EFFECT OF STANDARD POST-HARVEST INTERVENTIONS ON THE SURVIVAL AND RE-GROWTH OF ANTIBIOTIC-RESISTANT BACTERIA ON FRESH PRODUCE

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SCIENTIFIC ABSTRACT

Raw vegetables can sometimes be the source of outbreaks of human illness; however the potential for fresh vegetables to serve as a vehicle for antibiotic -resistant bacteria is poorly understood. Antibiotics and antibiotic-resistant bacteria have been shown to persist in manure of animals administered antibiotics, and in compost generated from this manure, where there is the potential for their transfer to produce. The purpose of this study was to determine the survival of antibiotic-resistant bacteria on raw, peeled, carrots after washing with commonly used chemical sanitizers.

Multi-drug resistant *E. coli* O157:H7 and *Pseudomonas aeruginosa* were inoculated into a compost slurry of composted manure from dairy cattle, with and without prior administration of antibiotics, and used to inoculate carrot surfaces prior to the washing studies. This approach provided defined model antibiotic-resistant pathogens present within a background microbial community simulating potential carry over from manure-derived fertilizer. Carrots (n=3, 25g) were air-dried and stored at 4°C until washing with tap water, XY-12 (sodium hypochlorite, 50 ppm free chlorine) or Tsunami 100 (peroxyacetic acid/hydrogen peroxide, 40 ppm free paracetic acid), according to manufacturer's directions. A second batch of carrots representing each inoculation x wash condition (n=3) were individually packaged for storage at 2°C for 1,7, and 14 days, or 10°C for 7 days and enumerated on those day intervals to recover bacteria from the surfaces of washed carrots. The resulting previously washed and stored carrots were subject to serial dilution and plated onto corresponding agar to enumerate total aerobic bacteria (R2A),

aerobic bacteria tolerant or resistant to antibiotics (antibiotic-supplemented R2A), *E. coli* (Eosin Methylene Blue), and *Pseudomonas* spp. (*Pseudomonas* Isolation Agar). In addition, the *tet*A gene was quantified from the carrot samples as a measure of the effect of sanitizers and storage on an antibiotic resistance gene known to be carried by the inoculated bacteria.

Inclusion of sanitizer in the wash water significantly reduced the absolute numbers of inoculated bacteria (*E.coli* and *Pseudomonas*) as well as populations of bacteria capable of growth on the R2A media containing cefotaxime (10μg/mL), sulfamethoxazole (100μg/mL), or tetracycline (3μg/mL). Comparable reductions in the inoculated *P. aeruginosa* resistant to tetracycline (PIA T, 4μg/mL), bacteria resistant to cefotaxime (10μg/mL) and tetracycline (3μg/mL) occurred after washing with XY-12 or Tsunami 100. The sanitizer effectiveness may be bacterial dependent, as evident by larger absolute reductions of the inoculated *E. coli* (EMB) and bacteria grown on sulfamethoxazole (100μg/mL)-amended plates after washing with Tsunami 100 compared to washing with tap water or XY-12.

Re-growth of both the inoculated and native compost-associated bacteria was inhibited by storage at 2°C, as there were no significant differences in the log CFU/g values on the various media (total aerobic bacteria, bacteria on antibiotic-amended plates, *E. coli* inoculum, *P. aeruginosa* inoculum) during the 14-day storage period. However, temperature abuse at 10°C resulted in significant re-growth of native *Pseudomonas*, compared to storage at 2°C. A sanitizer-associated interaction between re-growth and temperature was also observed for bacteria resistant to clindamycin (25μg/mL) and cefotaxime (10μg/mL), with substantial re-growth occurring only on carrots washed with Tsunami 100. There was no significant re-growth of the inoculated *E. coli* O157:H7 at either temperature. Results indicate that some bacterial

populations are reduced by post-harvest washes and that temperature abuse of fresh produce may result in increases in antibiotic-resistant bacterial populations.

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PUBLIC ABSTRACT

Fresh vegetables are frequently washed to remove soil and pests before shipment to suppliers, with the goal of creating a ready-to-eat- product for consumers. The inclusion of a chemical sanitizer in the wash water has the benefit of killing or reducing the number of bacteria in the wash water. Chemical sanitizers also have the potential to reduce spoilage bacteria and human pathogenic bacteria on the vegetable and prevent cross-contamination from one vegetable to another. While the intention of sanitizers is to reduce bacterial numbers in wash water, there can be added benefit of also reducing bacterial numbers on vegetable surfaces. Given the rising problem of antibiotic resistance, in this study we sought to determine the effectiveness of two commonly used wash water sanitizers for reducing antibiotic-resistant bacterial pathogens and other antibiotic-resistant bacteria on carrots. It was not possible to completely eliminate all bacteria on the carrots by washing, a frequent misconception. However, washing in water that included a food-grade sanitizer, Tsunami 100 (peroxyacetic acid/hydrogen peroxide) or XY-12 (sodium hypochlorite), numbers of *E.coli* and *Pseudomonas* that had been pre-inoculated on the carrots were reduced. Despite the reduction in numbers after washing, the surviving bacteria on the carrot surfaces grew significantly when stored improperly at warm temperatures (10°C instead of 2 °C). Bacteria that could grow in the presence of antibiotics were reduced by the sanitizer wash and did not re-grow when stored at 2°C. The use of food-grade sanitizers does reduce the numbers of some bacteria on carrots, but it is equally important that consumers store produce at chilled temperatures to prevent re-growth of potentially harmful bacteria.

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DEDICATION

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ATTRIBUTION

Multiple contributions were made to this research from the following:

Monica A. Ponder, PhD, (Food Science & Technology Department at Virginia Tech) is currently an Associate professor and served as the principal investigator of this project. Dr. Ponder provided guidance, direction, and funding for this research, assisted with data analysis as well as experimental design. She is a co-author on the manuscript in Chapter 3.

Amy Pruden, PhD, (Civil and Environmental Engineering Department at Virginia Tech) is currently the W. Thomas Rice Professor in the Via department of Civil and Environmental Engineering, and is the associate dean for Interdisciplinary Graduate Education. She is the project director for the USDA grant funding the work presented in this thesis and served on the advisory committee.

Kim M. Waterman, MS, (Food Science & Technology Department at Virginia Tech) is currently the food microbiology lab manager, supporting departmental and graduate research. Ms. Waterman contributed significant technical assistance throughout the project.

Giselle Guron, PhD, (Food Science & Technology at Virginia Tech) is currently a post-doc in Dr. Ponder and Dr. Pruden's labs. Dr. Guron provided technical assistance and knowledge for the molecular work in this project.

Jian Wu, PhD, (Food Science & Technology Department at Virginia Tech) is currently a project associate for the food safety and food microbiology lab. Dr. Wu provided technical assistance whenever needed.

Renee R. Boyer, PhD, (Food Science & Technology at Virginia Tech) is currently an associate professor and extension agent in food safety. Dr. Boyer has provided guidance on experimental design and served on the advisory committee.

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LIST OF ABBREVIATIONS

ARB: Antibiotic Resistant Bacteria

ARG: Antibiotic Resistance Gene

EMB: Eosin Methylene Blue

PIA: *Pseudomonas* Isolation Agar

PIA T: *Pseudomonas* Isolation Agar supplemented with tetracycline

R2A CO: R2A supplemented with cefotaxime

R2A CL: R2A supplemented with clindamycin

R2A S: R2A supplemented with sulfamethoxazole

R2A V: R2A supplemented with vancomycin

R2A T: R2A supplemented with tetracycline

DC: Dairy control compost

DCAB: Dairy Antibiotic Compost

INOC: Inoculated

NON: Non-inoculated

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Fresh produce has been associated with multiple outbreaks of food-borne illness every year. Between 1998-2008, 51% of all food-borne outbreaks could be attributed to plant commodities, with 41% of all food-borne hospitalizations and 25% of deaths being attributed to plants [1]. While leafy vegetables, in particular, are associated with food-borne illness, food-borne illness outbreaks have been attributed to other vegetables. . Between 2000-2007 celery was responsible for 6 food-borne illness outbreaks, cabbage was associated with 9 food-borne outbreaks, and onions accounted for 15 food-borne illness outbreaks [2]. However, of all the vegetables investigated carrots were responsible for the most food-borne illness outbreaks, causing a total of 18 outbreaks and 405 total cases of illness [2]. The most common pathogens associated with fresh produce are *Norovirus*, *Salmonella* spp., and *Escherichia coli* O157:H7 [3]. *E. coli* O157:H7 was the etiological agent found in 350 outbreaks between the years 1982-2002 in the United States, in which 52% of those outbreaks were food-borne [3]. The source of these microbial contaminants is often linked to surface contamination with animal manure, improperly processed compost, or contaminated water that come into contact with the raw produce [4-6]. In fact, an upsurge in intensive farming practices is strongly correlated with the increase of food-borne illness outbreaks associated with fresh produce, along with other factors [4].

In order to try and mitigate this risk, food processors often put in place post-harvest interventions that aim to prevent cross-contamination from vegetable to vegetable [7]. These post-harvest interventions are often in the form of chemical sanitizers such as sodium hypochlorite or chlorine washes, and peroxyacetic acid washes. However, while chemical sanitizers do sanitize the wash water and reduce microbial load on produce to some degree, they are only effective on the surface of the produce [7, 8]. However, the use of sanitizers may be

contributing to the increase of antibiotic-resistant bacterial populations and the likelihood of transfer of antibiotic-resistant genes.

