and tryptic soy agar (TSA) following incubation at room temperature (20-22°C) for 2-4 days (Tables 1 and 2). Bacterial isolates were transferred onto fresh plates every 6-8 weeks.

Table 1: Leaf vegetables from which the bacterial isolates used in this study were obtained. (modified from Randolph 2011)

Sample	Cultivation Method	Brand	Packaging	Date Acquired	Expiration Date
Romaine Lettuce	Conventional	Kroger Brand "3 Jumbo Romaine Hearts"	Prepackaged in a bag, not "prewashed"	9/26/2010	10/04/2010
Romaine Lettuce	Organic	Private Selection "Organic Romaine Hearts"	Prepackaged in a bag, not "prewashed"	9/26/2010	10/07/2010
Baby Spinach	Conventional	Fresh Express "Baby Spinach"	Prepackaged in a bag, noted to be "prewashed thoroughly"	9/26/2010	9/28/2010
Baby Spinach	Organic	Private Selection "Organic Baby Spinach"	Prepackaged in a plastic container, "prewashed and ready to eat"	9/26/2010	10/04/2010
Green Leaf Lettuce	Conventional	Kroger Brand "Green Leaf Lettuce"	Bought as head of lettuce, not bagged	10/24/2010	N/A
Green Leaf Lettuce	Organic	Lakeside Organic Gardens "Green Leaf Lettuce"	Bought as head of lettuce, not bagged	10/24/2010	N/A
Iceberg Lettuce	Conventional	Kroger Brand "Iceberg Lettuce"	Bought as head of lettuce, not bagged	10/24/2010	N/A
Iceberg Lettuce	Organic	Lakeside Organic Gardens "Iceberg Lettuce"	Bought as head of lettuce, not bagged	10/24/2010	N/A
Red Leaf Lettuce	Conventional	Kroger Brand "Red Leaf Lettuce"	Bought as head of lettuce, not bagged	10/31/2010	N/A
Red Leaf Lettuce	Organic	Lakeside Organic Gardens "Red Leaf Lettuce"	Bought as head of lettuce, not bagged	10/31/2010	N/A

Table 2: R2A bacterial isolates, including what type of fresh salad green it derived from.

Isolate Number	Lettuce Type	Isolate
1	Romaine conventional unsterilized	*Pantoea*
2	Romaine conventional unsterilized	*Pseudomonas*
4	Romaine conventional unsterilized	*Janthinobacterium lividum*
5	Romaine conventional sterilized	*Pseudomonas viridiflava*
6	Romaine conventional sterilized	*Flavobacterium succinicans*
7	Romaine conventional sterilized	*Janthinobacterium lividum*
9	Romaine conventional sterilized	*Flavobacterium succinicans*
11	Romaine conventional sterilized	*Pseudomonas*
13	Romaine conventional sterilized	*Pseudomonas rhodesiae*
14	Romaine organic unsterilized	*Stenotrophomonas*
15	Romaine organic unsterilized	*Arthrobacter*
16	Romaine organic unsterilized	*Arthrobacter*
18	Romaine organic sterilized	*Arthrobacter*
19	Romaine organic sterilized	*Bacillus flexus*
20	Romaine organic sterilized	*Pseudomonas*
21	Romaine organic sterilized	*Sphingobium yanoikuyae*
23	Spinach conventional unsterilized	*Pseudomonas fragi*
24	Spinach conventional unsterilized	*Pseudomonas*
25	Spinach conventional sterilized	*Flavobacterium succinicans*
27	Spinach conventional sterilized	*Pseudomonas*
30	Spinach organic unsterilized	*Acinetobacter*
31	Spinach organic unsterilized	*Sejongia*
32	Spinach organic unsterilized	*Shewanella sp. ANA-3*
33	Spinach organic unsterilized	*Flavobacterium succinicans*
35	Spinach organic sterilized	*Pantoea*
36	Spinach organic sterilized	*Curtobacterium flaccumfaciens*
41	Green Leaf conventional unsterilized	*Pseudomonas*
43	Green Leaf conventional unsterilized	*Stenotrophomonas*
44	Green Leaf conventional unsterilized	*Janthinobacterium lividum*
46	Green Leaf conventional sterilized	*Arthrobacter*
47	Green Leaf conventional sterilized	*Pedobacter*
48	Green Leaf conventional sterilized	*Sphingobacterium*
49	Green Leaf conventional	*Leifsonia poae*

	sterilized	
51	Green Leaf conventional sterilized	*Agrobacterium*
52	Green Leaf conventional sterilized	*Xanthomonadaceae* (family)
53	Green Leaf organic unsterilized	*Serratia*
55	Green Leaf organic unsterilized	*Chryseobacterium*
59	Green Leaf organic sterilized	*Pseudomonas*
60	Green Leaf organic sterilized	*Pseudomonas rhodesiae*
61	Green Leaf organic sterilized	*Chryseobacterium*
62	Green Leaf organic sterilized	*Pseudomonas rhodesiae*
63	Iceberg conventional unsterilized	*Pseudomonas*
64	Iceberg conventional unsterilized	*Xanthomonas*
65	Iceberg conventional unsterilized	*Acinetobacter*
66	Iceberg conventional unsterilized	*Massilia timonae*
68	Iceberg conventional sterilized	*Pedobacter*
70	Iceberg conventional sterilized	*Chryseobacterium*
71	Iceberg conventional sterilized	*Sphingomonas*
72	Iceberg conventional sterilized	*Erwinia*
74	Iceberg organic unsterilized	*Stenotrophomonas*
76	Iceberg organic unsterilized	*Paenibacillus amylolyticus*
78	Iceberg organic sterilized	*Pseudomonas*
79	Iceberg organic sterilized	*Pseudomonas*
80	Iceberg organic sterilized	*Microbacterium*
81	Iceberg organic sterilized	*Chryseobacterium*
82	Iceberg organic sterilized	*Erwinia*
83	Red Leaf conventional unsterilized	*Methylobacterium adhaesivum*
84	Red Leaf conventional unsterilized	*Sphingomonas*
88	Red Leaf conventional unsterilized	*Pseudomonas veronii*
89	Red Leaf conventional unsterilized	*Pseudomonas*
91	Red Leaf conventional sterilized	*Pseudomonas*
92	Red Leaf conventional sterilized	*Pseudomonas*
96	Red Leaf organic unsterilized	*Pseudomonas*
97	Red Leaf organic unsterilized	*Flavobacterium succinicans*
101	Red Leaf organic unsterilized	*Pseudomonas*
102	Red Leaf organic unsterilized	*Pseudomonas*
105	Red Leaf organic sterilized	*Frigoribacterium*
106	Red Leaf organic sterilized	*Pseudomonas*
107	Red Leaf organic sterilized	*Microbacterium*
108	Red Leaf organic sterilized	*Curtobacterium flaccumfaciens*
109	Red Leaf organic sterilized	*Pseudomonas rhodesiae*
110	Red Leaf organic sterilized	*Pseudomonas viridiflava*

111	Red Leaf organic sterilized	*Devosia*

Table 3: TSA bacterial isolates, including what type of fresh salad green it derived from.

Isolate Number	Lettuce Type	Isolate
2	Romaine conventional unsterilized	*Pseudomonas*
3	Romaine conventional unsterilized	*Pseudomonas rhodesiae*
7	Romaine conventional unsterilized	*Xanthomonas*
10	Romaine conventional sterilized	*Pseudomonas rhodesiae*
11	Romaine conventional sterilized	*Pseudomonas viridiflava*
13	Romaine conventional sterilized	*Pseudomonas*
15	Romaine organic unsterilized	*Pseudomonas*
17	Romaine organic unsterilized	*Arthrobacter*
19	Romaine organic unsterilized	*Bacillus flexus*
21	Romaine organic sterilized	*Pseudomonas*
22	Romaine organic sterilized	*Arthrobacter*
23	Romaine organic sterilized	*Pantoea*
24	Spinach conventional unsterilized	*Flavobacterium succinicans*
25	Spinach conventional unsterilized	*Pseudomonas rhodesiae*
27	Spinach conventional unsterilized	*Pseudomonas fragi*
29	Spinach conventional sterilized	*Pseudomonas*
30	Spinach conventional sterilized	*Pseudomonas*
32	Spinach conventional sterilized	*Pseudomonas*
34	Spinach organic unsterilized	*Pseudomonas*
35	Spinach organic unsterilized	*Pseudomonas*
36	Spinach organic unsterilized	*Pseudomonas fragi*
39	Spinach organic sterilized	*Pantoea*
40	Spinach organic sterilized	*Pantoea*
41	Spinach organic sterilized	*Pseudomonas fragi*
42	Spinach organic sterilized	*Pseudomonas fragi*
43	Spinach organic sterilized	*Microbacterium*
45	Green Leaf conventional unsterilized	*Chryseobacterium*
46	Green Leaf conventional unsterilized	*Sphingobacterium faecium*
47	Green Leaf conventional unsterilized	*Pantoea*
48	Green Leaf conventional unsterilized	*Mycetocola*
49	Green Leaf conventional unsterilized	*Pseudomonas*
52	Green Leaf conventional unsterilized	*Arthrobacter*
53	Green Leaf conventional unsterilized	*Pseudomonas*
55	Green Leaf conventional sterilized	*Agrobacterium*
56	Green Leaf conventional sterilized	*Pantoea*
57	Green Leaf conventional sterilized	*Sphingobacterium faecium*
58	Green Leaf conventional	*Pseudomonas*

	sterilized	
59	Green Leaf conventional sterilized	*Microbacterium*
60	Green Leaf conventional sterilized	*Aeromicrobium*
61	Green Leaf conventional sterilized	*Chryseobacterium*
63	Green Leaf conventional sterilized	*Microbacterium*
66	Green Leaf organic unsterilized	*Stenotrophomonas*
68	Green Leaf organic unsterilized	*Serratia*
70	Green Leaf organic unsterilized	*Pseudomonas*
73	Green Leaf organic sterilized	*Pseudomonas syringae*
74	Green Leaf organic sterilized	*Pseudomonas*
75	Green Leaf organic sterilized	*Pseudomonas*
76	Green Leaf organic sterilized	*Serratia*
77	Green Leaf organic sterilized	*Pseudomonas*
78	Green Leaf organic sterilized	*Ewingella Americana*
79	Iceberg conventional unsterilized	*Xanthomonas*
81	Iceberg conventional unsterilized	*Pseudomonas rhodesiae*
82	Iceberg conventional unsterilized	*Pantoea*
87	Iceberg conventional sterilized	*Chryseobacterium*
88	Iceberg conventional sterilized	*Agrobacterium*
89	Iceberg conventional sterilized	*Chryseobacterium*
90	Iceberg conventional sterilized	*Sphingobium yanoikuyae*
91	Iceberg organic unsterilized	*Erwinia rhapontici*
92	Iceberg organic unsterilized	*Pseudomonas*
93	Iceberg organic unsterilized	*Pantoea*
95	Iceberg organic unsterilized	*Pseudomonas*
97	Iceberg organic unsterilized	*Stenotrophomonas*
98	Iceberg organic unsterilized	*Erwinia rhapontici*
102	Iceberg organic sterilized	*Paenibacillus amylolyticus*
104	Iceberg organic sterilized	*Pseudomonas*
106	Iceberg organic sterilized	*Erwinia*
110	Red Leaf conventional unsterilized	*Curtobacterium flaccumfaciens*
111	Red Leaf conventional unsterilized	*Microbacterium*
112	Red Leaf conventional unsterilized	*Pantoea*
113	Red Leaf conventional sterilized	*Pseudomonas*
114	Red Leaf organic unsterilized	*Flavobacterium succinicans*
119	Red Leaf organic unsterilized	*Pseudomonas*
121	Red Leaf organic unsterilized	*Pseudomonas rhodesiae*
122	Red Leaf organic unsterilized	*Pseudomonas*
129	Red Leaf organic sterilized	*Pseudomonas*
130	Red Leaf organic sterilized	*Flavobacterium succinicans*
131	Red Leaf organic sterilized	*Curtobacterium flaccumfaciens*
132	Red Leaf organic sterilized	*Pseudomonas*
133	Red Leaf organic sterilized	*Flavobacterium succinicans*

Antibiotic testing

Bacterial isolates were tested for resistance to four different antibiotics: ampicillin, erythromycin, streptomycin, and tetracycline. These specific antibiotics were chosen to represent four different classes of antibiotics: ampicillin represents the beta-lactam class, erythromycin represents the macrolides class, streptomycin represents the aminoglycoside class, and tetracycline represents the aminoclygcosidic class. Resistances were tested using a microscale broth culture technique, which allowed for the growth of isolates in liquid media (R2A broth or trypticase soy broth) appropriate to a particular isolate. R2A broth was prepared using 0.5 g yeast extract, 0.5 g proteose peptone No. 3, 0.5 g casamino acids, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g dipotassium phosphate, and 0.05 g magnesium sulfate per 1 L of reverse osmosis (RO) water. Tryptic soy broth (TSB) was prepared using commercial TSB powder (Bacto; Becton, Dickinson and Company, Sparks, MD) dissolved in RO water. Both broths were divided into 50 mL batches and autoclaved at 121°C for sterilization. Antibiotic stock solutions were prepared concentrations of 5 and 50 mg per mL. Ampicillin, streptomycin, and tetracycline stock solutions were made in RO water, whereas erythromycin was made in 100% ethanol.