Standard post-harvest chemical washes combined with common agricultural practices may be contributing to antibiotic-resistant bacterial populations on fresh produce. Common agricultural practices, such as the abuse and use of both clinical and sub-therapeutic doses of antibiotics in animal husbandry, such as chlortetracycline, pirlimycin, etc. can often be associated with an increase in antibiotic-resistant bacterial populations [9]. This can, in part, be attributed the fact that antibiotics often undergo incomplete metabolism within the animal body, resulting in 40-90% of intact antibiotics being excreted into the feces or urine of cattle [10, 11]. Bacteria, both in the environment and cattle feces, are then repeatedly exposed to low doses of antibiotics, which may assist bacterial populations in gaining antibiotic resistance [9]. Antibiotic-resistance may develop in a variety of ways. Exposure to low doses of antibiotics can select for bacteria that possess mutations or lead to spontaneous mutations that are then selected for because it gives those bacteria a competitive edge over there other bacterial counterparts [9, 12]. Adaptive resistance has also been documented in cases where bacteria have been exposed to antibiotics in low concentrations. In the case of adaptive resistance bacteria adapt to changing environmental stressors as a mechanism to survive. *Pseudomonas* sp. have demonstrated adaptive resistance in the face of antibiotic use and even form biofilms and exhibit swarming behavior as both a way to increase virulence and gain adaptive resistance [12]. Bacteria have multiple other mechanisms for the acquisition of antibiotic-resistance including drug inactivation or modification often activated by penicillin-resistant bacteria, alteration of a target site, alteration of a metabolic pathway often seen in sulfonamide-resistant bacteria, and reduced drug

accumulation by decreasing cell permeability or increasing the number of efflux pumps [13]. These mechanisms of resistance are often associated with altered gene expression.

The spread of antibiotic-resistance genes (ARGs) are of great concern in the realm of human medicine. ARGs can be passed via horizontal gene transfer from bacterium to bacterium, often between different bacterial species [14, 15]. This means that non-pathogenic bacteria have the ability to transfer resistance to pathogenic bacteria. This is alarming because 95% of *Pseudomonas* isolates taken from ready-too-eat produce possessed resistance to multiple classes of antibiotics including aminoglycosides, fluoroquinolones, and beta-lactam antibiotics [12]. While *Pseudomonas* sp. usually do not present a health threat to the general population, its prevalence on produce, antibiotic-resistance profiles, and ability to transfer antibiotic-resistance genes to closely-related human pathogens means it still may present a health threat and needs to be investigated further [16, 17].

Antibiotic-resistant bacterial infections are more difficult to treat, often require an increased recovery period, result in greater healthcare costs for diagnostics and treatments, and frequently result in healthcare complications [18-20]. People infected with antibiotic-resistant strains of bacteria cost an additional ~$6,000-$30,000 dollars to treat than patients with antibiotic-sensitive strains [19]. According to the Centers for Disease Control and Prevention, ~2 million people acquire antibiotic-resistant infections per year, and 23,000 people die as a result of those infections. Beyond the cost of human lives, studies suggest that antibiotic-resistance costs an additional $20 billion in extra direct healthcare costs annually in the United States [20]. The loss of lives and money demonstrate the importance of mitigating the spread of antibiotic-resistance.

Further research is needed to evaluate whether common post-harvest produce chemical washes, such as chlorine and peroxyacetic acid, in conjunction with farming practices are contributing to an increase in antibiotic-resistant bacteria.

The objectives of this project include:

1. Are the numbers of antibiotic resistant bacteria transferred to carrots from compost made from manure collected during administration of antibiotics to dairy cattle different compared to compost generated from non-dosed cattle manure?.
    - Tested hypothesis: Compost made from dairy cattle manure that have been administered antibiotics (cephapirin and pirlimycin) will have a larger number of bacteria (log CFU/g) recovered on media containing antibiotics compared to compost made from dairy cattle manure that did not receive antibiotics.

2. Determine the effectiveness of chemical sanitizers against pathogenic and native antibiotic-resistant bacterial populations recovered from compost-dipped carrots.
    - Tested hypothesis 1: Bacterial populations from baby carrots dipped in compost made from the manure of dairy cattle with a history of antibiotic administration (cephapirin and pirlimycin) will have larger numbers of bacteria (log CFU/g) survive chemical washes compared to the no antibiotic dairy compost control-dipped baby carrots.

    - Tested hypothesis 2: Exposure to chemical sanitizers (40ppm free paracetic acid in the form of Tsunami 100 or 50ppm free chlorine as XY-

12) will result in reductions of total aerobic bacteria. However, a greater amount of the inoculated strains of *Pseudomonas* and *E. coli* O157:H7 will survive the sanitizer treatments compared to a control wash with water alone. This means that there will be an increase in the overall proportion and absolute amount of antibiotic-resistant bacteria (ARBs) post-intervention.

3. Determine the effect that storage temperature and time has on the re-growth of inoculated pathogenic and native antibiotic-resistant bacterial populations recovered from washed carrots.
    - Tested hypothesis: Increased storage time under temperature abuse conditions will result in greater log CFU/g of all aerobic, inoculated, and antibiotic-resistant bacteria compared to the initial day of washing. Carrots stored under optimal temperature conditions will have fewer log CFU/g of aerobic, inoculated, and antibiotic-resistant bacterial populations compared to carrots stored under temperature abuse conditions.

4. Determine the effect that storage temperature and wash type have on the levels of the *tet*A gene recovered from washed carrots.
    - Tested hypothesis: The proportion of *tet*A/16S rDNA will be greater in carrots stored under temperature abuse conditions compared to optimal storage conditions for all wash types.

This research can serve to inform development of future post-harvest interventions, including implementation of commonly used food-grade sanitizers, that decrease the numbers and persistence of antibiotic resistant bacteria on fresh produce, thus increasing consumer safety and decreasing healthcare, productivity loss, and human costs associated with food-borne illness.

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CHAPTER 2: LITERATURE REVIEW

Produce Consumption

Fruit and vegetables are commonly consumed foods among many people all over the world. In recent years, consumption of produce has increased dramatically both in the United States and worldwide [1-3]. In the United States between 1997 and 1999 consumption of fruits and vegetables increased dramatically, with a total 8% increase in fruits and a 24% increase in vegetable consumption [1, 4]. Interestingly, produce consumption is positively correlated with an increase in income, both at a nation level and an individual level [1, 5, 6]. Some possible explanations for this correlation are improved food security, food choice, and increased availability [1]. In the United States, which is considered a high income country, consumers tend to choose highly-perishable produce items, while those in low-income countries tend to choose less-perishable, high calorie options, such as tubers, roots, and legumes [1].

The reason for this increase in produce consumption can be attributed to many factors. One large factor is the increased awareness of the health benefits of fruits and vegetables [1]. Consumption of fruits and vegetables have many health benefits including reducing the risk of cancer and other chronic diseases, such as heart disease, obesity, type 2 diabetes, and risk of stroke [7, 8]. Consuming vegetables also provide important vitamins, nutrients, and fiber, while still being low in fat and calories, and containing no cholesterol [8]. This increases satiety while still providing invaluable health benefits [8]. Vegetables containing different substances confer diverse health benefits; potassium helps control blood pressure, fiber helps promote healthy digestion, folic acid aids the body's production of red blood cells, and Vitamin A and C help promote a healthy immune system [8].

Other factors that contribute to this increased consumption include increased market availability, and increased international trade. The shelf life of produce within supermarkets has dramatically increased in comparison to previous decades. This is in part due to international trade, which allows import of off-season fruits or vegetables or produce with domestic shortages in the United States market [1]. Also due to increased international trade, there is a greater selection of produce products available. Fruits and vegetables not previously available in U.S. markets due to climate and other growth conditions that cannot be met in the United States or cannot be met during certain times of the year, can now be imported and consumed year-round [1]. These products include grapes, peaches, and plums from Chile, tomatoes from Mexico, clementines from Spain, and other tropical fruits from all over the world [1]. This increased selection peaks interest in different produce varieties and allows for year-round consumption.

Beyond these factors, increased technology and modern farming has done wonders to expand the produce market and increase intake. Fruits and vegetables have increased shelf-life, and are able to retain their quality and appearance, even after long transportation has occurred [1]. This can be attributed to better packaging, storage options, and shipping and transportation methods. This includes atmospheric storage, such as that used with apples. Beyond this, new farming technologies, such as plant breeding techniques can increase shelf-life, or even make a product more desirable or convenient to consume [1].

One last factor that contributes to increased consumption, especially within the United States is increased convenience. In the United States, there has been an increase in consumption of fresh and frozen produce versus canned produce for exactly this reason [1]. Frozen produce is often available pre-cut, pre-peeled, with seasoning, etc. and is available year-round while still maintaining both freshness and nutritional content [1]. In addition to frozen foods, processed or

ready-to-eat produce is more likely to be consumed, due to the lack of preparation needed. For example, 43% of fruits are consumed as juice, and fresh produce is more likely to be consumed if it is in the ready-to-eat form such as bagged salads or baby carrots [1]. However, increased intake of fresh produce likely increases the risk of food-borne disease, due to the lack of any type of intervention step, such as heating.