Aliquots of stock solutions were added to a 50 mL batch of R2A broth or TSB to obtain working broth solutions of the desired antibiotic concentration. 200µL of working broth solution was dispensed into wells of a sterile 96-well microplate. Wells were then inoculated with bacterial isolates using sterile toothpicks. Following inoculation, microplates were shaken (200 rpm) in an incubator for 24 hours at 25°C. After this time, microplates were examined visually to determine whether the wells were cloudy or clear.

Cloudiness was taken as an indicator of growth and resistance to that particular antibiotic at that particular concentration. Clearness was taken as an indicator of no growth and sensitivity to that antibiotic at that particular concentration. Each bacterial isolate was tested for resistance to each of the four antibiotics, with three replicate tests per antibiotic and antibiotic concentration. Any replicates showing positive growth were taken as an indication of antibiotic resistance. Each isolate was tested for resistance to each antibiotic at a concentration of 100, 200, 400, 800, 1200, 1600, 2000, 3000, 4000, and 5000 μg/mL. Overall resistance of a particular isolate to a particular antibiotic was reported as the highest concentration that still permitted any growth. Testing continued at increasing antibiotic concentrations until all isolates showed no resistance to the four antibiotics.

RESULTS

Antibiotic resistance was observed in isolates cultured in both R2A broth and TSB, and in isolates obtained from all samples whether sterilized or unsterilized, organically or conventionally grown, or from different lettuce types. The level of antibiotic resistance varied depending on the particular bacterial isolate and antibiotic tested (Table 4 and 5).

The greatest amount of antibiotic resistance among bacterial isolates was found in the presence of ampicillin (Figure 1). While fewer isolates generally showed resistance as ampicillin concentrations increased, this pattern was not clearly defined, and there were still isolates capable of growth at higher ampicillin concentrations. Isolates from R2A included 12 bacterial isolates that showed ampicillin resistance to over 1200 μg/mL, while isolates from TSA included 16 bacterial isolates that showed ampicillin resistance to over 1200 μg/mL. Bacterial isolates grown on TSA media generally showed the most resistance to ampicillin, with a mean (± SD) resistance of 800 ± 1334 μg/mL (standard deviations are large because of high variation in the concentration of antibiotics to which isolates were resistant). Five bacterial isolates grown in TSB showed ampicillin resistance to 5000 μg/mL, including *Pseudomonas rhodesiae* (isolate number 3), and four other isolates identified as *Pseudomonas* sp. (isolate numbers 49, 70, 75, and 77).

Table 4: Antibiotic resistance of bacterial isolates from different varieties of salad produce detected in R2A broth. Isolate number refers to the naming system used to identify the bacterial isolates during testing. Isolate represents the specific bacteria identified by 16S rRNA gene sequencing. Lettuce type represents the original source from which the bacterial isolates were collected. Antibiotic resistance shows the highest concentrations (µg/mL) at which the bacterial isolates showed growth.

Isolate Number	Isolate	Lettuce Type	Antibiotic Resistance (µg/mL)			
			Ampicillin	Erythromycin	Streptomycin	Tetracycline
1	*Pantoea* sp.	Romaine conventional unsterilized	0	0	0	0
2	*Pseudomonas* sp.	Romaine conventional unsterilized	400	100	100	0
4	*Janthinobacterium lividum*	Romaine conventional unsterilized	400	100	0	0
5	*Pseudomonas viridiflava*	Romaine conventional sterilized	400	0	0	0
6	*Flavobacterium succinicans*	Romaine conventional sterilized	0	0	0	0
7	*Janthinobacterium lividum*	Romaine conventional sterilized	800	0	400	0
9	*Flavobacterium succinicans*	Romaine conventional sterilized	200	100	0	0
11	*Pseudomonas* sp.	Romaine conventional sterilized	400	100	0	0
13	*Pseudomonas rhodesiae*	Romaine conventional sterilized	400	100	100	0
14	*Stenotrophomonas* sp.	Romaine organic unsterilized	1200	0	100	800
15	*Arthrobacter* sp.	Romaine organic unsterilized	0	1200	0	800
16	*Arthrobacter* sp.	Romaine organic unsterilized	2000	400	100	800
18	*Arthrobacter* sp.	Romaine organic	400	100	100	800

		sterilized				
19	*Bacillus flexus*	Romaine organic sterilized	100	0	0	0
20	*Pseudomonas* sp.	Romaine organic sterilized	400	200	0	0
21	*Sphingobium yanoikuyae*	Romaine organic sterilized	800	200	400	0
23	*Pseudomonas fragi*	Spinach conventional unsterilized	400	100	0	100
24	*Pseudomonas* sp.	Spinach conventional unsterilized	400	100	0	0
25	*Flavobacterium succinicans*	Spinach conventional sterilized	100	0	0	0
27	*Pseudomonas* sp.	Spinach conventional sterilized	400	100	0	0
30	*Acinetobacter* sp.	Spinach organic unsterilized	0	0	0	0
31	*Sejongia* sp.	Spinach organic unsterilized	400	0	100	0
32	*Shewanella sp. ANA-3*	Spinach organic unsterilized	400	0	0	0
33	*Flavobacterium succinicans*	Spinach organic unsterilized	100	0	100	0
35	*Pantoea* sp.	Spinach organic sterilized	0	200	0	0
36	*Curtobacterium flaccumfaciens*	Spinach organic sterilized	100	100	0	0
41	*Pseudomonas* sp.	Green Leaf conventional unsterilized	400	100	0	0
43	*Stenotrophomonas* sp.	Green Leaf conventional unsterilized	2000	400	400	800
44	*Janthinobacterium lividum*	Green Leaf conventional unsterilized	400	0	0	0
46	*Arthrobacter* sp.	Green Leaf conventional sterilized	0	0	100	0
47	*Pedobacter* sp.	Green Leaf conventional sterilized	800	0	200	0

48	*Sphingobacterium* sp.	Green Leaf conventional sterilized	100	0	0	0
49	*Leifsonia poae* sp.	Green Leaf conventional sterilized	400	0	0	100
51	*Agrobacterium* sp.	Green Leaf conventional sterilized	200	0	100	0
52	*Xanthomonadaceae* family	Green Leaf conventional sterilized	400	0	0	0
53	*Serratia* sp.	Green Leaf organic unsterilized	2000	100	0	100
55	*Chryseobacterium* sp.	Green Leaf organic unsterilized	400	0	0	0
59	*Pseudomonas* sp.	Green Leaf organic sterilized	800	400	400	0
60	*Pseudomonas rhodesiae*	Green Leaf organic sterilized	400	800	100	0
61	*Chryseobacterium* sp.	Green Leaf organic sterilized	200	100	0	0
62	*Pseudomonas rhodesiae*	Green Leaf organic sterilized	1200	200	100	0
63	*Pseudomonas* sp.	Iceberg conventional unsterilized	400	400	400	0
64	*Xanthomonas* sp.	Iceberg conventional unsterilized	400	100	400	0
65	*Acinetobacter* sp.	Iceberg conventional unsterilized	800	200	400	0
66	*Massilia timonae*	Iceberg conventional unsterilized	1200	100	400	0
68	*Pedobacter* sp.	Iceberg conventional sterilized	400	0	0	100
70	*Chryseobacterium* sp.	Iceberg conventional sterilized	0	0	0	0
71	*Sphingomonas* sp.	Iceberg conventional sterilized	200	0	0	0
72	*Erwinia* sp.	Iceberg conventional sterilized	400	100	0	0
74	*Stenotrophomonas*	Iceberg	800	400	100	0

	sp.	organic unsterilized				
76	*Paenibacillus amylolyticus*	Iceberg organic unsterilized	0	0	100	0
78	*Pseudomonas* sp.	Iceberg organic sterilized	400	800	0	0
79	*Pseudomonas* sp.	Iceberg organic sterilized	2000	200	400	0
80	*Microbacterium* sp.	Iceberg organic sterilized	0	0	0	0
81	*Chryseobacterium* sp.	Iceberg organic sterilized	100	0	0	0
82	*Erwinia* sp.	Iceberg organic sterilized	400	100	2000	0
83	*Methylobacterium adhaesivum*	Red Leaf conventional unsterilized	0	0	0	0
84	*Sphingomonas* sp.	Red Leaf conventional unsterilized	100	0	0	0
88	*Pseudomonas veronii*	Red Leaf conventional unsterilized	3000	400	400	100
89	*Pseudomonas* sp.	Red Leaf conventional unsterilized	400	200	100	0
91	*Pseudomonas* sp.	Red Leaf conventional sterilized	400	100	100	0
92	*Pseudomonas* sp.	Red Leaf conventional sterilized	400	200	100	0
96	*Pseudomonas* sp.	Red Leaf organic unsterilized	400	100	100	0
97	*Flavobacterium succinicans*	Red Leaf organic unsterilized	100	0	0	0
101	*Pseudomonas* sp.	Red Leaf organic unsterilized	1200	200	400	0
102	*Pseudomonas* sp.	Red Leaf organic unsterilized	2000	200	400	0
105	*Frigoribacterium* sp.	Red Leaf organic sterilized	0	0	0	0
106	*Pseudomonas* sp.	Red Leaf organic	1600	100	0	100

		sterilized				
107	*Microbacterium* sp.	Red Leaf organic sterilized	0	100	0	0
108	*Curtobacterium flaccumfaciens*	Red Leaf organic sterilized	100	0	0	0
109	*Pseudomonas rhodesiae*	Red Leaf organic sterilized	2000	400	100	0
110	*Pseudomonas viridiflava*	Red Leaf organic sterilized	800	100	0	0
111	*Devosia* sp.	Red Leaf organic sterilized	0	0	0	0

Table 5: Antibiotic resistance of bacterial isolates from different varieties of salad produce detected in TSB. Isolate number refers to the naming system used to identify the bacterial isolates during testing. Isolate represents the specific bacteria identified by 16S rRNA gene sequencing. Lettuce type represents the original source from which the bacterial isolates were collected. Antibiotic resistance shows the highest concentrations (μg/mL) at which the bacterial isolates showed growth.

Isolate Number	Isolate	Lettuce Type	Antibiotic Resistance (μg/mL)			
			Ampicillin	Erythromycin	Streptomycin	Tetracycline
2	*Pseudomonas* sp.	Romaine conventional unsterilized	800	200	0	0
3	*Pseudomonas rhodesiae*	Romaine conventional unsterilized	5000	100	0	0
7	*Xanthomonas* sp.	Romaine conventional unsterilized	0	0	0	0
10	*Pseudomonas rhodesiae*	Romaine conventional sterilized	1600	200	100	100
11	*Pseudomonas viridiflava*	Romaine conventional sterilized	100	0	0	0
13	*Pseudomonas* sp.	Romaine conventional sterilized	400	800	0	0
15	*Pseudomonas* sp.	Romaine organic unsterilized	400	800	200	100
17	*Arthrobacter* sp.	Romaine organic unsterilized	0	0	800	800
19	*Bacillus flexus*	Romaine organic unsterilized	200	0	0	0
21	*Pseudomonas* sp.	Romaine organic sterilized	400	100	0	0
22	*Arthrobacter* sp.	Romaine organic sterilized	100	100	400	400
23	*Pantoea* sp.	Romaine organic sterilized	200	100	200	0
24	*Flavobacterium succinicans*	Spinach conventional	100	0	0	0

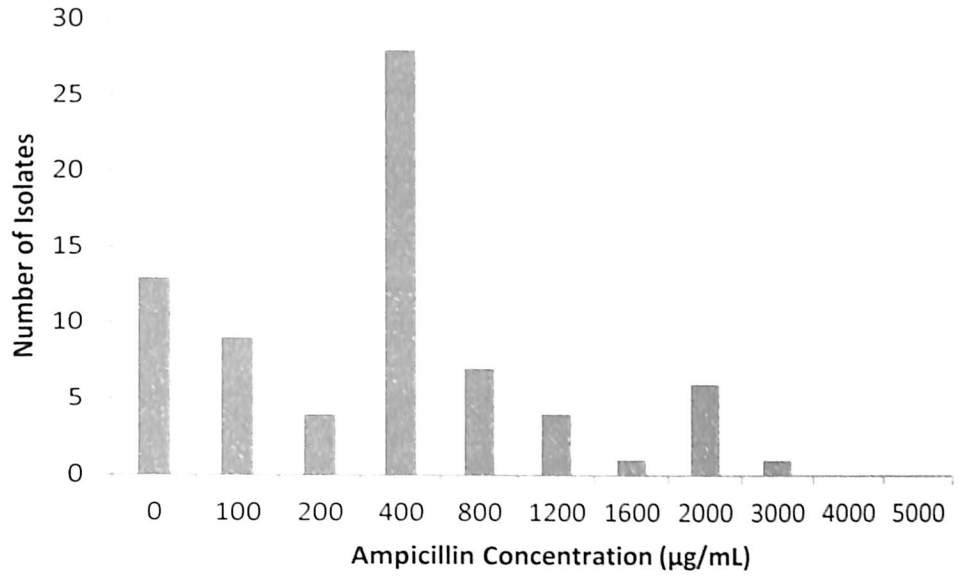
		unsterilized				
25	*Pseudomonas rhodesiae*	Spinach conventional unsterilized	3000	400	200	100
27	*Pseudomonas fragi*	Spinach conventional unsterilized	800	200	800	100
29	*Pseudomonas* sp.	Spinach conventional sterilized	1200	100	100	0
30	*Pseudomonas* sp.	Spinach conventional sterilized	3000	100	200	0
32	*Pseudomonas* sp.	Spinach conventional sterilized	400	0	0	0
34	*Pseudomonas* sp.	Spinach organic unsterilized	400	100	100	100
35	*Pseudomonas* sp.	Spinach organic unsterilized	100	100	0	0
36	*Pseudomonas fragi*	Spinach organic unsterilized	100	0	100	100
39	*Pantoea* sp.	Spinach organic sterilized	200	200	100	0
40	*Pantoea* sp.	Spinach organic sterilized	200	100	100	0
41	*Pseudomonas fragi*	Spinach organic sterilized	100	100	100	200
42	*Pseudomonas fragi*	Spinach organic sterilized	100	100	0	0
43	*Microbacterium* sp.	Spinach organic sterilized	0	0	0	0
45	*Chryseobacterium* sp.	Green Leaf conventional unsterilized	0	0	100	100
46	*Sphingobacterium faecium*	Green Leaf conventional unsterilized	100	0	400	100
47	*Pantoea* sp.	Green Leaf conventional unsterilized	100	100	100	0
48	*Mycetocola* sp.	Green Leaf conventional unsterilized	0	0	0	0
49	*Pseudomonas* sp.	Green Leaf conventional unsterilized	5000	0	400	100

52	*Arthrobacter* sp.	Green Leaf conventional unsterilized	0	0	0	100
53	*Pseudomonas* sp.	Green Leaf conventional unsterilized	800	100	0	100
55	*Agrobacterium* sp.	Green Leaf conventional sterilized	100	0	0	0
56	*Pantoea* sp.	Green Leaf conventional sterilized	200	0	100	100
57	*Sphingobacterium faecium*	Green Leaf conventional sterilized	100	0	400	400
58	*Pseudomonas* sp.	Green Leaf conventional sterilized	3000	200	400	800
59	*Microbacterium* sp.	Green Leaf conventional sterilized	0	0	0	0
60	*Aeromicrobium* sp.	Green Leaf conventional sterilized	0	100	0	0
61	*Chryseobacterium* sp.	Green Leaf conventional sterilized	100	0	0	0
63	*Microbacterium* sp.	Green Leaf conventional sterilized	100	0	0	0
66	*Stenotrophomonas* sp.	Green Leaf organic unsterilized	800	200	1200	100
68	*Serratia*	Green Leaf organic unsterilized	400	1600	2000	1200
70	*Pseudomonas* sp.	Green Leaf organic unsterilized	5000	400	800	200
73	*Pseudomonas syringae*	Green Leaf organic sterilized	100	100	0	0
74	*Pseudomonas* sp.	Green Leaf organic sterilized	3000	400	1200	100
75	*Pseudomonas* sp.	Green Leaf organic sterilized	5000	100	800	0
76	*Serratia* sp.	Green Leaf organic sterilized	400	200	100	100
77	*Pseudomonas* sp.	Green Leaf organic sterilized	5000	100	800	100
78	*Ewingella*	Green Leaf	1600	1600	1200	800

	americana	organic sterilized				
79	*Xanthomonas* sp.	Iceberg conventional unsterilized	0	0	0	0
81	*Pseudomonas rhodesiae*	Iceberg conventional unsterilized	3000	0	0	100
82	*Pantoea* sp.	Iceberg conventional unsterilized	0	200	800	0
87	*Chryseobacterium* sp.	Iceberg conventional sterilized	0	0	0	100
88	*Agrobacterium* sp.	Iceberg conventional sterilized	0	0	100	0
89	*Chryseobacterium* sp.	Iceberg conventional sterilized	0	0	0	100
90	*Sphingobium yanoikuyae*	Iceberg conventional sterilized	0	0	100	0
91	*Erwinia rhapontici*	Iceberg organic unsterilized	400	400	200	100
92	*Pseudomonas* sp.	Iceberg organic unsterilized	1200	200	0	100
93	*Pantoea* sp.	Iceberg organic unsterilized	100	200	0	0
95	*Pseudomonas* sp.	Iceberg organic unsterilized	800	200	0	100
97	*Stenotrophomonas* sp.	Iceberg organic unsterilized	400	200	400	0
98	*Erwinia rhapontici*	Iceberg organic unsterilized	800	200	200	100
102	*Paenibacillus amylolyticus*	Iceberg organic sterilized	0	0	2000	0
104	*Pseudomonas* sp.	Iceberg organic sterilized	400	400	0	100
106	*Erwinia* sp.	Iceberg organic sterilized	800	100	200	100
110	*Curtobacterium flaccumfaciens*	Red Leaf conventional unsterilized	100	0	0	0
111	*Microbacterium* sp.	Red Leaf conventional	0	0	0	0

		unsterilized				
112	*Pantoea* sp.	Red Leaf conventional unsterilized	100	200	0	0
113	*Pseudomonas* sp.	Red Leaf conventional sterilized	1200	400	100	100
114	*Flavobacterium succinicans*	Red Leaf organic unsterilized	0	0	0	0
119	*Pseudomonas* sp.	Red Leaf organic unsterilized	800	200	800	400
121	*Pseudomonas rhodesiae*	Red Leaf organic unsterilized	1200	200	0	0
122	*Pseudomonas* sp.	Red Leaf organic unsterilized	800	1200	0	200
129	*Pseudomonas* sp.	Red Leaf organic sterilized	800	200	100	0
130	*Flavobacterium succinicans*	Red Leaf organic sterilized	0	0	0	0
131	*Curtobacterium flaccumfaciens*	Red Leaf organic sterilized	0	100	0	0
132	*Pseudomonas* sp.	Red Leaf organic sterilized	400	100	0	0
133	*Flavobacterium succinicans*	Red Leaf organic sterilized	100	0	100	200

A



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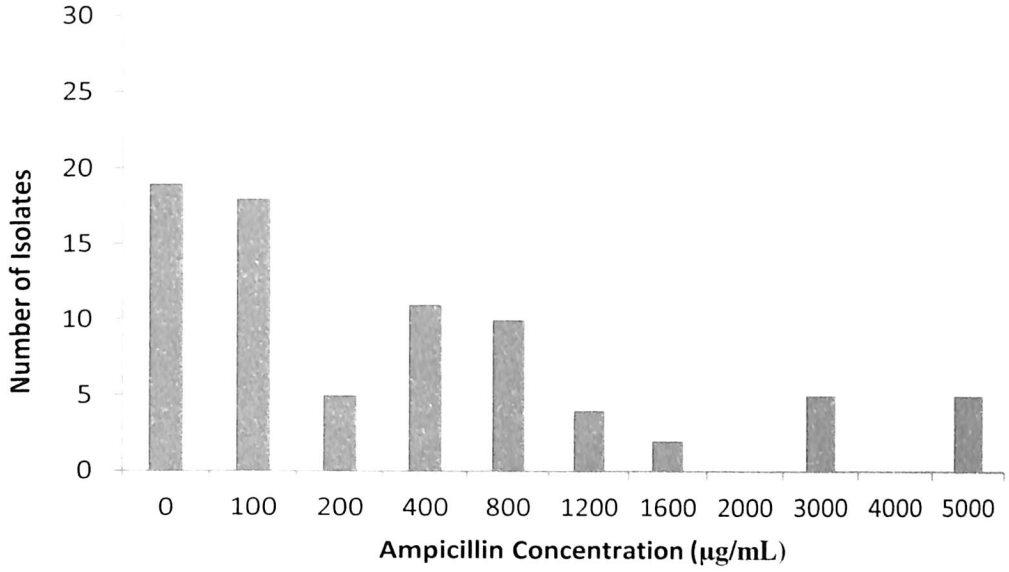

Figure 1: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of ampicillin. Isolates were tested for antibiotic resistance using either R2A (A) or TSB (B) media. Ampicillin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 73 isolates were tested using R2A broth and 79 isolates were tested using TSB.

Resistance to erythromycin, streptomycin, and tetracycline followed a more expected pattern of growth - as the concentrations of antibiotic increased, the number of isolates showing resistance steadily decreased (Figure 2-4). Antibiotic resistance to erythromycin did not differ greatly between isolates obtained from the different media types, with the mean (±SD) resistance in R2A broth being 134 ± 209 µg/mL and in TSB being 175 ± 305 µg/mL. One bacterial isolate from R2A showed resistance to over 1200 µg/mL of erythromycin, while three isolates from TSA showed resistance to at least this this concentration of erythromycin (Figure 2). Although no bacterial isolate showed erythromycin resistance up to 5000 µg/mL, two isolates (*Serratia* sp (isolate number 68) and *Ewingella americana* (isolate number 78)) grown on TSA media showed resistance to 1600 µg/mL.

As with ampicillin, resistance to streptomycin was greatest in bacterial isolates grown in TSB which showed a mean (± SD) resistance to 235 ± 421 µg/mL streptomycin, compared to isolates grown in R2A broth which exhibited a mean (± SD) resistance to 121 ± 266 µg/mL. Isolates from R2A included one that showed streptomycin resistance to over 1200 µg/mL, while Five bacterial isolates from TSA were resistant to greater than 1200 µg/mL (Figure 3). In total, three isolates displayed a resistance of 2000 µg/mL- *Erwinia* sp. (isolate number 82) from R2A, and *Serratia* sp. (isolate number 68) and *Paenibacillus amylolyticus* (isolate number 102) from TSA. Isolates showed the least amount of resistance to tetracycline, with the mean (± SD) resistance of R2A and TSA derived isolates being 63 ± 203 µg/mL and 104 ± 209 µg/mL, respectively. No isolates from R2A grew at a tetracycline concentration of 1200 µg/mL,

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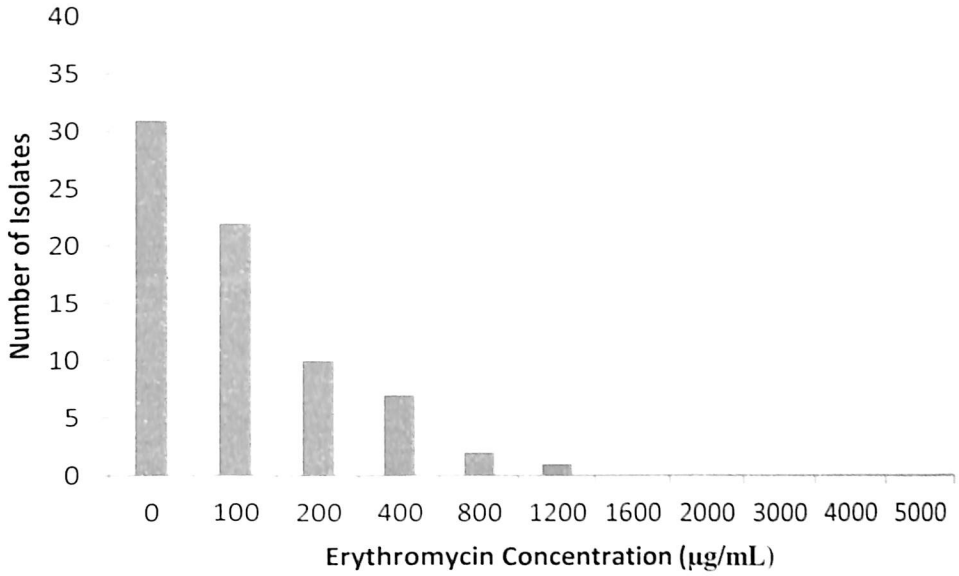

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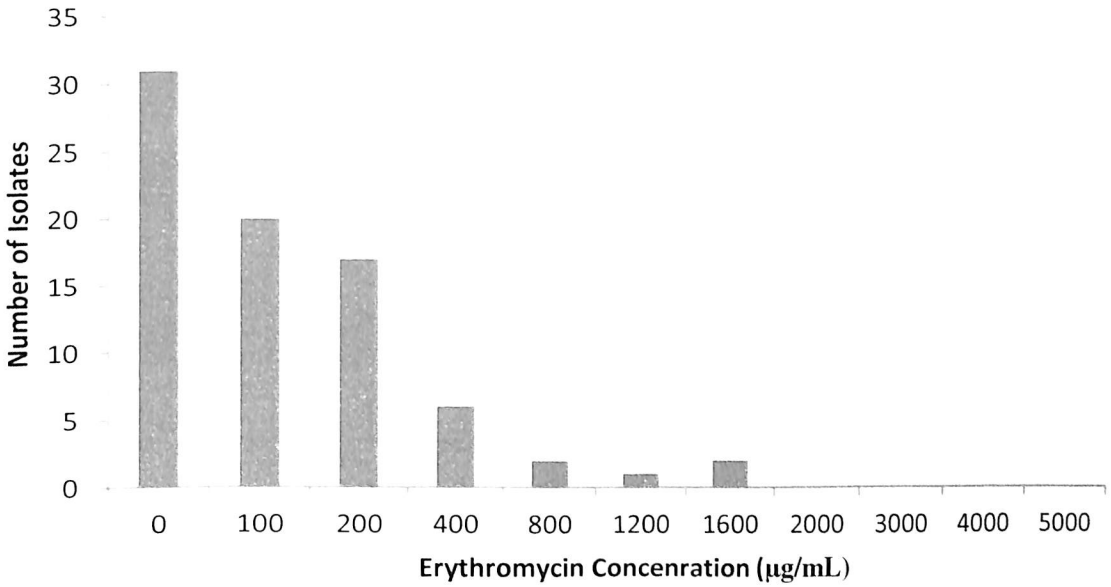

Figure 2: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of erythromycin. Isolates were tested for antibiotic resistance using either R2A (A) or TSB (B) media. Erythromycin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 73 isolates were tested using R2A broth and 79 isolates were tested using TSB.