Microbiological Safety of Produce/Produce Outbreaks

Beyond all of the health benefits of consumption, there are a number of potential hazards when choosing to eat vegetables, with one of the largest being the acquisition of a food-borne illness due to the fact that most produce is consumed raw. In the United States, between 1998 and 2008 the fruits and vegetables commodity was associated with the most food-borne outbreaks, accounting for 51% of all outbreaks [9]. Plant commodities also accounted for 41% of food-borne hospitalizations, and 25% of deaths [9]. In the United States between the years 2000-2015, there have been 6 food-borne illness outbreaks, resulting in 125 illnesses due to carrots [10]. There were an additional 5 outbreaks and 61 illness due to products containing carrots [10]. However, the biggest culprit is leafy vegetables, which were associated with an increase in illness, being responsible for 22% of all outbreaks, 35% of all food-borne viral illnesses, 14% of hospitalizations, and being the 5th cause of death [9]. In particular leafy vegetables such as spinach and lettuce were highly associated with *Norovirus* and *Escherichia coli* O157 [9].

The number of outbreaks associated with fresh produce in the United States varies annually, with a range between 23 and 60 outbreaks per year, with 13% of the outbreaks being multi-state between the years 2004-2012 [11, 12]. In the European Union there were between 10 and 42 outbreaks caused by fresh produce annually between 2004-2012 [11, 13]. In Canada,

between the years 2001 and 2009 there were 27 food-borne illness outbreaks due to produce, with *E. coli* being the implicated etiological agent in 33% of the outbreaks [14].

Enterohemorrhagic *E. coli* (EHEC) were responsible for being the second most common etiological agent in multi-state outbreaks within the United States, with *E. coli* O157 being the most prevalent strain [15]. One very notable outbreak associated with fresh produce in the United States was the multi-state spinach outbreak of 2006. In September of 2006 there were 5 days where no sale of fresh spinach occurred in the United States. The FDA told consumers to avoid fresh spinach before extensive recalls occurred. In this single spinach outbreak, there were 204 cases of *E. coli* O157:H7 infections, resulting in 104 hospitalizations, 31 cases of hemolytic uremic syndrome (HUS), and 3 deaths. The outbreak spanned 26 states and into Canada [16]. It was concluded that the microbial contamination occurred at the farm level—*E. coli* O157:H7 was identified in river water on the farm and in cattle feces near the spinach field [16]. This outbreak resulted in huge economic losses for farmers due to a public warning issued by the U.S. Food and Drug Administration to not consume fresh spinach, which lasted a total of 15 days [17].

In the last five years (2010-2015) there have been 195 outbreaks, resulting in 2,790 cases of food-borne illness, 605 hospitalizations, and 10 deaths due to contamination of food with *Escherichia* [12]. Half of produce-associated food-borne outbreaks due to *E. coli* O157 were due to contamination with the etiological agent prior to purchase, while the other half was due to cross-contamination during preparation [18]. Beyond this, 47% of outbreak tracebacks in food-borne illness cases due to produce were attributed to contamination sources at the farm-level [19]. This indicates that one of the causes of contamination prior to purchase may be due to improper fertilizer practices, including the use of raw manure, or improperly composted manure.

Other possible routes of contamination include contaminated irrigation water, wash water, equipment, farm run-off, or poor handling practices and transportation [18]. These possible routes of contamination are also of note in an important produce outbreak in the European Union that was associated with sprouted fenugreek seeds due to contamination with *E. coli* O104:H4 in 2011 [13]. As a result of this outbreak there were 3000 people with bloody diarrhea, 852 cases of hemolytic uremic syndrome, and a total of 53 deaths [20]. These outbreaks are indicative of the increasing association between food-borne illnesses and produce worldwide. This large association between food-borne illness and produce can be attributed to a number of reasons including raw consumption of produce, a general increase in the consumption of produce, the availability of produce year round, increased transportation including international trade, and an increase in intensive farming practices [1].

Table 2.1.1. Examples of fresh vegetable-related food safety recalls due to pathogen detection (2012-2016).

Date of Recall	Company	Product	Etiological Agent	Amount of product recalled	Reference
August 19, 2012	Tanimura & Antle Inc.	Romaine lettuce	*E. coli* O157:H7	2,095 cases	FDA. (2012). "Tanimura & Antle Voluntarily Recalls Single Lot of Romaine Lettuce Because of Possible Health Risk." Recalls, Market Withdrawals, & Safety Alerts Search Retrieved August 29, 2016, from http://www.fda.gov/Safety/Recalls/ucm316256.htm. [21]
November 01, 2012	Bolthouse Farms	Chip-cut style carrots	*Salmonella*	5,600 cases	FDA. (2012). "Bolthouse Farms Voluntarily Recalls Limited Quantity of 16-Ounce Carrot Chips." Recalls, Market Withdrawals, & Safety Alerts Search Retrieved August 29, 2016, from http://www.fda.gov/Safety/Recalls/ucm326785.htm.
November 02, 2012	Wegmans	Spinach	*E. coli* O157:H7	31,000 lbs	FDA. (2012). "Wegmans Food Markets, Inc. Announces Recall of 5 oz. and 11 oz. Organic Spinach & Spring Mix Blend - May be Contaminated with E.coli O157:H7." Recalls, Market Withdrawals, & Safety Alerts Search Retrieved August 29, 2016,

					from http://www.fda.gov/Safety/Recalls/ucm326782.htm.
November 09, 2013	Atherstone	RTE salads and wraps	*E. coli* O157:H7	Not listed	FDA. (2016). "Atherstone Foods Voluntarily Recalls Salads and Wraps Because of Possible Health Risk." Retrieved August 29, 2016, from http://www.fda.gov/Safety/Recalls/ucm374400.htm.
November 26, 2015	Multiple-supplier Taylor Farms Pacific	Celery and onion products	*E. coli* O157:H7	> 148.000 products	FDA. (2016). "Taylor Farms Pacific, Inc. Recalls Celery Products Because Of Possible Health Risk." Retrieved August 29, 2016, from http://www.fda.gov/Safety/Recalls/ucm474601.htm.
February 29, 2016	Jack and the Green Sprouts	Sprouts	*E. coli* O157:H7	Not listed	FDA. (2016). "Jack and the Green Sprouts, Inc. is Voluntarily Recalling Alfalfa and Alfalfa Onion Sprouts." Recalls, Market Withdrawals, & Safety Alerts Search Retrieved August 29, 2016, from http://www.fda.gov/Safety/Recalls/ucm488347.htm.

Sources of Produce Contamination with Enteric Pathogens

Food-borne illness associated with produce is usually due to surface contamination with animal manure or contaminated water [22]. Bacteria isolated from irrigation water and from lettuce had a high degree of genetic relatedness, especially when it came to antibiotic-resistance gene profiles, indicating that irrigation water may be a likely source of transmission [23]. The other major route of contamination is the application of improperly processed compost, or raw manure to soils used for growth of fruit and vegetable produce crops [24]. The application of manure or composted manure is a common practice worldwide, especially the application of cattle manure, because it is both economical and provides valuable nutrients [25]. Due to this practice many different pathogens can be transferred to soil and the surrounding environment, including produce surfaces. One major human pathogen associated with the application of cattle manure is *E. coli* O157:H7, which can persist in improperly processed compost for up to 5 months at a temperature of 7°C [24]. Proper composting validated to inactivate *E. coli* and

Salmonella involves maintaining a temperature of at least 55°C for 3 days [25]. However, even in these special conditions other factors, such as if the organic matter is not stable, or if the composting environment has a pH outside of the 5.5-8 range it could cause the process to be inefficient, and *E. coli* may persist [25]. A natural reservoir of EHEC are cattle which are unaffected by bacterial presence, but shed bacterial cells into their feces, therefore proper composting and monitoring of composting conditions are crucial [26]. Improper composting, the use of bovine feces as fertilizers, or the use of irrigation water that has come into contact with bovine manure for produce crops have been linked to many outbreaks of *E. coli* O157:H7 illnesses [27]. The first identified outbreaks of *E. coli* O157:H7 were attributed to contaminated ground beef products. However, due to a recent upsurge in intensive farming practices it has been increasingly associated with fresh produce [26]. *E. coli* O157:H7 in produce is especially a problem on leafy greens, due to contamination of the fields with manure from farm run-off, free-roaming livestock, or the application of raw manure or improperly processed compost [26].

Vegetables are exposed to dirt, soil, animal manure, etc. and as a result are often contaminated with different bacteria. Out of 420 samples of vegetables and restaurant salad, 49.5% were found to be contaminated with *E. coli,* with 84% of the *E. coli* found on salads and 87% found on vegetables identified as STEC [28]. In addition, the *E. coli* that was found displayed antibiotic resistance; 96.6% of isolates resistant to ampicillin, 87% resistant to tetracycline, and 90% resistant to gentamicin. This indicates that not only leafy greens, but that other vegetables may also harbor and be an under-recognized source of antibiotic-resistant bacteria.

Good Agricultural Practices (GAPs) and Composting Standards

In order to minimize microbial contamination of fresh produce, Good Agricultural Practices (GAPs) is highly recommended in the United States, although suggested practices are not always followed nor is certification currently mandatory [2]. GAP certification currently requires an individual or group to participate in GAP food safety training, develop and implement a farm food safety plan, and have a USDA third-party audit of their farm [29].