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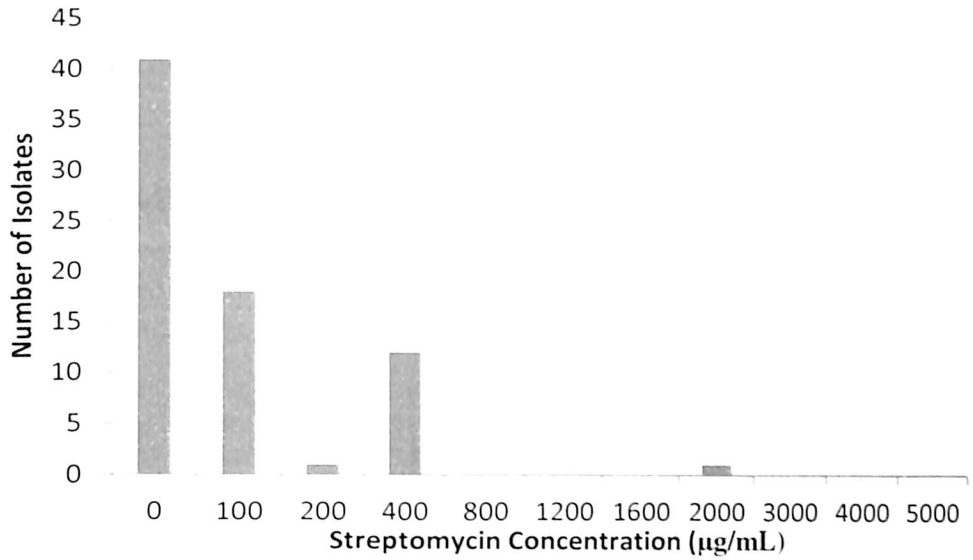

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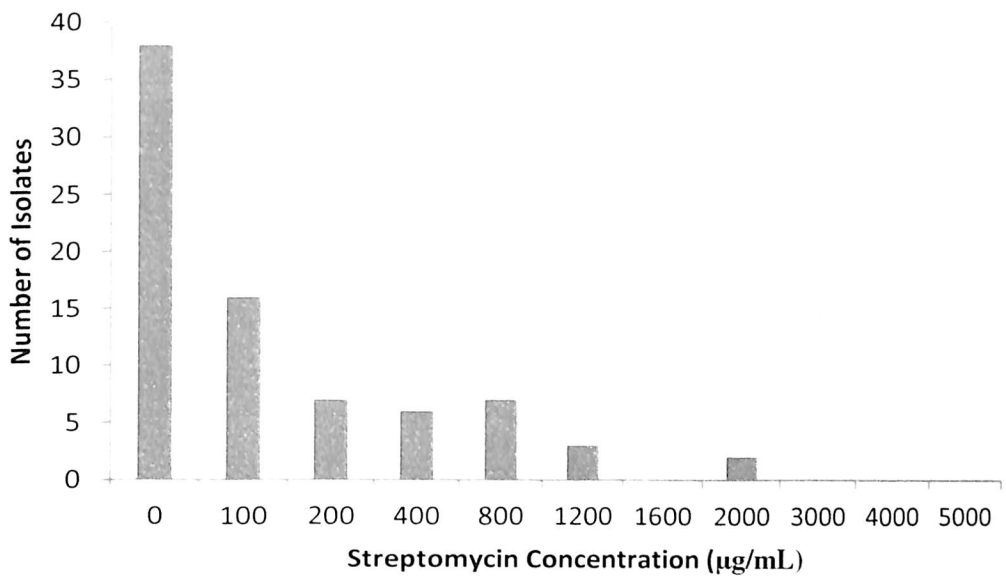

Figure 3: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of streptomycin. Isolates were tested for antibiotic resistance using either R2A (A) or TSB (B) media. Streptomycin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 73 isolates were tested using R2A broth and 79 isolates were tested using TSB.

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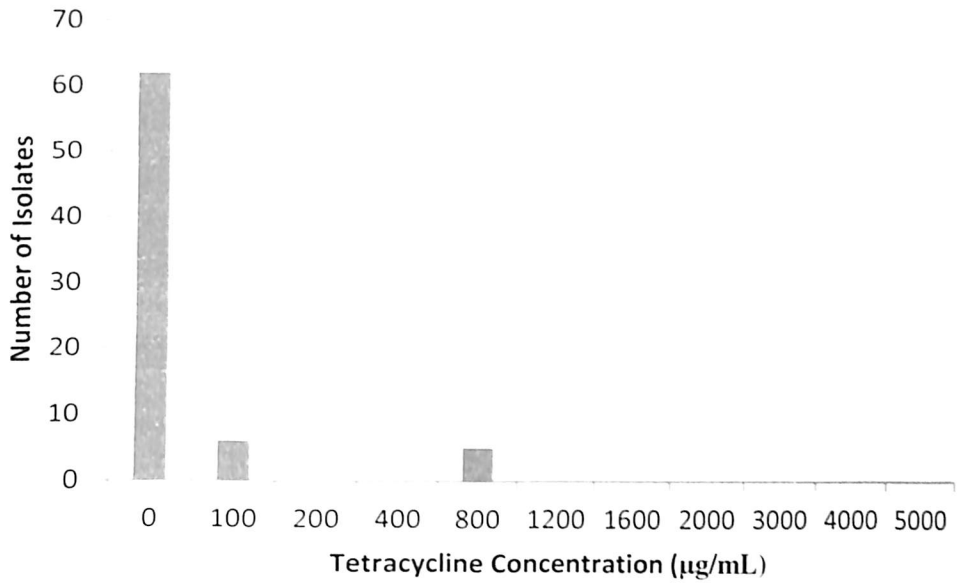

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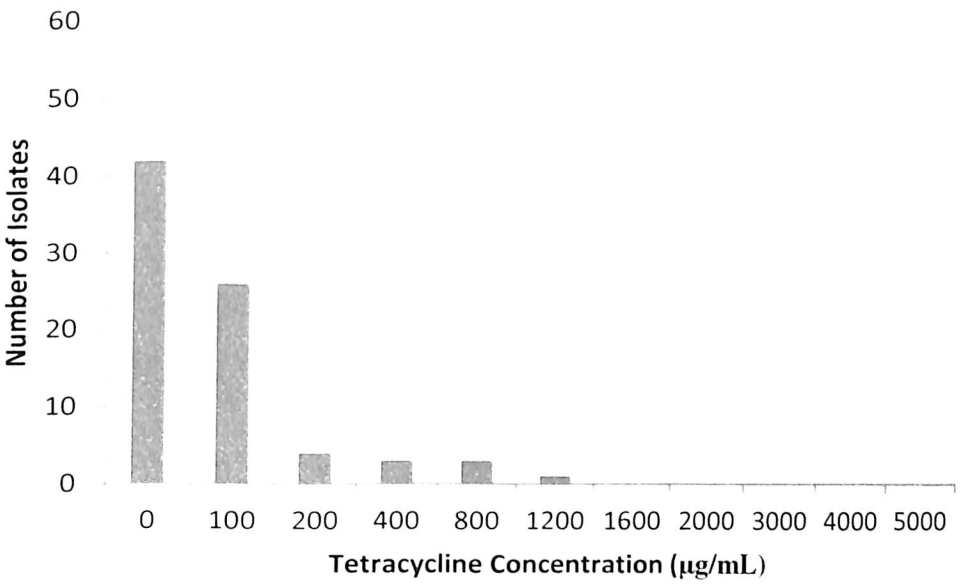

Figure 4: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of tetracycline. Isolates were tested for antibiotic resistance using either R2A (A) or TSB (B) media. Tetracycline concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 73 isolates were tested using R2A broth and 79 isolates were tested using TSB.

while a single isolate (*Serratia* sp. (isolate number 68)) from TSA was resistant to 1200 µg/mL (Figure 4).

When isolates were separated into those obtained from surface sterilized or unsterilized samples, the greatest amount of resistance in each group of isolates was also seen for ampicillin (Figure 5). Isolates from unsterilized samples showed a mean (± SD) resistance to ampicillin of 784 ± 1153 µg/mL, while isolates from surface sterilized samples showed a mean (± SD) ampicillin resistance of 588 ± 974 µg/mL. Isolates from surface sterilized samples included 13 that showed ampicillin resistance to over 1200 µg/mL, while 15 isolates from unsterilized samples showed ampicillin resistance to over 1200 µg/mL. Of the five bacterial isolates that could resist 5000 µg/mL of ampicillin, two *Pseudomonas* sp. (isolate numbers 75 and 77) came from surface sterilized samples, while the other three isolates, *Pseudomonas rhodesiae* (isolate number 3) and two *Pseudomonas* sp. (isolate numbers 49 and 70) came from unsterilized samples.

When resistance patterns to the other three antibiotics were separated by isolates from sterilized verses unsterilized samples, the same patterns were seen as when isolates were grouped together. Mean (± SD) erythromycin resistance in isolates obtained from surface sterilized and unsterilized samples was 134 ± 236 and 180 ± 292 µg/mL, respectively. Isolates from surface sterilized samples included one bacterial isolate that showed antibiotic resistance to over 1200µg/mL, while isolates from unsterilized samples included three bacterial isolates that showed antibiotic resistance to this concentration or higher (Figure 6). The highest erythromycin resistance observed (1600 µg/mL) was exhibited by two isolates: *Ewingella americana* (isolate number 78) from a surface sterilized sample and *Serratia* sp. (isolate number 68) from an unsterilized sample.

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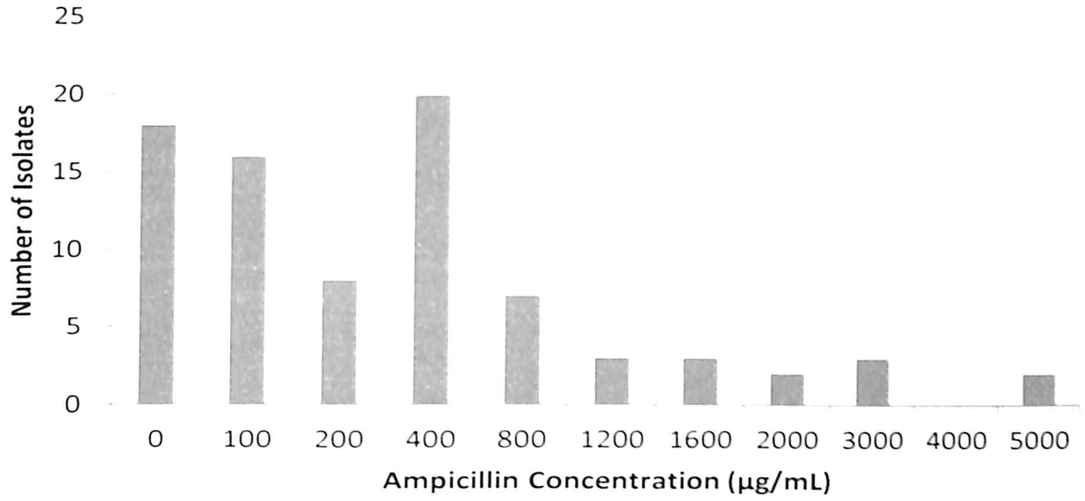

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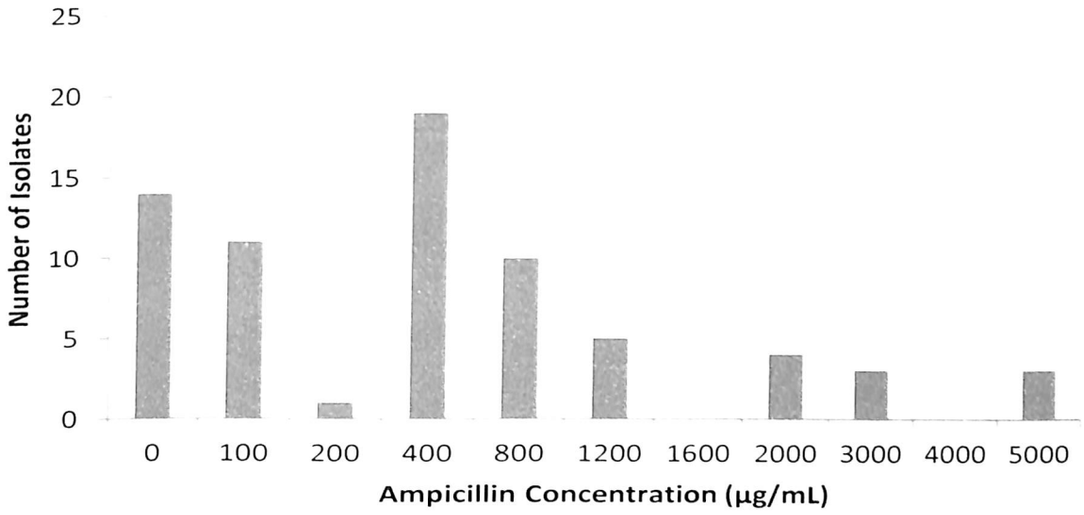
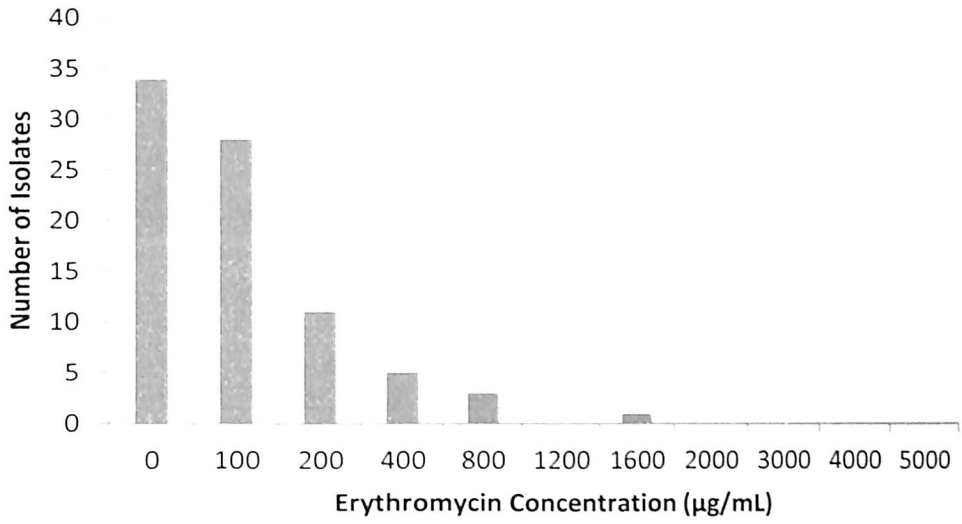


Figure 5: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of ampicillin. Isolates tested for antibiotic resistance were obtained from either surface sterilized (A) or unsterilized (B) leaf vegetables. Ampicillin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 82 isolates were tested from surface sterilized produce and 70 isolates were tested from unsterilized produce.

A



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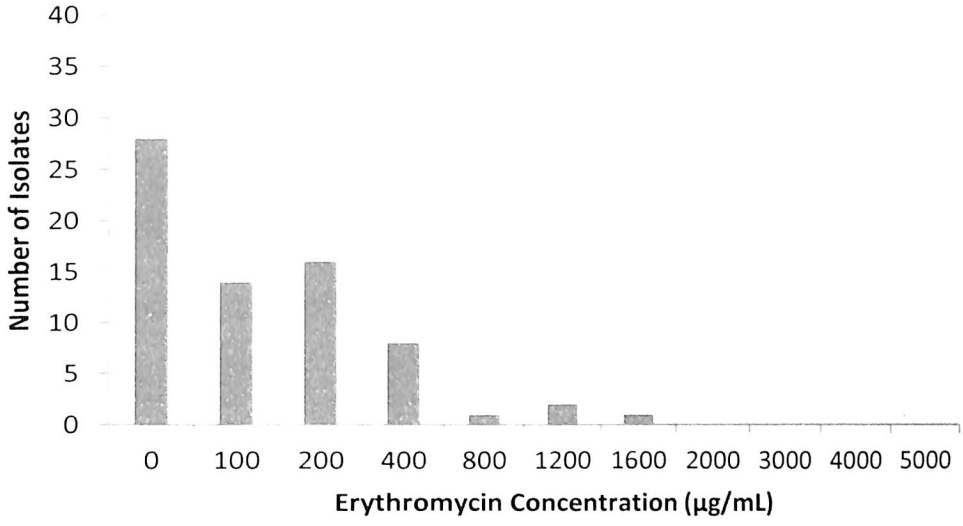

Figure 6: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of erythromycin. Isolates tested for antibiotic resistance were obtained from either surface sterilized (A) or unsterilized (B) leaf vegetables. Erythromycin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 82 isolates were tested from surface sterilized produce and 70 isolates were tested from unsterilized produce.

Isolates obtained from surface sterilized and unsterilized produce showed little differences in streptomycin resistance, with the mean resistance (± SD) being 167 ± 375 and 196 ± 341 µg/mL, respectively. Isolates obtained from surface sterilized samples included four that showed antibiotic resistance to over 1200 µg/mL, while isolates from unsterilized samples included two bacteria that showed resistance to over 1200 µg/mL (Figure 7). Three bacterial isolates could resist 2000 µg/mL of streptomycin: two from surface sterilized samples, *Erwinia* sp. (isolate number 82) and *Paenibacillus amylolyticus* (isolate number 102), and one, *Serratia* sp. (isolate number 68), from an unsterilized sample. In terms of tetracycline resistance, isolates from surface sterilized and unsterilized samples showed mean (± SD) resistances of 60 ± 163 and 113 ± 246 µg/mL, respectively. No isolates from surface sterilized samples showed tetracycline resistance to 1200 µg/mL, while one isolate (*Serratia* sp. (isolate number 68)) from an unsterilized sample was resistant to 1200 µg/mL of tetracycline (Figure 8). Overall, there did not appear to be any clear difference in resistance to any of the antibiotics tested based on whether isolates came from surface sterilized or unsterilized samples.

When isolates were grouped into those obtained from produce that was either organically or conventionally grown, the greatest amount of resistance in each group of isolates was still seen for ampicillin (Figure 9). Overall, organic and conventional methods of growth yielded isolates with similar ampicillin resistance (means (± SD) resistance of 713 ± 1063 µg/mL for isolates derived from organic produce vs. 642 ± 1066 µg/mL for isolates from conventional produce). Isolates from organically grown samples included 16 bacterial isolates that showed ampicillin resistance to over 1200 µg/mL, while isolates from conventionally grown samples included 12 bacterial isolates that

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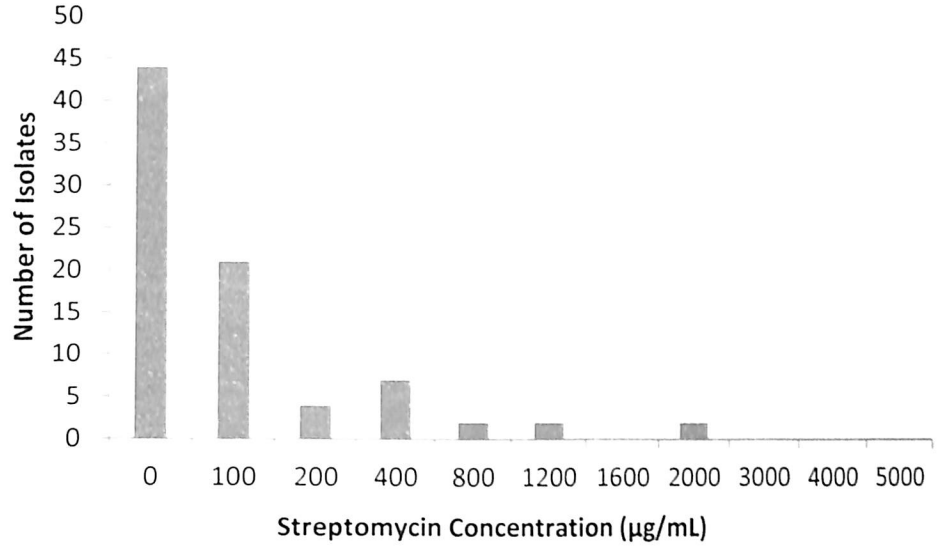

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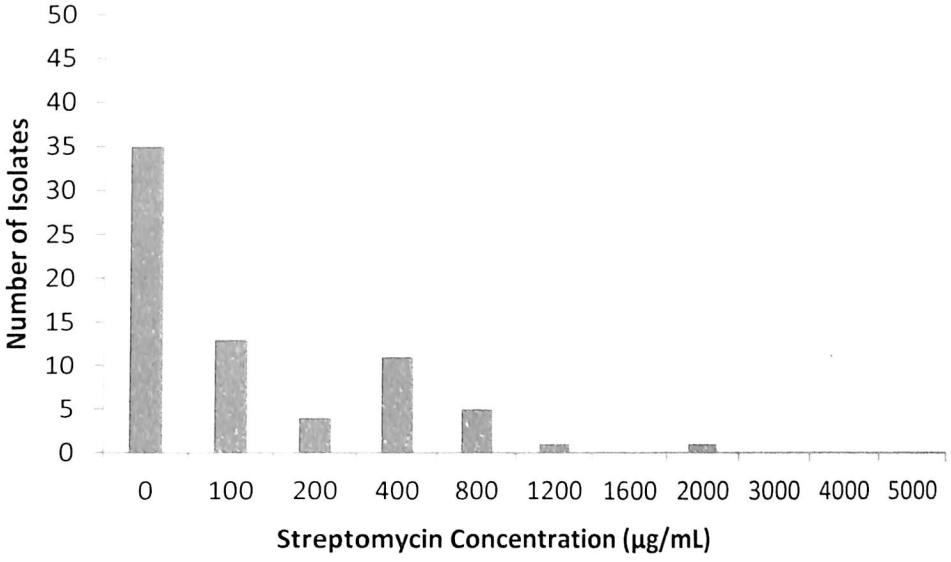
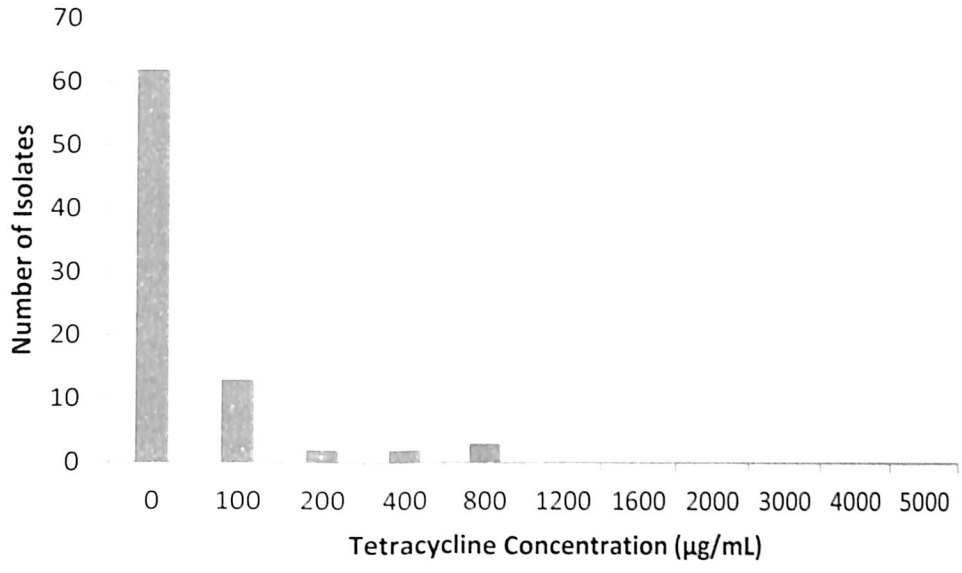


Figure 7: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of streptomycin. Isolates tested for antibiotic resistance were obtained from either surface sterilized (A) or unsterilized (B) leaf vegetables. Streptomycin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 82 isolates were tested from surface sterilized produce and 70 isolates were tested from unsterilized produce.