GAPs are recommendations put into place to try and reduce the risk for microbial contamination of fresh produce, and include regulations for soil, water, and animal health. They provide recommendations for proper manure application, prevention of farm run-off, prevention of water contamination, minimizing the non-therapeutic use of antibiotics, and prevention of medicinal residues from entering the food chain [30]. In reference to manure application, GAP recommendations state that farmers should perform high temperature aerobic composting, which means that the internal temperature of the compost remains at 131-170°F for at least 3 days, and is well-aerated with sufficient mixing, which must occur at least 5 times [31]. Raw manure prior to composting should be stored properly, as to avoid run-off onto crops, and once it is composted it should be stored to avoid recontamination. If using raw manure, it must be applied 2 weeks before planting and/or you must allow a minimum of 120 days from the application of manure to the harvest date [31]. Manure should be properly mixed into soil so that other soil microbes can aid in the breakdown of pathogens [31].

Farm run-off is also a large problem that can result in food-borne pathogens making it into the food supply. Recommendations to prevent farm run-off include planting some sort of barrier or diversion to prevent run-off from reaching crops, as well as altering the geography of your crops if necessary, such as not having crops downhill from livestock areas [31]. Beyond

this, water quality should be tested periodically and drip-irrigation is preferred to sprinkler systems that may cause soil dispersal [32].

During harvesting and post-harvest, GAPs are still key to prevent contamination, since no heating intervention usually occurs when consuming produce. Worker's hands should be clean and workers should be free of illness. In terms of produce, farmers and processors should keep the processing area clean, damaged produce should not enter the processing facility, and containers used to transport products should be easily cleanable and sanitized before and after use [32]. Equipment should also be easily cleanable and sanitized regularly. Sanitizers as an addendum to wash water, that are food-grade, are also strongly recommended in order to reduce cross-contamination between individual pieces or produce [32]. Water used to wash produce must meet potable water standards, should be changed when dirty, and monitored for free chlorine concentrations using either test strips or an oxidation reduction potential (ORP) meter [33]. Products should also be properly stored and transported to prevent re-entry of pathogens onto produce surfaces.

Keeping our food supply safe truly requires farm-to-table precautions and monitoring. However, noncompliance is often an issue. For example, one GAP recommendation offers information on how to keep farms from contamination by minimizing contact of cropland and livestock or wild animals. Recommendations include fences to prevent livestock from roaming onto crop fields, and regular monitoring of grounds for contamination with wild animal feces. If a problem is spotted then additional measures need to be taken, such as netting or some sort of repellent or physical barrier [31]. However, in a study conducted in Minnesota, 81% of farmers that responded to the mail-in survey replied that they took "measures to reduce the risk of wild and/or domestic animals entering into fruit and vegetable growing areas", however when on-

farm investigation was conducted, only 70% had actually taken measures to prevent animals from roaming into produce fields [34]. This demonstrates noncompliance with GAPs in spite of voluntary certification.

When these recommendations are not followed, contamination of the food supply may be more likely and cause subsequent infections. For example, in the spinach outbreak of 2006, in spite of regulations an outbreak occurred which likely means that growers did not follow protocols consistently or correctly, or perhaps the outbreak occurred because of outside factors not addressed by those regulations [16]. In the case of GAPs, there are no monetary benefits from following good safety practices although some foodservice buyers require that growers be GAP compliant, however with the implementation of FSMA—compliance to improve food safety will now be required [16, 32].

As of October 31, 2015, the new produce safety rule, as part of the Food Safety and Modernization Act will go into effect, and compliance with some of these standards will no longer be voluntary [35]. These new requirements include "growing, harvesting, sorting, packing, and storage operation, and science based minimum standards related to soil amendments, hygiene, packaging, temperature controls, animals in the growing area, and water [36]." The goal is to minimize food safety risks and prevent food-borne illness associated with fresh produce.

Food Safety Modernization Act (FSMA)

In an additional effort to decrease incidences of food-borne illness and keep the food supply safe for consumers the Food Safety Modernization Act (FSMA) was signed into law in the United States on January 4, 2011 [37]. As an addendum to FSMA, the FSMA Produce Safety

rule was proposed in January of 2013 and is now currently in effect. It sets forth regulations, rather than guidance documents, and requires compliance, meaning its impact on food safety is likely to be greater than that of GAPs. It emphasizes prevention of food-borne illness by stressing safe growing, harvesting, and post-harvest practices. It sets strict numerical criteria for water used in agriculture based on the presence of *E. coli*. If agricultural water is being used for the growing of produce, it must be less than 126 CFU/g of generic *E. coli* per 100mL water if based off the geometric mean, versus if water is being used for hand-washing, washing of produce post-harvest, or if it is being used for sprout irrigation then there must be no detectable amount of generic *E. coli* [37]. If water does not meet these criteria corrective action must be taken as soon as possible with a maximum of a year to make necessary corrections [37]. Corrective actions can include treating the water, which may include the use of sanitizers or filtration, or allowing time for die-off of pathogenic microbes, or even washing of produce [37].

Beyond this, the Produce Rule also lays out requirements for soil amendments. If applying raw manure, there must be a 120-day interval from application to soil to harvest of crops, or a 90-day interval if the crop is not in direct contact with the soil [37]. If utilizing compost, microbial standards must be met for certain microbes and valid composting methods must be used. Examples of this include composting methods put forth in the Good Agricultural Practices (GAPs) documents which state that farmers should perform high temperature aerobic composting, which means that the internal temperature of the compost remains at 131-170°F for at least 3 days, in a well-aerated composter with sufficient mixing, which must occur at least 5 times [31]. In both cases of manure and compost application, the farmer should "minimize contact with the crop both during and after application [37]." In addition, farmers are required to monitor their fields for evidence of both domesticated and wild animals, and if potential

contamination by animals is found, they must take corrective action, which may include marking the area, etc. in order to prevent contamination of the produce at harvest [37].

This rule goes beyond growing requirements and extends into post-harvest practices in order to ensure food safety until fresh produce meets the consumer. Post-harvest, farm workers and commercial processors must utilize hygienic practices. This includes workers notifying their supervisors if they are ill, proper hand washing, and training and education of farm workers on health and hygiene practices. In addition, proper storage and transport of produce should occur, and equipment or buildings in which produce may make contact must meet proper sanitation standards [37].

However, there are certain crops and farms that are exempt from this rule. Crops that are exempt include produce and grain crops that are rarely consumed raw, while farm exemptions include farms that make less than $25,000 a year from produce sold [37]. In addition, there are exemptions for produce that undergoes commercial processing that decreases the presence of pathogenic microorganisms, however specific requirements must be met in order for this exemption to apply, which includes making less than $500,00/year and having no active investigations into food-borne illness outbreaks on that farm [37].

Beyond this, FMSA provides a Food Safety technical Assistance Network and other educational materials to better assist growers, harvesters, and post-harvest workers with information to help them comply and keep fresh produce safe for consumers.

Shiga-toxin Producing *E. coli* (STEC) Infection

Despite these guidance documents and rules, which take steps to mitigate the risk of food-borne illness, food-borne illness is always a real and present risk on fresh produce. Human infection with *E. coli* O157:H7 presents with clinical signs such as grossly bloody diarrhea, and

severe abdominal cramps due the production of Shiga-toxin [38]. The incubation from the time of exposure to the presentation of symptoms can be anywhere from 1-8 days, and the illness can last 5-10 days if no further complications occur [39]. The infectious dose of *E. coli* O157:H7 is very low, and is estimated to be between 50-100 cells [22]. While infectious dose is dependent on many factors including the food matrix in which it exists, state of the cells, host factors, etc., even a low level of contamination may result in illness [40]. Illness caused by *E. coli* O157:H7 can affect people of any age, however the elderly, young children, and immunocompromised people are more likely to get ill and develop further complications. These complications can have deadly implications, such as the development of Hemolytic Uremic Syndrome especially in young children, which leads to kidney failure and anemia [38]. Risk factors for the development of hemolytic uremic syndrome include being a child, the use of anti-diarrheal medication, and the use of antibiotics to treat the infection [41, 42]. Children administered antibiotics were 14.3 times more likely to develop hemolytic uremic syndrome when compared to those children not treated with antibiotics [42]. HUS develops in approximately 2-7% of *E. coli* O157:H7 infections, which is significant considering the amount of infections per year [43]. *E. coli* O157:H7 causes approximately 73,000 illnesses in the United States every year, and between the years 1982 and 2002 caused a total of three-hundred and fifty outbreaks, 52% of which were food-borne [18]. While *E. coli* O157:H7 does not cause the most food-borne illnesses, due to its clinical symptoms, complications, and increasing prevalence it is an important emerging food-borne pathogen.

Veterinary Usage of Dairy Cattle Antibiotics and Modes of Action

In addition to increased prevalence on produce, *E. coli* O157:H7 has the ability to develop resistance to antibiotics due to its exposure to clinical and subclinical therapeutic doses of antibiotics used in animal husbandry, which is then excreted into its natural source, cattle manure. Common antibiotics associated with dairy cattle husbandry include cephapirin and pirlimycin. In the dairy cow industry cephapirin, a first generation cephalosporin antibiotic, is often used as a single intramammary dose during the dry off period as a therapeutic preventative measure for mastitis [44]. Pirlimycin, a lincosamide, is often used to treat cases of mastitis caused by Gram-positive bacteria, administered as two intramammary doses given 24 hours apart [45]. Both antibiotics are used to prevent or treat mastitis, however their use, mechanism of action, and demonstrated effectiveness against groups of bacteria differ. Due to their different mechanism of actions, the way in which bacteria acquire resistance to these antibiotics vary, as does the risk of transferability of resistance between bacteria. Different classes of antibiotics degrade at different rates and may exhibit different bioavailability in soil, which should be considered when determining which antibiotics can be used on the farm [46].

Table 2.1.2 Common antibiotics used in dairy cattle husbandry.

Antibiotic Name	Use	Antibiotic Class	Mechanism of Action	Effectiveness	Mode of Resistance	Transferability of Resistance	Reference
Cephapirin	Dry cow therapy for the prevention of mastitis.	1st-generation cephalosporin.	Inhibits cell wall synthesis (via changing the affinity for penicillin-binding proteins).	Bactericidal. Only effective against rapidly growing cells. Effective against Gram-negative and Gram-positive bacteria.	Gram-negative bacteria may hydrolyze the drug, may cause reduced affinity of existing PBPs, or may acquire supplementary B-lactam-insensitive PBP.*E. coli* and *Pseudomonas* have developed resistance. Cross-resistance may occur with penicillins.	Mediated by plasmids and chromosomes.	AAVPT (2003). Cephapirin, American Academy of Veterinary Pharmacology and Therapeutics. (2015). "Cephalosporins and Cephamycins: Antibacterial Agents." 2015.
Pirlimycin	Therapeutic treatment for	Lincosamide.	Inhibits protein synthesis by	Bacteriostatic. Effective against	Enzymatic inactivation of the drug, methylation of	Risk of transferable	AAVPT, *Pirlimycin*. 2003,

	mastitis.		binding the 50S ribosomal subunits of bacterial ribosomes and preventing peptide bond formation.	Gram-positive bacteria.	the 23S binding site, which alters the drug's binding site.	elements is low.	American Academy of Veterinary Pharmacology and Therapeutics.

Interestingly, Pol and Ruegg (2006) found that intramammary infusion of cephapirin was not associated with an increase in antibiotic resistance in the pathogens studied, which included *Staphylococcus aureus* and *Streptococcus* spp. which are common etiological agents of both clinical and subclinical mastitis [47]. However, it is important to note that the samples used for bacterial analysis were from milk, rather than feces, and more research needs to be conducted in the area of cephapirin use and antibiotic resistance. On the other hand, increased pirlimycin exposure was correlated with increased Minimum Inhibitory Concentrations (MICs) of pirlimycin required to kill off all mastitic pathogens studied [47]. However, further research needs to be conducted about the use of intramammary antibiotic usage used in dairy cattle and corresponding antibiotic resistance.

Antibiotic resistance genes (ARGs) were quantified on farms in Finland, which is considered a country that uses relatively low levels of antibiotics. In spite of this low level of usage, it was found that the amount of antibiotic resistance genes increased within soil samples after manure had been applied, indicating that manure is likely a source of antibiotic resistance [48]. Use of stored manure further increased the relative abundance of antibiotic resistance genes, and ARGs were found across all farms, indicating that ARGs are widely dispersed [48]. *E. coli* isolated form dairy cattle in South Africa were found to have multiple antibiotic-resistance genes: 90% of isolates possessed the bla_{ampC}, 65% $bla_{CTX\text{-}M}$, 27% bla_{TEM}, 70% *tet*A, and 80% *str*A genes [49]. This indicates that dairy cattle can act as a reservoir for antibiotic-resistance genes. Shiga-toxin producing *E. coli* from varying environments in Argentina

possessed either the stx_{1a} or stx_{2a} genes, and 17% of the isolates possessed genes that encoded for antimicrobial resistance [50]. This indicates that bacterial pathogens from animals, food, and clinical environments may be a large source of antibiotic-resistant infections.

Veterinary Usage of Beef Cattle Antibiotics and Modes of Action

In the beef industry, many antibiotics are used as feed additives in order to increase profit margins by increasing feed-conversion rate and decreasing incidence of disease including chlortetracycline, sulfamethazine, and tylosin [51]. They have a variety of uses, mechanisms of action, and demonstrate differences in effectiveness against bacterial infections. In addition, the way bacteria acquire resistance to each of the antibiotics vary, and as a result the likelihood of transfer of resistance from one bacterium to another also varies greatly.

Table 2.1.3 Common antibiotics used in beef cattle husbandry.

Antibiotic Name	Use	Antibiotic Class	Mechanism of Action	Effectiveness	Mode of Resistance	Transferability of Resistance	Reference
Chlortetracycline	Accelerates weight gain, many other uses.	Tetracycline	Binds to the bacterial 30S ribosomal subunit, preventing the binding of aminoacyl-tRNA, which inhibits protein synthesis.	Bacteriostatic. Mostly effective against Gram-positive bacteria. Minimal action against Gram-negative bacteria based off the loss of cell wall permeability.	Enzymatic inactivation of tetracycline via acetylation, efflux pumps (via resistance gene—plasmid pBR322), ribosomal protection (resistance gene encodes proteins that can alter via many different mechanisms dependent on which gene is transferred ex: blocking tet binding to ribosome, distorting the ribosomal structure, dislodging tet).	Plasmid-mediated: resistance gene on plasmid pBR322.	AAVPT, Tetracyclines, in Micromedex. 2003, Thomson. Chopra, I. and M. Roberts, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev, 2001. 65(2): p. 232-60 ; second page, table of contents.
Sulfamethoxazole	Prevent and treat respiratory diseases (Shipping Fever).	Sulfonamide	Inhibits specific enzymes (dihydropterate synthetase), acts as a	Bacteriostatic. Effective against both Gram-positive and Gram-negative	Chromosomal and R-factor mediated resistance. Emergence of resistance is	Chromosomal and R-factor mediated resistance.	Gadberry, S., Feed Additives for Beef Cattle, US Department of Agriculture. AAVPT. Sulfamethazine. 2007; Available from: http://c.ymcdn.com/sites/www.aavpt.org/resource/resmgr/imported/sulfonamides.pdf.

			structural analog for para-aminobenzoic acid (PABA) to prevent synthesis of folic acid. Acts as an antimetabolite for a variety of co-enzymes to prevent the formation of nucleic acids, methionine, and glycine to prevent DNA synthesis.	bacteria.	gradual and widespread, cross-resistance across sulfonamides may occur, plasmid-mediated resistance often linked with ampicillin and tetracycline resistance (in gram(-) bacteria).		
Tylosin	Prevent liver abscesses in feedlot cattle.	Macrolide	Binds to the 50S ribosomal subunit at the donor site to prevent translocation, which interferes with protein synthesis.	Bacteriostatic. Only effective against rapidly dividing cells, works better at an increased pH (7.8-8). Not effective against Gram-negative bacteria because their cell wall prevents penetration into the cell.	In Gram-positive bacteria due to alterations in ribosomal structure and loss of macrolide affinity, genetic changes (erythromycin ribosome methylase genes) that cause modification of the 50S ribosomal subunit or methylates the 23S rRNA.	Genetic (erm genes), can be plasmid-borne, may be present in transposons, integrons, or in gene cassettes. Can be acquired via transduction and conjugation (HGT), and transformation. Resistance gene likely to be transferred in the human intestines from a food source. Very likely to be transferable.	Chopra, I. and M. Roberts, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev, 2001. 65(2): p. 232-60 ; second page, table of contents. Gadberry, S., Feed Additives for Beef Cattle, US Department of Agriculture. Schlecht, H. and C. Bruno, Vancomycin. 2016: Merck Manual.

Each of these antibiotics have been associated with an increase in antibiotic-resistant bacteria, although the increase in antibiotic-resistance is often multi-factorial. Chlortetracycline use in livestock has been suggested to cause an increase in antibiotic resistance, and use of it at sub-therapeutic doses have been banned in the European Union, however no such ban in the United States exists today [52, 53]. Alexander et. al (2008), found that sub-therapeutic inclusion of chlortetracycline plus sulfamethazine administered in feed to steers resulted in increased shedding of tetracycline and ampicillin-resistant *E. coli* in both the silage-based and grain-based diets by 5 log CFU and 5.36 log CFU, respectively [54]. Interestingly, cattle fed a grain-based diet, common in feedlot operations, led to increased shedding of antibiotic-resistant *E. coli* compared to cattle fed a silage-based diet regardless of the treatment [54]. Beukers et. al (2015), found that tylosin use at feedlots increased the prevalence of both erythromycin and tylosin

resistance in *Enterococcus* spp, but that once tylosin was removed 28 days prior to slaughter there was no difference in antibiotic resistance versus the control group [55]. This suggests that an antibiotic withdrawal period prior to slaughter would decrease antibiotic resistance within our food chain, however more research needs to be conducted on macrolide use and antibiotic resistance. While antibiotic resistance may not be present in immediate fecal samples taken in studies, it is important to note that in many instances there is incomplete metabolism of antibiotics that are used in animal husbandry, which results in up to 40-90% of the antibiotics themselves being excreted into the feces or urine of cattle [56, 57].

Antibiotic Resistance in Agroecosystems

The use of manure on fields has been correlated with an increase in antibiotic-resistant bacteria and genes, whether the manure contains antibiotic or not, but studies using different ecosystems have reported varied results [58]. In addition, pre-antibiotic era resistance have been noted in pristine soils, and the added layer of intrinsic resistance makes it hard to quantify resistance with respect to antibiotic use [59]. However, composting for 30 days decreased antibiotic concentrations by 85-99%, which indicates that composting may be an effective way to mitigate antibiotic resistance [60]. Composting also significantly decreased the number of copies of antibiotic-resistance genes and 16S rRNA genes compared to stockpiled manure, suggesting composting might help decrease ARGs on the farm [61].