A



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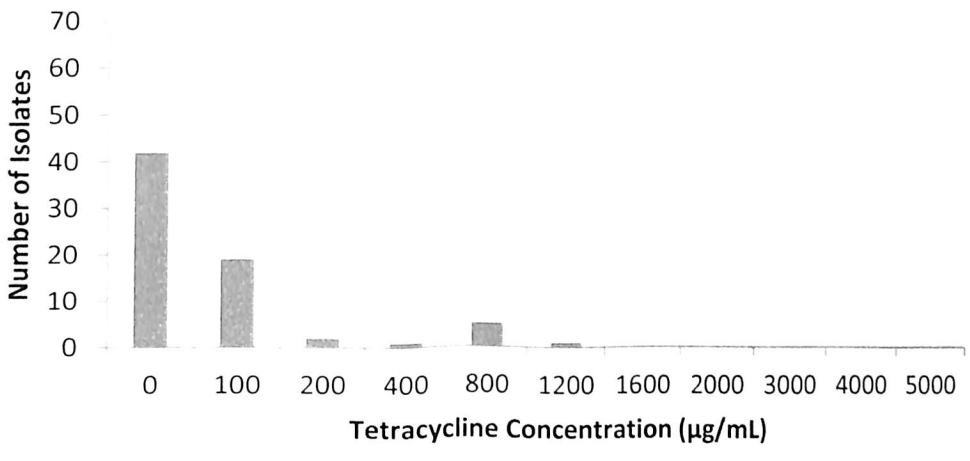
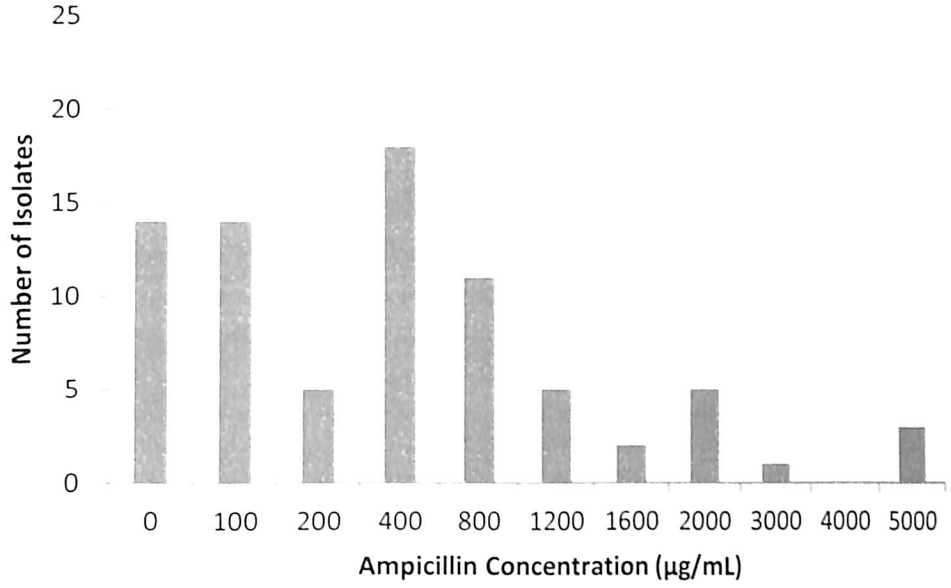


Figure 8: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of tetracycline. Isolates tested for antibiotic resistance were obtained from either surface sterilized (A) or unsterilized (B) leaf vegetables. Tetracycline concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 82 isolates were tested from surface sterilized produce and 70 isolates were tested from unsterilized produce.

A



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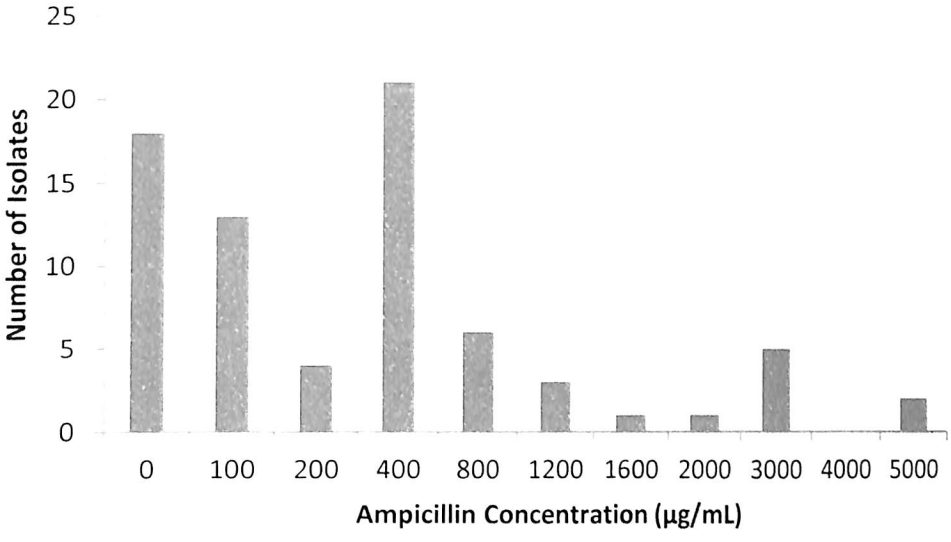


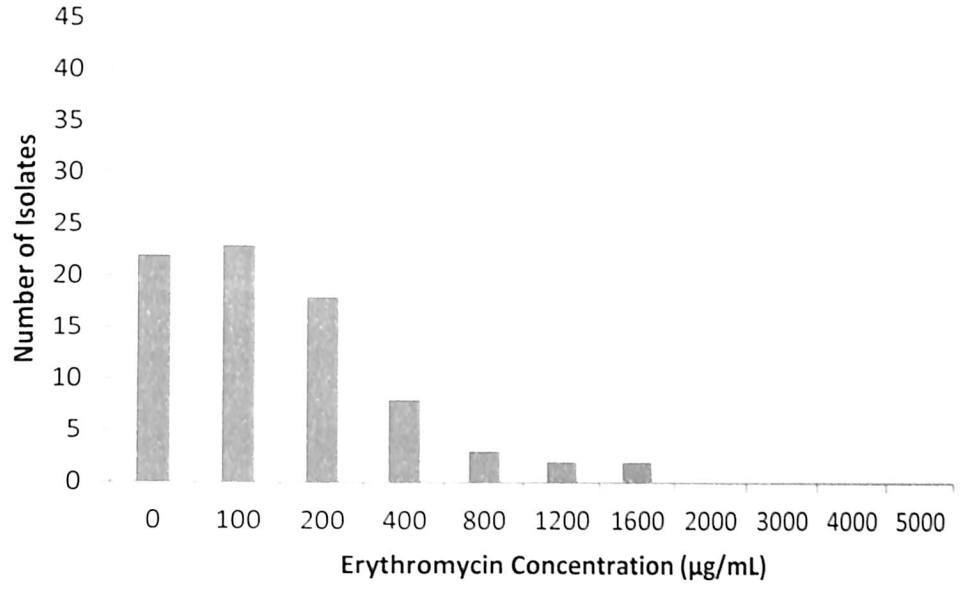
Figure 9: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of ampicillin. Isolates were tested for antibiotic resistance from either organic (A) or conventional (B) grown produce. Ampicillin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 78 isolates were tested from organically grown produce and 74 isolates were tested from conventionally grown produce.

showed ampicillin resistance to this concentration of ampicillin or greater. Of the five bacterial isolates that could resist 5000 µg/mL of ampicillin, three *Pseudomonas* sp. (isolate numbers 70, 75, and 77) came from organically grown samples, while the other two, *Pseudomonas rhodesiae* (isolate number 3) and *Pseudomonas* sp. (isolate number 49), came from conventionally grown samples.

When resistance patterns to the other three antibiotics were examined the same way (by distinguishing isolates based on whether they were obtained from organically or conventionally grown produce), organically grown produce generally yielded isolates with higher erythromycin resistance (mean (± SD) of 219 ± 330 µg/mL), compared to isolates from conventionally grown samples (mean (± SD) of 88 ± 139 µg/mL). Four isolates from organically grown samples showed erythromycin resistance to over 1200µg/mL, while no isolates from conventionally grown samples could resist 1200 µg/mL erythromycin (Figure 10). The highest erythromycin resistance observed (1600 µg/mL) was exhibited by two isolates from organically grown samples: *Ewingella americana* (isolate number 78) and *Serratia* sp. (isolate number 68).

Isolates from organically grown samples showed slightly higher streptomycin resistances samples than those from conventionally grown samples, with the mean (± SD) streptomycin resistance of the isolates being 247 ± 459 and 109 ± 182 µg/mL, respectively. Isolates obtained from organically grown samples included six that showed streptomycin resistance to over 1200 µg/mL, while no isolates from conventionally grown samples showed resistance to that concentration (Figure 11). Three of the bacterial isolates derived from organic produce displayed streptomycin resistance of 2000 µg/mL: *Serratia* sp. (isolate number 68), *Erwinia* sp. (isolate number 82) and

A



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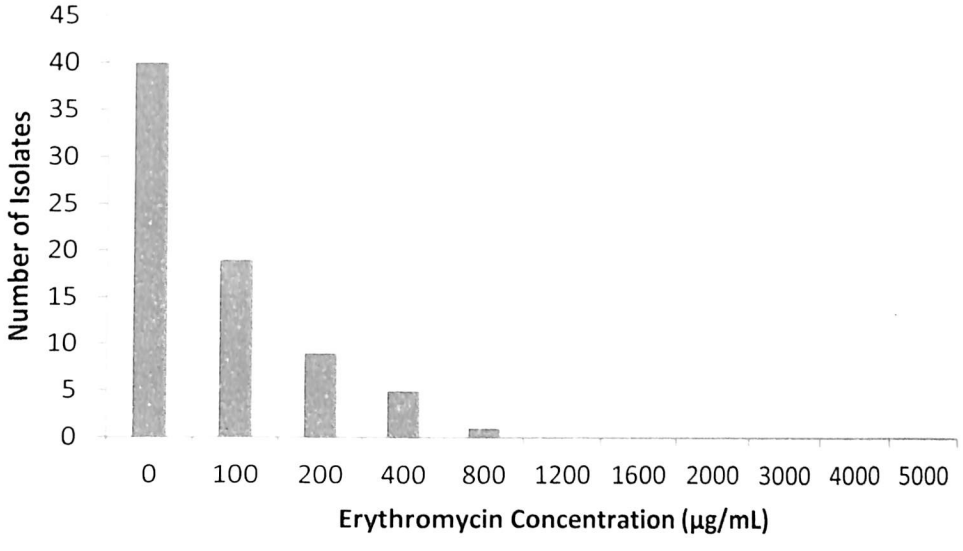
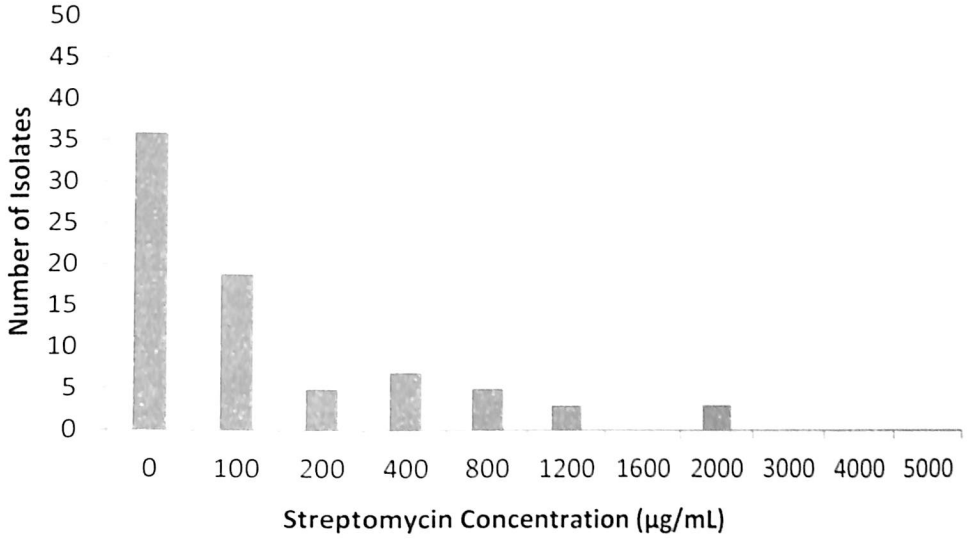


Figure 10: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of erythromycin. Isolates were tested for antibiotic resistance from either organic (A) or conventional (B) grown produce. Erythromycin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 78 isolates were tested from organically grown produce and 74 isolates were tested from conventionally grown produce.

A



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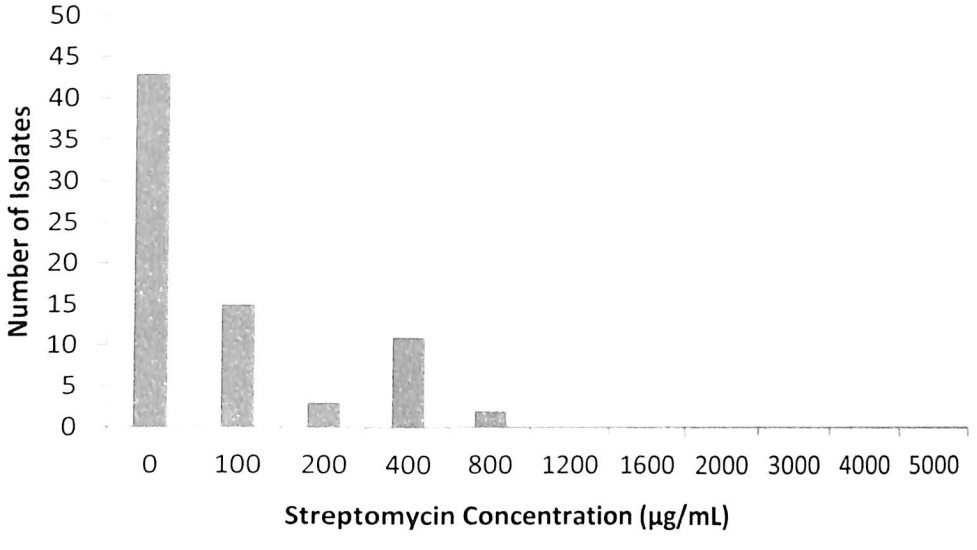
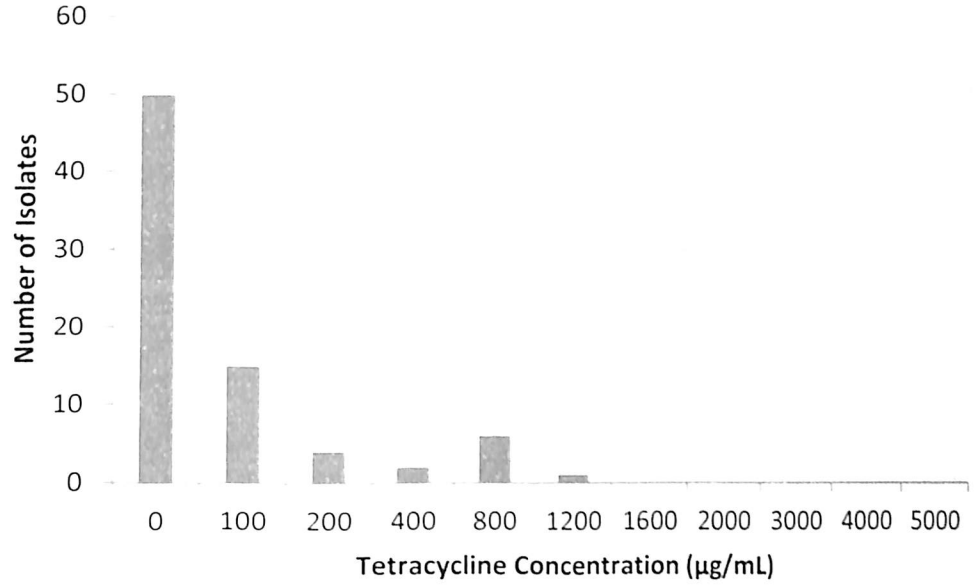


Figure 11: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of streptomycin. Isolates were tested for antibiotic resistance from either organic (A) or conventional (B) grown produce. Streptomycin concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 78 isolates were tested from organically grown produce and 74 isolates were tested from conventionally grown produce.

Paenibacillus amylolyticus (isolate number 102). In terms of tetracycline resistance, means (± SD) of isolates obtained from organically and conventionally grown samples were 117 ± 251 and 50 ± 140 µg/mL, respectively. One isolate (*Serratia* sp. (isolate number 68)) from an organically grown sample showed tetracycline resistance to over 1200 µg/mL, while no isolates from conventionally grown samples were that resistant (Figure 12). Overall, while there was no clear difference in antibiotic resistance based on whether isolates came from organically or conventionally grown samples, there was a suggestion that isolates from organically grown produce may be more resistant to three (erythromycin, streptomycin, tetracycline) of the four antibiotics tested.

Patterns in antibiotic resistance were also evaluated on more detailed scale, by separating isolates into five groups based on the specific plant type that they were isolated from, regardless of sterilized, unsterilized, organic, or conventional (Table 6). Isolates from green leaf lettuce showed the greatest mean (±SD) resistance to ampicillin (1041 ± 1554 µg/mL), while isolates from other plant types showed mean ampicillin resistances that were roughly half of that. Erythromycin resistance was more similar in isolates obtained from the five lettuce types than ampicillin resistance, ranging from isolates obtained from spinach which showed the lowest mean (± SD) erythromycin resistance (88 ± 95 µg/mL), to isolates obtained from green leaf lettuce which, as with ampicillin, showed the greatest mean (± SD) erythromycin resistance (187 ± 373 µg/mL). As a group, green leaf lettuce isolates also showed the greatest resistance to streptomycin, with a mean (± SD) streptomycin resistance of 292 ± 458 µg/mL. Isolates from iceberg lettuce showed similar mean streptomycin resistance to those obtained from green leaf lettuce, while isolates from the other three produce types showed mean streptomycin

A



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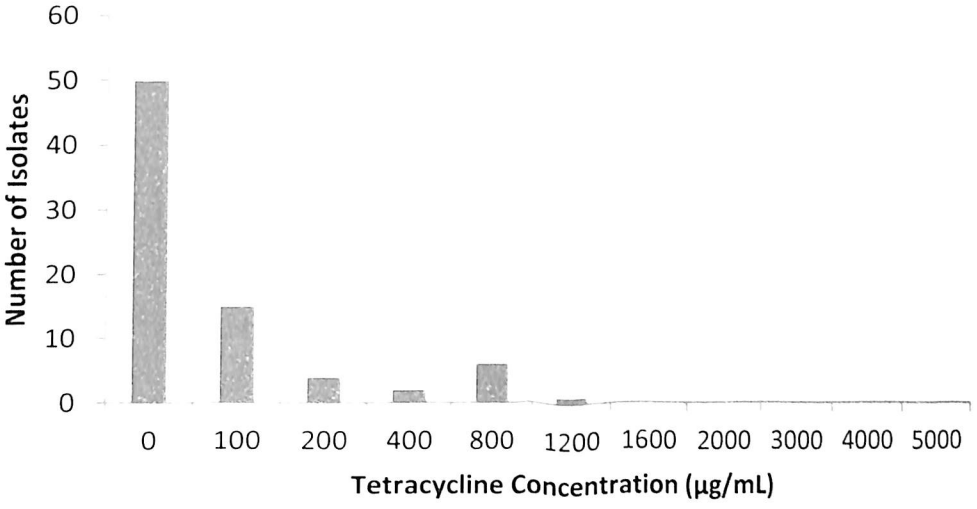

Figure 12: Numbers of bacterial isolates obtained from salad produce that showed resistance to different concentrations of tetracycline. Isolates were tested for antibiotic resistance from either organic (A) or conventional (B) grown produce. Tetracycline concentration is the highest concentration at which a bacterial isolate still showed growth. A total of 78 isolates were tested from organically grown produce and 74 isolates were tested from conventionally grown produce.

Table 6: Antibiotic resistance of bacterial isolates from five different varieties of salad produce. Lettuce type represents the original source from which the bacterial isolates were collected. Mean and standard deviation of antibiotic resistance represents the average concentration (μg/mL) at which isolates from a particular lettuce type showed resistance to various antibiotics. Numbers of isolates tested for each produce type were green leaf (39), iceberg (31), romaine (28), red leaf (30), and spinach (24).

Produce Type	**Mean and Standard Deviation of Antibiotic Resistance (μg/mL)**			
	Ampicillin	**Erythromycin**	**Streptomycin**	**Tetracycline**
Green Leaf	1041 ± 1554	187 ± 373	292 ± 458	138 ± 278
Iceberg	497± 656	145 ± 182	265 ± 503	32 ± 48
Romaine	611 ± 986	179 ± 288	107 ± 186	164 ± 312
Red Leaf	600 ± 754	157 ± 233	93 ± 180	37 ± 89
Spinach	500 ± 817	88 ± 95	83 ± 166	29 ± 55

resistances approximately one third of that concentration. Unlike the other antibiotics, the highest mean resistance to tetracycline was seen in isolates obtained from romaine lettuce, although green leaf lettuce isolates also showed appreciable tetracycline resistance. Lower resistance to tetracycline was seen in isolates obtained from red leaf lettuce, iceberg lettuce, and baby spinach. In terms of general patterns between produce types, isolates from baby spinach tended showed the lowest antibiotic resistance, while isolates from green leaf lettuce were the most resistant.

Most isolates (67.7%) showed resistance to multiple antibiotics, although 16 of the 152 bacterial isolates (10.5%) showed no resistance to any of the four antibiotics tested (Figure 13). 33 isolates (21.7%) showed resistance to one antibiotic. For the multidrug resistant isolates, 23.0% of bacterial isolates showed resistance to two of the tested antibiotics, 28.9% of bacterial isolates showed resistance to three of the tested antibiotics, and 15.8% of bacterial isolates showed some level of resistance to all four antibiotics. Of the multidrug resistant isolates, several showed high levels of resistance to the four antibiotics tested. All of these isolates were obtained using TSA media. *Serratia* sp. (isolate number 68) came from unsterilized organically grown green leaf lettuce and showed an ampicillin resistance to 400 µg/mL, erythromycin resitance to 1600 µg/mL, streptomycin resistance to 2000 µg/mL, and tetracycline resistance to 1200 µg/mL. *Pseudomonas* sp. (isolate number 70) also came from unsterilized organically grown green leaf lettuce and could withstand 5000 µg/mL of ampicillin, 400 µg/mL of erythromycin, 800 µg/mL of streptomycin, and 200 µg/mL of tetracycline. A third highly resistant isolate, *Pseudomonas* sp. (isolate number 74), also came from organic green leaf lettuce, but from a surface sterilized sample. This isolate showed ampicillin

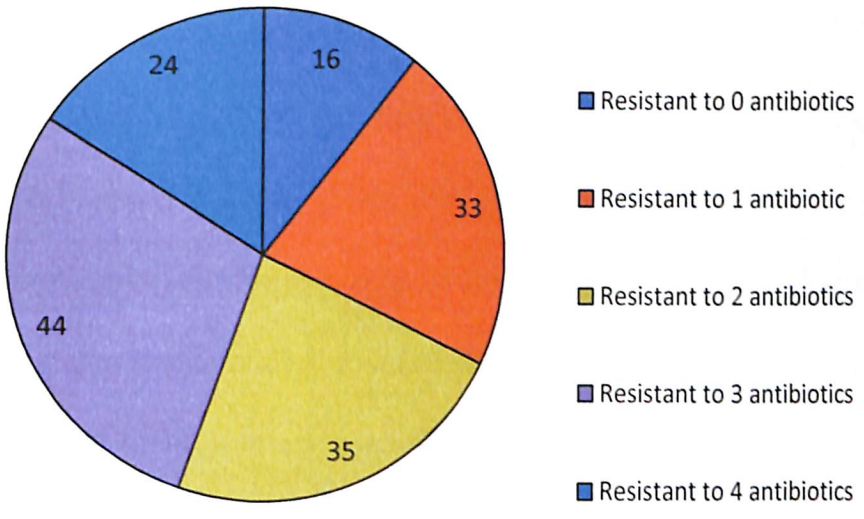

Figure 13: Numbers of bacterial isolates obtained from salad produce that showed multidrug resistance. Antibiotics tested were ampicillin, erythromycin, streptomycin, and tetracycline, and isolates were obtained from green leaf, iceberg, romaine, red leaf, and spinach produce. A total of 152 bacterial isolates were tested for antibiotic resistance.

resistance of 3000 µg/mL, erythromycin resitance of 400 µg/mL, streptomycin resistance of 1200 µg/mL, and tetracycline resistance of 100 µg/mL. *Ewingella americana* (isolate number 78) also came from a surface sterilized organic green leaf lettuce sample, and showed resistances to 1600, 1600, 1200, and 800 µg/mL for ampicillin, erthromycin, streptomycin, and tetracycline, respectively. Together, these four bacterial isolates showed the greatest overall amount of antibiotic resistance, and all four were isolated from the organically grown green leaf lettuce sample.

DISCUSSION

An increase in the popularity of nutritional and convenient food such as prepackaged salad produce has led to an increased awareness of food microbiology. Pathogen contamination of food has been identified as a transmitter of many known diseases (Bryan, 1982), and is responsible for a vast number of illnesses, hospitalizations, and deaths within the United States each year (Mead et al., 1999). With the increased demand for prepackaged produce have come large outbreaks of pathogens associated with leafy salad vegetables, possibly because cut surfaces on the leaves release nutrients that can stimulate the growth of pathogenic microorganisms (Heaton and Jones, 2007). As health concerns become more prevalent, efforts have been made to offer a wider variety of fresh produce through improvements in the transportation and preservation of such food (Glanz and Yarock, 2004). This has allowed for consumers to add more produce into their diet, obtained from markets which stock minimally processed and ready-to-consume produce (Everis, 2004). These ready-to-consume products have the potential to contain pathogenic bacteria, which is a major interest in regards to understanding foodborne illnesses. This study focused on bacterial isolates found on samples obtained from prepackaged leafy green vegetables and the antibiotic resistance shown by those isolates. Findings from this study could help to better understand the relationship between antibiotic resistance and bacteria found in food, as well as influence others to appreciate the importance of foodborne pathogenic outbreaks.

The bacterial isolates tested were obtained from a previous study that determined plate counts of various leafy green salad vegetables to be from 10^2 to 10^8 CFU/g fresh leaf material (Randolph, 2011). The highest colony counts were obtained from baby spinach (10^8 CFU/g), although few of those bacterial isolates were identified (Randolph, 2011). The lowest counts (10^2-10^3 CFU/g) were generally found on iceberg lettuce, although the diversity of bacterial species isolated from lettuce samples appeared to be much greater than those found on baby spinach. Organically grown samples of baby spinach, red lettuce, and green leaf lettuce showed higher CFU counts compared to the same vegetables grown conventionally. As would be expected, unsterilized samples, which were simply washed with sterile water, yielded higher CFU counts than surface sterilized samples, typically by two or three orders of magnitude (Randolph, 2011).