However, many studies use manure or compost fortified with antibiotics compared to antibiotics excreted from animals, which do not always follow the same dissipation rate [60]. On a dairy farm in California the potential for leaching of antibiotics, specifically monesin, from concentrated animal feeding operations (CAFOs) to irrigation and groundwater was evaluated

[62]. Levels as high as 1.6 ppb was found in irrigation water and was detected in wells near the waste storage lagoon, which suggests irrigation water may be a source of antibiotics, and that location of waste has a significant impact on potential risk of antibiotic leaching [62]. Antibiotics in concentrations between 0.87-1.23g were detected in farm run-off from piles of compost, generated from beef cattle administered antibiotics, following simulated rainfall events [63]. This suggests that farm run-off may not only be a source of pathogenic microbes on the farm, but may also be an under-recognized factor in contamination of water sources with antibiotics [63]. Pirlimycin has been shown to be the most susceptible to leaching from manure directly after manure application following a rainfall event, indicating that applying manure in drier seasons or using injection methods for fertilization may help decrease antibiotic concentrations in farm run-off [64].

Environment plays a key role in adaptation and acquisition of antibiotic resistance. Bacterial isolated from water displayed a much higher conjugation frequency compared to isolates from lettuce [23]. This indicates the importance of maintaining high wash water quality standards and indicates that wash water may facilitate an increase in antibiotic-resistance genes by providing an environment in which trans-conjugants can be formed [23].

Difficulties of Measuring Antibiotic Resistance in Environmental Samples

Recently, different models have been put forth to evaluate the impact of antibiotics on the environment, however the models require information such as metabolism of that specific antibiotic, environmental information, and antibiotic usage information which is often not reported accurately or is widely unknown [59]. Low levels of antibiotics in the environment can contribute to antibiotic resistance, even levels that may not be detectable by current methods,

especially from environmental samples that exist as complex matrices that may make isolation of antibiotics or their metabolites difficult [65]. Culturing is often used to quantify antibiotic-resistant bacteria because it allows for phenotypic identification of antibiotic-resistance [66]. In addition, it allows researchers to link genotypes to functional expression, and provides an easy way to distinguish specific antibiotic-resistant isolates, and identify which of those isolates possess resistance to multiple antibiotics [66]. In clinical settings Minimum Inhibitory Concentrations are used to identify breakpoints of resistance, however this presents a challenge when applied to agroecosystems [66]. Another issue when trying to utilize culturing methods to identify antibiotic resistance is difference in antibiotic mechanisms of action, uniformity of an environmental sample, and culture bias which includes cells that may not produce colonies but are present such as cells in the VBNC or persister state [66, 67]. Often times, molecular methods are utilized to assess antibiotic resistance from the environment. Specific methods include Polymerase Chain Reaction (PCR), quantitative PCR (qPCR), and next-generation sequencing. While effective, there are detection limits and genetic information does not necessarily translate to functional genes of phenotypic expression, thus a combination of culturing and molecular methods may be the most useful approach [68].

Bacterial Development of Antibiotic-Resistance

These antibiotics can survive in the surrounding environment, and the exposure of bacteria to these antibiotics at low concentrations may select for antimicrobial resistance over time [69]. *E. coli* isolated from conventional swine systems were found to be more resistant to a wide range of antibiotics and were able to attach to very fine silica sand than *E. coli* isolated from organic farming systems [70]. This may mean that the use of antibiotics may provide competitive advantages such as increased ability to attach to soil particles and antibiotic

resistance[70]. Development of antimicrobial-resistance influenced by environmental factors include spontaneous mutations that randomly occur and are selected for because those bacteria are able to outcompete their counterparts for nutrients and space [71]. Spontaneous mutations can occur as a result of exposure to antibiotics and while frequencies of mutations differ based on antibiotic, they can occur at a rate of 10^6 to 10^9 [71]. Spontaneous mutations may also increase in frequency if bacterial cells are stressed, or under conditions that may damage DNA, such as exposure to sub-lethal doses of antibiotics [71]. Tanimoto et. al (2008) found that mutation frequency for carbapenem-resistance in a *Pseudomonas aeruginosa* strain increased by 5.6-6.8 fold when grown in an environment containing sub-inhibitory concentrations of fluouroquinolones [72]. *Pseudomonas aeruginosa* also had an increased frequency of mutation when exposed to another antibiotic, meropenem, which resulted in the bacterial strain being able to survive twice the amount of the Minimum Inhibitory Concentration (MIC) of antibiotics (0.4 μg/mL) compared to the standard MIC of the parent strain (0.4μg/mL) [72]. Other conditions that may contribute to spontaneous mutations include growth patterns. *Pseudomonas aeruginosa* that grew in a biofilm had an increase of 100-fold in mutation frequency for ciprofloxacin-resistance when compared to cells that grew outside of a biofilm, thought to be as a result of down-regulation of enzymes that help protect DNA [73]. These mutations allow for antimicrobial-resistance in a variety of ways. In *Pseudomonas aeruginosa* spp. these mutations may lead to altered antibiotic target sites, an up-regulation in the amount of efflux pumps, a decrease in antibiotic uptake, or the production of enzymes that may aid the bacteria in the breakdown of antibiotics [71].

Another type of antibiotic-resistance is adaptive resistance in *Pseudomonas* sp., which is when the bacteria adapt to changing stress and environments as a mechanism to survive without

a genetic mutation [71]. Quorum sensing, a type of cell-to-cell communication method that regulates gene expression based on cell density, of *Pseudomonas* sp. help aid the acquisition of this adaptive resistance [74, 75]. Adaptive resistance requires an ongoing exposure to the stressor, such as an antibiotic. It allows for an inducible change that aids the bacteria in survival, such as increasing the level of Beta-lactamase [71]. Biofilm formation and swarming behavior, two distinct growth behaviors, may also be a form of adaptive resistance. Bacteria that exist in swarms have been shown to have increase in antibiotic-resistance due to an increase in expression of virulence factors in response to increased stress [76]. It is important to note that in cases of adaptive resistance, once the stressor is removed from the environment surrounding the bacteria, the bacteria no longer express adaptive resistance, but rather revert back to their original state and respective sensitivity [71].

Beyond this, antibiotic-resistance and how it is attained can be heavily influenced by the class of antibiotics to which it is acquiring resistance. One example of this includes drug inactivation or modification activated by penicillin-resistant bacteria. [77]. This type of drug modification also occurs in the presence of aminoglycosides, where bacteria will alter enzymes at a hydroxyl or amine group, by adding a phosphate, adenine, or acetyl group, thus changing its molecular form, and thus rendering the antibiotic ineffective [78, 79]. Resistance to cephapirin and its human analog, cefotaxime, occurs via this mechanism. Hydrolysis occurs via B-lactamase alteration of penicillin-binding proteins (PBPs) and in turn reduces affinity of existing PBPs [44, 80]. However, the mode of resistance to these types of cephalosporins can occur via multiple mechanisms like resistance to most antibiotics. Other modes of resistance for cephalosporins include acquisition of a supplementary B-lactam-insensitive PBP, such as occurs in *E. coli* and *Pseudomonas* sp., as well was plasmid, or chromosomally-mediated mechanisms [80]. For third-

generation cephalosporins used in human medicine, such as cefotaxime and ceftazidime, resistance can occur via increased efflux pump mechanisms, intrinsic resistance which can be observed in *Enterococcus* sp., but mainly via hydrolysis by Beta-lactamases, conformational changes in PBP structure, and decreased permeability [81-85]. In the case of ceftazidime, spontaneous mutations that caused the deletion of a gene that encodes for penicillin-binding protein 3 led to ceftazidime resistance in *Burkholderia pseudomallei* [77].

Enzymatic inactivation is also a common mechanism of resistance and is seen in lincosamide and macrolide resistance. Resistance to pirlimycin and its human analog, clindamycin, as well as tylosin and erythromycin, occurs via enzymatic inactivation and methylation of the 23S binding site [86]. However, in the case of macrolides, resistance can occur if the erythromycin ribosome methylase genes (*erm*) are present, which in turn cause modification of the 50S ribosomal subunit or can result in methylation of the 23S rRNA site [87]. *Erm* genes can be plasmid-borne, inducible, or constitutive, and thus have a high probability of transference [87]. In addition, gene transfer of *erm* genes that confer resistance from food to bacteria within the human intestines is very likely, especially in the case of *Enterococcus* sp. and *Bacteroides* sp. [88, 89]. Tetracycline resistance also occurs via enzymatic inactivation, however it occurs via acetylation. In addition, efflux pumps via the resistance gene pBR322, ribosomal protection genes, and point mutations are important to note in tetracycline resistance [90]. The resistance genes that encode for ribosomal protection can alter the way in which resistance can occur in a variety of ways, including blocking the binding of tetracycline to the ribosome, causing a conformational change to the ribosome that prevents binding, and even dislodging the tetracycline molecule [4, 91, 92].

Another type of resistance gained based on antibiotic class is the alteration of a target site in penicillin-resistant bacteria, which can often result from a spontaneous gene mutation [81]. Sulfonamide-resistant bacteria utilize alteration of a metabolic pathway as a way to acquire resistance. Sulfonamides, such as sulfamethazine used in beef cattle, work by acting as a competitive inhibitor to a pre-cursor, paraaminobenzoic acid (PABA), for nucleic acid and folic acid synthesis that is usually required for bacterial cells, however some bacteria can alter their metabolic pathway and begin using a preformed version, thus eliminating the efficacy of sulfonamide antibiotics [93, 94]. In addition, environments that are PABA-rich, such as necrotic tissue can competitively bind sulfonamides and thus increase resistance [95].