The purpose of this particular project was to test the bacterial isolates obtained from the previously discussed study for resistance to different concentrations of four antibiotics: ampicillin, erythromycin, streptomycin, and tetracycline. The concentrations of antibiotics used ranged from 100 µg/mL to 5000 µg/mL. Isolates generally showed the highest overall resistance to ampicillin, with five bacterial isolates showing ampicillin resistance to 5000 µg/mL. By comparison, the highest levels of streptomycin and erythromycin resistance were 2000 and 1600 µg/mL, respectively, and the maximum resistance to tetracycline was even lower (1200 µg/mL exhibited by just one isolate). One possible reason for the higher ampicillin resistance could be methodological, for example if the ampicillin did not fully dissolve in the stock solutions used this would result in the bacteria being exposed to lower concentrations than believed. However, from visual examination of the stock solutions, all antibiotics appeared to be fully dissolved at the

time of use so that differences in solubility between antibiotics are unlikely to be the cause of different resistance patterns. Ampicillin is in the penicillin class of antibiotics, the first type of antibiotics developed in the 1930's and 1940's (Bennett et al., 2001). Given that this group of antibiotics has been in use for over 70 years, there has been a longer time period over which bacteria could develop resistance to them compared to other classes of antibiotics. Another potential factor accounting for overall higher resistance to ampicillin could be in the way that this antibiotic functions. Ampicillin impairs the ability of bacteria to form cross links in peptidoglycan as they assemble the cell wall, affecting Gram positive bacteria more than Gram negative bacteria (Sauvage et al., 2011). This was illustrated in that the bacteria with the most resistance were indeed Gram negative, suggesting that they were less affected by ampicillin than Gram positive bacteria.

Generally, the concentrations of antibiotics used in this study are comparable to or exceed the typical clinical dose recommended for human use. The typical oral human dose of ampicillin, erythromycin, and tetracycline normally does not exceed 500 mg (Satoskar et al., 2009). Given that an average human body holds approximately six liters of blood, a typical dose of these antibiotics would result in a blood titer of 500 mg/ 6L or 83 mg/L (assuming 100% efficiency of uptake into the bloodstream). The lowest antibiotic concentration tested (100 μg/mL, equivalent to 100 mg/L) in this study is comparable to this number, although many bacterial isolates were resistant to much higher amounts. The highest resistance to ampicillin that was found was 5000 μg/mL; around 60 times that of an actual dose. Even the highest resistances seen for erythromycin and tetracycline (to 1600 μg/mL) are around 19 times that which might be

encountered in the human body. The typical human dose of streptomycin is roughly 1/10 of that of the other antibiotics (50 mg), so that even the lowest concentrations tested in this study are 10 times the concentration of streptomycin that would be expected in human blood, and the highest resistance to streptomycin observed (2000 µg/mL) would be around 200 times what a bacterial pathogen could be exposed to within a human being treated with this antibiotic. As a result, the isolates tested within this study are significant in that some exhibit resistance to extremely high concentrations of antibiotics, which are much greater than doses of those same antibiotics used to treat human pathogens.

As a group, isolates obtained from green leaf lettuce tended to be the most antibiotic resistant, although the colony counts on green leaf lettuce determined in the previous study were not particularly high or low (ranging from 10^3 to 10^7 CFU/g; Randolph 2011). Isolates from green leaf lettuce showed the greatest resistance to ampicillin, erythromycin, and streptomycin, and although green leaf lettuce isolates were not generally the most resistant to tetracycline (isolates from romaine lettuce were), these isolates did show appreciable tetracycline resistance. The four bacterial isolates (two species of *Pseudomonas*, one species of *Serratia*, and an isolate identified as *Ewingella americana*) that were the most resistant to all of the antibiotics tested were from green leaf lettuce samples. Green leaf lettuce is a loose leaf lettuce, meaning that it is not grown, stored, or packaged in a way that the leaves are tightly packed together, potentially resulting in opportunities for bacteria to be transferred to the surface of the lettuce leaf in a multitude of locations. The presence of potentially highly antibiotic resistant bacteria residing on and within green leaf lettuce does suggest that consumption

of this vegetable could result in the transfer of the bacteria to the human body, which could in turn lead to illness and disease.

Isolates obtained from non-surface sterilized produce typically showed slightly higher resistances to antibiotics than those obtained from surface sterilized material, but these were only minor differences. In the previous study, surface sterilized samples were washed with tap water, followed by three separate washes of both 1.3% sodium hypochlorite solution and 70% ethanol solution before culturing (Randolph, 2001). Given this stringent sterilization procedure, the bacterial isolates obtained from sterilized samples are almost certainly endophytes living within the vegetable. Colony counts from unsterilized samples were consistently higher than surface sterilized samples for all produce types, although surface sterilized samples did yield an appreciable number of colonies, up to a third of what was found on unsterilized samples (Randolph 2011). This indicates that although the sterilization methods were likely effective, they could not be used to completely rid the plant of all bacteria, especially those which are endophytes. Many endophytic bacteria are not transient populations but are true endosymbionts that live within a plant for at least part of their life, forming relationships with their hosts (Sturz et al., 2000). Because endophytes are inside the plant tissue, there is no washing method that can be used to remove these bacteria before ingesting the vegetable. Therefore, prewashing carried out prior to packaging or washing carried out by consumers before ingesting the leafy vegetable of choice are useless in regards to eliminating or minimizing the presence of these endophytes. Although many endophytic bacteria are unlikely to be pathogenic to humans, the consumption of antibiotic resistant

endophytes could facilitate the transfer of their antibiotic resistance to bacteria residing in humans, ultimately resulting in increased antibiotic resistance of human pathogens.

Bacterial isolates obtained from organically grown samples generally yielded higher resistances to all antibiotics, although this was only a minor difference with regards to ampicillin. The suggestion of higher antibiotic resistance in isolates obtained from organic produce could be a result of growing practices in the field, such as the use of animal manure instead of the fertilizers that are used within conventional growing methods (Pimentel et al., 2005). Animal manure is a recognized carrier of both plant-associated bacteria, which are passed through the animal, and bacteria that can potentially live within humans (Cotta et al., 2003). When animal manure is used to fertilize plants, it can result in the transfer of potentially harmful bacteria directly onto the plant surface. There is the possibility that antibiotic resistant bacteria within the manure could move onto or into crops, or transfer their antibiotic resistance to other environmental bacteria. The spread of antibiotic resistant bacteria within the agricultural production chain is directly affected by the way manure is handled and applied (Kudva et al., 1998). Due to recent foodborne outbreaks, in the United States, composting is suggested for all manure before it is applied to an organic farm; however, in other parts of the world, manure is still used in its raw state (Semenov et al., 2007). While it could be assumed that only composted manure was used on the organic produce from which these isolates were obtained, there is no way of verifying that assumption.

Over two thirds of the bacterial isolates tested showed resistance to multiple antibiotics, with several of the multidrug resistant isolates withstanding high concentrations of all of the antibiotics tested. Resistance to specific antibiotics is

normally due to an adaptation acquired through mutation or gene transfer (Bennett, 2008). Typically, resistance is initially built up to a specific drug, followed by cross-resistance to several structurally related drugs (Pearce et al., 1989). The four antibiotics used in this study represent different classes of antibiotics: beta-lactam antibiotics (ampicillin), macrolide antibiotics (erythromycin), aminoglycoside antibiotics (streptomycin), and polyketide antibiotics (tetracycline). The mechanisms for resistance differ for each of these classes. Resistance to ampicillin occurs either through enzymatic hydrolysis of the antibiotic (the most common method of resistance) or through alteration of penicillin-binding proteins in the cell wall (Garcia-Cobos et al., 2007). The mechanisms for erythromycin resistance are typically target-site modification or an efflux mechanism (Pinheiro et al., 2009). Similarly, streptomycin resistance also arises from efflux systems, although these are typically expressed through chromosomal changes rather than the acquisition of new genetic elements (Islam et al., 2008). Tetracycline resistance can arise from genes that code for energy-dependent efflux systems or from proteins that protect bacterial ribosomes from the blockage of protein synthesis, which is thought to be one of the most frequent types of antibiotic resistance, passed through gene transfer (Ammor et al., 2008). Bacterial isolates that are multidrug resistant must be exhibiting more than one of these resistance mechanisms. There are few other studies that have examined the presence of multidrug resistant isolates in produce, although multidrug resistance has been found in lactose fermenting bacteria obtained from samples of several vegetables, including lettuce (Levy, 1984). Also, vegetarians have been found to carry more resistant fecal flora than meat eaters, further suggesting that these resistant bacteria reside on uncooked produce, such as lettuce (Levy, 2001). Decreases in the

numbers of antibiotic resistant bacteria have also been seen in vegetarians that have been switched to a sterilized diet, suggesting that food either carries resistant bacteria or has the ability to harbor residues of antibiotics (Levy, 2001).

The four specific bacterial isolates that showed high levels of resistance to all antibiotics tested were obtained using TSA media, and all are found within the phylum Gammaproteobacteria. Because of their Gram negative cell wall, bacteria within this group are generally more resistant to penicillin-derived antibiotics (such as the ampicillin used in this study) than Gram positive bacteria (Ryan and Ray, 2004). Two of these isolates were identified as species of *Pseudomonas* and came from organic green leaf lettuce (one from an unsterilized lettuce sample and one from a sterilized sample). Many species of *Pseudomonas* have long been recognized to possess the ability to grow at low temperatures, allowing them to cause food spoilage even while food is being refrigerated (Pereira and Morgan, 1957). One species, *Pseudomonas aeruginosa*, is considered an opportunistic pathogen to humans, and some strains are highly antibiotic resistant, acquiring resistance through mutation or gene transfer (Poole, 2004). *P. aeruginosa*, which is a major cause of nosocomial infections, also resists antibiotics intrinsically; when these mechanisms are present together, multidrug resistance is displayed, making many drug treatments ineffective (Mesaros et al., 2007). A third multidrug resistant isolate (obtained from unsterilized organically grown green leaf lettuce) was identified as a species of *Serratia*, another genus that can be an opportunistic pathogen of humans and which also causes nosocomial infections (Ligozzi et al., 2010). The species of *Serratia* that is the most commonly encountered as a human pathogen is *Serratia marcescens*, which can be found in damp conditions in bathrooms and hospitals (Hejazi and Falkiner,

1997). Strains of *S. marcescens* have been shown to be highly antibiotic resistant to multiple antibiotics, and to possess the capability to transfer their antibiotic resistance to other bacteria (Zhang et al., 2007).

A fourth highly resistant isolate (obtained from surface sterilized organic green leaf lettuce) was identified as *Ewingella americana*, another potential pathogen. *E. americana* is the only known species in the genus *Ewingella*, which was only described in 1983. Although the presence of *E. americana* as a human pathogen is rare, it has been found in clinical specimens taken from blood, urine, stools, and conjunctiva, and is thought to be normally transmitted in hospital settings (Ryoo et al., 2005). *E. americana* has been tested for its susceptibility to numerous antibiotics, and some strains appear to be naturally resistant or show just intermediate susceptibility to many antibiotics, including erythromycin (Stock et al., 2003). It is interesting that the four bacterial isolates that showed high resistance to all four antibiotics tested are also potentially pathogens of humans. Ingestion of produce containing such highly resistant pathogenic bacteria could be extremely dangerous for the immunocompromised, especially if the bacterial strains are multidrug resistant and can no longer be targeted by the common antibiotics used to fight such infections.

Both the original source of the antibiotic resistant isolates obtained from salad produce, and how these bacterial isolates acquired such antibiotic resistance are interesting points to consider. It is possible that the bacteria had some level of natural resistance that increased over time, perhaps through exposure to background levels of antibiotics used in agriculture. Alternatively, some isolates could have been the recipients of antibiotic resistant genes passed from other, more resistant, bacteria. If antibiotic

resistance is indeed being transferred between bacteria, the transfer process could be happening between populations in close proximity on the leaf surface, in which case sterilization methods might minimize its spread. On the other hand, if resistance is being passed between endophytic bacteria within the leaves, then any surface sterilizing or washing procedures would be ineffective at reducing the spread of antibiotic resistance. Further studies on the potential transmission of resistance between endophytic populations, and between endophytes and bacteria associated with humans are certainly needed.

Because of an increased demand for healthy and convenient food, the presence of antibiotic resistant bacteria that are potential pathogens in salad produce is of vital interest. With an estimated one in four Americans being affected by a foodborne pathogen each year, it is obvious that foodborne illnesses are a significant problem (Tauxe, 2002). Bacteria that are antibiotic resistant not only have the ability to become more resistant to multiple antibiotics, but can also transfer their resistance to other species of bacteria. Ingestion of antibiotic resistant foodborne bacteria could result in the transmission of resistance to pathogens or commensal bacteria already residing within the human body. Thus, while humans may not be affected directly by the ingestion of plant-associated bacteria, the ability of these microorganisms to transfer antibiotic resistance to other bacteria could be a significant problem. While currently there are only a few pathogens that have been recognized as being resistant to a wide variety of antibiotics, increased use and misuse of antibiotics exerts a continued pressure for the development of further antibiotic resistance. An increased focus on the detection of antibiotic resistant

bacteria in food products could become extremely important in the coming years, as more and more pathogens become increasingly more antibiotic resistant.

The following implications should be taken away from this study:

- Salad vegetables may serve as vehicles to transport antibiotic resistance to other bacteria.
- Producers of salad vegetables need to be aware of antibiotic resistance concerns and monitor the antibiotics used within the growing process, whether it is through fertilizer or manure.
- Consumers of salad vegetables need to be aware of the origin of lettuce consumed and the fact that bacteria, which could possibly be antibiotic resistant, may reside on or within the food source.

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