Lastly, reduced-drug accumulation by decreasing permeability or increasing the number of efflux pumps can occur across many different microbial species and antibiotic classes, although quinolone resistance is most common [93]. This type of antibiotic-resistance is due to a chromosomal mutation, which can then increase the number of efflux pumps to more efficiently pump out antibiotics from within the bacterial cells, and thus prevent damage or death [96]. While these different modes of action of antibiotic-resistance all can lead to antibiotic-resistant bacteria, it is important to note that they are heavily influenced by environmental conditions that can alter gene expression.

Pseudomonas aeruginosa **and the plant phyllosphere**

Another concern related to antibiotic resistance is the acquisition of antibiotic-resistant genes (ARGs) from other non-pathogenic bacteria present on the surfaces of fresh produce [97]. One of the bacterial genera of largest concern is *Pseudomonas*. *Pseudomonas* sp. are aerobic, Gram-negative rod-shaped bacteria that can thrive in a variety of environments including soil,

plants, water, and even animals [98]. *Erwinia* sp., and *Pseudomonas* sp. are among the most widespread bacterial genera on plants, including vegetable surfaces [99]. *Pseudomonas* sp., in particular, are prevalent on the epiphytic surfaces of plants, are abundant throughout the plant phyllosphere, and are known for being able to adapt to a variety of ecological niches [100, 101].

Most *Pseudomonas* sp. are non-pathogenic to humans, however some species such as *Pseudomonas aeruginosa* are opportunistic pathogens, meaning that they present a health threat if their host is immunocompromised, but both opportunistic and non-pathogenic species are of concern because of their ability to transfer antibiotic resistance genes [100]. Recently, *Pseudomonas aeruginosa* has gained a lot of clinical significance due to its increasing prevalence in disease systems [102]. It has been implicated in necrotizing entercolitis, gastroenteritis, skin/wound infections, and respiratory infections in patients with cystic fibrosis [102-104]. These infections are often difficult to treat due to multi-drug resistance [102].

While the concern is growing with regards to *Pseudomonas* infections in general, it is its widespread antibiotic resistance that is of greatest concern. In one study, up to 95% of *Pseudomonas* isolates taken from ready-to-eat produce possessed resistance to multiple classes of antibiotics including aminoglycosides, fluoroquinolones, and beta-lactam antibiotics (penicillins and cephalosporins). This antibiotic-resistance was partially dependent on the environment to which the *Pseudomonas* isolates were exposed [71]. These environmental conditions include exposure to sub-inhibitory concentrations of antibiotics, temperature, oxygen availability, cation-limited environments that can lead to biofilm formation, low iron, available nutrients, and other stressors which may lead to swarming [71]. For example, in chronic Cystic Fibrosis infections *Pseudomonas* will often form biofilms, exhibit swarming behavior, increase production of exoproducts, and lose their LPS O-antigen [105]. This occurs in response to the

increased level of mucous and hypoxic environment in which they live, and in response to repeated dosing with antibiotics, which in turn allow the bacteria to become resistant to a broad range of different antibiotics and increase their virulence [71, 105].

In instances when environmental conditions play such a key role, the resistance is often adaptive resistance, in which the bacteria will revert back to their "wild-type" non-resistant state once the stressor is removed [71]. In the food industry, washing of produce often occurs in the presence of sanitizers that may select for antibiotic resistance as a result of stress. This paired with the fact that *Pseudomonas* sp. are present in large numbers on produce surfaces, can survive post-harvest practices, and are often present in re-circulating wash water creates a cause for concern in our food chain. Their potential to transfer antibiotic resistance to other bacteria via horizontal gene transfer within this re-circulating wash water, makes *Pseudomonas* sp. of great importance [100, 101]. *Pseudomonas* sp. are resident members of the human gut microbiome and are closely related to many human bacterial pathogens, which increases the likelihood of transferring antibiotic resistance genes (ARGs) to bacteria that are known pathogens [71]. This means that while *Pseudomonas* itself may not present a health risk to the general population, its presence on produce, antibiotic resistance profiles, and the ability to transfer antibiotic-resistance genes to closely-related human pathogens such as *E. coli* O157:H7 means it still presents a health threat [106]. The main mechanism and driving factor of the development of antibiotic resistance is horizontal gene transfer from the surrounding environment and other bacteria [93, 97].

Acquisition of Antibiotic-Resistance Genes

Horizontal gene transfer is the acquisition of genetic material by one bacterium from another, which can often be between different species or even different genera of bacteria [107].

Many antibiotic resistance genes are acquired in this manner. The resistance genes may be within or transferred via plasmids, transposons (mobile DNA segments), integrons, or gene cassettes and then can be passed using these molecules from one bacterium to another. Horizontal gene transfer may occur via three different mechanisms: conjugation, transformation, or transduction.

Conjugation refers to a process that involves direct cell-to-cell contact or the utilization of a "bridge" connection, usually a sexual pilus, to transfer genetic material from a donor cell to a recipient cell [107]. Leverstein-van Hall et al. found high frequencies of multi-drug resistance across multiple strains of Enterobacteriaceae within hospital patients thought to be the result of conjugation [108]. This was evidenced by the presence of the same integron, which encodes multi-drug resistance, being found across species [108]. In another experiment, donor and recipient strains were incubated together and integron transfer was monitored. In all experiments but 2, integrons that encoded resistance to sulfamethoxazole, cotrimoxazole, gentamicin, tobramycin, and streptomycin were transferred, and thus transconjugants were produced [108]. This demonstrates interspecies genetic transfer, and is of great concern because it suggests that environment is a strong factor for antimicrobial resistance. Oppegaard et. al (2001) discovered that an R plasmid (pTMS1) that encoded for multidrug resistance was found both in bovine feces and human isolates of *E. coli*, however the serotypes were not identical [109]. This strongly suggests that the plasmid that encodes for that multi-drug resistance was horizontally transferred in the environment, but that the isolate itself was not the common source [109]. Farm and livestock management play a key role in preventing antibiotic resistance due to these plasmid being able to transfer plasmids from a bovine fecal sample to humans. In addition, new research indicates that ciliated protozoa can engulf bacterium and as a result the bacteria have increased virulence gene expression and increases the probability of conjugal genetic transfer of antibiotic

resistance plasmids [110]. Brewer et. al (2011), observed that conjugation events and hyperexpression of virulence factors based on natural protozoan presence were found within different ruminant animals, and discovered that only the bovine rumen facilitated virulence gene hyperexpression, but that all ruminant protozoans caused increased conjugation between bacterium [110]. This emphasizes the need for good agricultural practices, careful manure application to crops, and good waste disposal methods so as not to facilitate antibiotic resistance spread within the natural environment. West et al. (2010), found that environment did in fact play a large role in whether antibiotic resistance genes were transferred between different bacteria. Their study observed general waterways and waterways that were near confined animal feeding operations (CAFOs) and its overall quality, including its microbiological profile. Waterways that were near these CAFO farms had an increased proportion of multi-drug resistant bacterial populations (41.6% of isolates) versus other sites (16.5%) not near CAFO farms [111]. This multi-drug resistance included resistance to ampicillin, kanamycin, oxytetracycline, and streptomycin [111]. Beyond this, when conjugation assays were performed in a laboratory setting 83.3% of environmental isolates, mostly fecal coliforms, were able to transfer one of more antibiotic resistance genes to a laboratory strain of *Salmonella typhimurium* [111]. This high rate of conjugative gene transfer implies that these isolates possess conjugative plasmids and/or transposons [111]. Lastly, this study among others suggests that manure, which contains a high amount of nutrients, provides an environment conducive to horizontal gene transfer [111-113]. In addition, on-farm use of manure that led to run-off due to weather conditions increased the levels of fecal coliforms present, and water-rich environments led to increased plasmid transfer between bacterium [114-116]. These studies indicate that bovine operations, farm run-off, irrigation water, and produce wash water may help facilitate conjugation of antibiotic resistance

genes, and emphasizes the importance of controlling any environmental factors we can to limit the spread of antibiotic resistance.

Another type of horizontal gene transfer is transformation which involves a "competent" bacteria, meaning it possesses the ability to uptake extracellular DNA, or DNA fragments, from its surrounding environment. Competence is a unique physiological state, which is often induced in cells with damaged DNA. This allows the bacterial cells to pick up "naked" DNA from the environment, in order to repair itself, which may be from dead bacterial cells that have lysed or from living bacteria that have released that genetic fragment into the environment [107]. Often bacterial cells that have been damaged by antibiotics will enter a state of competence. Claverys et. al (2006) found that *Streptococcus pneumoniae,* an important human pathogen, entered a state of competence when its DNA was damaged by the use of the antibiotic fluoroquinolone [117]. This demonstrates how environmental factors may aid the transfer of antibiotic resistant genes among bacteria. This phenomenon has been observed in other bacteria species as well, such as *Bacillus subtilis* and *Helicobacter pylori*, although the mechanism used to enter a state of competence varies [117-119]. In *Escherichia coli* species transformation experiment conducted by Tsen et. al (2002), it was found that as more plasmids are put into the environment surrounding *E. coli*, the number of total transformants increased, despite the fact that *E. coli* is not normally naturally transformable [120]. In addition, other studies suggest that certain environments may lead to the development of natural competence, to allow for transformation to occur in *E. coli*. Baur et. al, (1996) found that even at low calcium concentrations present in river and spring water that *E. coli* was capable of natural transformation [121]. In another study that investigated the possibility of *E. coli* transformants in foodstuffs, it was found that *E. coli* was able to transform in carrot juice held at 37°C, providing good indication that transformation in *E.*

coli may be able to occur naturally [122]. Ando et. al (2009), found that biofilm formation within foodstuffs and/or in environments that provided available nutrients from food increased the frequency of natural occurrence of transformation in *E. coli* cells [123]. It was also found that transformation of wild-type *E. coli* and the transfer of antibiotic resistance genes between bacteria can occur in aqueous environments with available calcium, such as irrigation water, but does not occur in nitrogen-rich mediums such as human urine [124]. This means that nitrogen-rich soils may play a key role in preventing the transfer of antibiotic-resistance genes in *E. coli* by preventing transformation, but more research is needed to look at pathogenic strains of *E. coli*.

The last mechanism of horizontal gene transfer that can occur is transduction. Transduction is the transmission of genetic material from one bacterium to another, which requires a virus, such as a bacteriophage, as the intermediary to transfer the genetic material between the bacterium [125]. Transduction is a very important mechanism in the passing of genetic elements that may increase both a bacteria's resistance to antibiotics, as well as their virulence, via the transfer of exotoxins between species or genera [126]. This transduction of exotoxins has been observed between some strains of *Escherichia coli* and *Shigella sonnei*, where a bacteriophage of *Shigella sonnei* has transferred the shiga-toxin producing gene from *Shigella* to *E. coli*, and thus increasing *E. coli*'s virulence [127]. Shousha et al (2015) found that phages were key in the transfer of antibiotic resistant genes in *E. coli* on chicken meat carcasses [128]. The phages naturally present on chicken meat lead to the exchange of genetic material that resulted in kanamycin, chloramphenicol, tetracycline, and ampicillin resistance in *E. coli* [128]. While this suggest that transduction between bacteria in the environment and our food may play a key role in antibiotic resistance in our food supply, further research is needed in the field of

vegetables and naturally occurring phages that may lead to transduction of antibiotic resistance genes.

Antibiotic resistance genes are passed via these different forms of horizontal gene transfer. The key factor in all of these mechanisms is the environment to which the bacteria are exposed. Thus, being able to limit contact of bacteria, especially pathogenic bacteria, to other bacteria that may carry antibiotic resistance genes is key. This can be done by regulating the environment in which bacteria may be present in, such as limiting their exposure to antibiotics, where mutations that encode for antibiotic resistance would be selected for in order to ensure survival. As intensive farming and environmental exposure to stressors increases, more and more bacteria are becoming resistant to either one or more classes of antibiotics.

Antibiotic Usage in Human Medicine

The relationship between the usage of antibiotics in intensive farming systems and subsequent antibiotic-resistant bacterial infections in humans may be, in part, attributed to the fact that many antibiotics used in animal husbandry have human analogues [56, 93, 111]. The bacteria are exposed in low doses to antibiotics from farming systems and as a result the bacteria that survive the low doses survive and multiply [111]. These bacteria may have acquired this resistance in a variety of ways and may survive exposure to the antibiotic via a variety of mechanisms. However, if bacteria have acquired resistance to a certain antibiotic, often that same bacteria can utilize the same mechanism to demonstrate resistance to other antibiotics in that same antibiotic class [56, 93]. This is often seen in the case of human bacterial infections. Antibiotics used in human medicine often belong to a variety of antibiotic classes, are utilized in

different ways to treat a variety of infections, and demonstrate different effectiveness against different infections.

Carbapenems are often a last resort for antibiotic-resistant infections, and are therefore reserved for emergency cases. However, despite their little use in food-producing animals, bacterial isolates from cattle feces have been shown to possess carbapenem-resistance [129]. Six different isolates of *Pseudomonas* isolated from dairy cattle feces had conserved domains of carbapenemase-producing genes and displayed phenotypic resistance to carbapenem [129], This is cause for concern, as *Pseudomonads* have a high capacity for horizontal gene transfer, and may be able to confer resistance to pathogenic bacteria in their surrounding environment.

Table 2.1.4 Common Antibiotics Used in Human Medicine.

Antibiotic Name	Use	Antibiotic Class	Mechanism of Action	Effectiveness	Mode of Resistance	Transferability of Resistance	Reference
Tetracycline	Urinary tract infections, respiratory tract infections, intestinal infections, Chlamydia, acne, and rosacea.	Tetracycline	Inhibits protein synthesis by preventing the binding of aminoacyl-tRNA with the ribosome (30S ribosomal subunit of mRNA)	Bacteriostatic. Effective against Gram-positive bacteria, and has minimal effectiveness against Gram-negative bacteria.	Ribosomal protection proteins in which the mode of action is dependent on which gene is encoded. May work by blocking tet binding, ribosome distortion, or dislodging the drug. In addition, enzymatic inactivation of tetracycline (acetylation-rare), point mutations, and others may occur.	Genetic acquisition, efflux genes (plasmid pBR322).	Chopra I, Roberts M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65:232-260 ; second page, table of contents. Connell SR, Trieber CA, Stelzl U, Einfeldt E, Taylor DE, Nierhaus KH. 2002. The tetracycline resistance protein Tet(○) perturbs the conformation of the ribosomal decoding centre. Molecular microbiology 45:1463-1472. AAVPT. 2003. Tetracyclines. Thomson.
Vancomycin	Last resort antibiotic for life-threatening infections caused by Gram-positive bacteria. Used as a last resort to treat MRSA, MRSE, endocarditis, and colitis due to *Clostridium difficile* infections.	Glycopeptide	Inhibits cell wall synthesis in Gram-positive bacteria by binding D-ala to D-ala residues at the end of the peptide chains so they can't cross-link and form a cell wall.	Bactericidal. Only effective against Gram-positive bacteria. Not active against Gram-negative bacteria.	The last D-ala residue has been replaced with a D-lactate so that vancomycin can't bind and cross-links are formed. Alters the terminal amino acid residues of the NAM/NAG-peptide subunits (changes the last D-ala subunit to D-lactate which doesn't allow hydrogen bonding/vancomycin to bind →1000 fold decrease in affinity. May also work by changing the D-ala to D-serine →6 fold loss of affinity due to a steric change.	May be inducible. Resistance development may be related to the use of Avoparcin used in the poultry industry.	Pootoolal J, Neu J, Wright G. 2002. Glycopeptide antibiotic resistance. Annu Rev Pharmacol Toxicol doi:doi:10.1146/annurev.pharmtox.42.091601.142813:381-408. Schlecht H, Bruno C. 2016. Vancomycin. Merck Manual.
Cefotaxime	Used as a broad-spectrum antibiotic to treat a variety of infections including	3rd generation Cephalosporin	Inhibits cell wall synthesis by binding to PBPs (inhibits transpeptidation of last step)→ bactericidal	Bactericidal. Effective against both Gram-positive and Gram-negative bacteria.	Usually due to hydrolysis by B-lactamase alteration of PBPs, decreased permeability, efflux pump mechanisms, and *Enterococci*	Horizontal gene transfer likely. CTX-M-encoding genes able to be passed via conjugation and	Anonymous. 2015. Cephalosporins and Cephamycins: Antibacterial Agents. 2015. Livermore D. 1987. Mechanisms of Resistance to Cephalosporin Antibiotics. Drugs 34:64-88. Paterson DL, Bonomo RA. 2005. Extended-spectrum beta-

	infections of the lower respiratory tract, urinary tract, CNS, septicemia, bones, joints, and to treat meningitis.		because the cells will lyse if the cell wall is not complete.		may have intrinsic resistance.	transduction.	lactamases: a clinical update. Clin Microbiol Rev 18:657-686. Schlecht H, Bruno C. 2016. Cephalosporins. Merck Manual.
Clindamycin	Usually used to treat infections caused by anaerobes– including infections of the mouth, bones, joints, respiratory tract, and the skin. Special indications for use to treat MRSA, toxic shock syndrome, toxoplasmosis, and malaria.	Lincosamide	Binds the 50S rRNA of the large ribosomal subunit→ inhibits protein synthesis via inhibition of ribosomal translocation	Bacteriostatic. Effective against aerobic Gram-positive cocci and anaerobes. Not effective against Gram-negative aerobes and facultative anaerobic Enterobacteriaceae.	Enzymatic inactivation of clindamycin, methylation of the 23S binding site (alter the drug's binding site).	Plasmid-mediated conjugal transfer. Can also be chromosomally-encoded. Resistance genes (various *erm* genes) may be carried on plasmids or transposons (*Tn4400, Tn4351*). Likely to be transferred.	Schlecht H, Bruno C. 2016. Clindamycin. Merck Manual. Leclercq R. 2002. Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. Clinical Infectious Diseases 34:482-492.
Erythromycin	Used to treat infections of the skin and upper respiratory tract.	Macrolide	Binds the 50S subunit of the bacterial 70S rRNA complex (inhibits protein synthesis by preventing aminoacyl translocation).	Bacteriostatic. Bacteriostatic. Only effective against rapidly dividing cells. Not effective against Gram-negative bacteria because their cell wall prevents penetration into the cell.	In Gram-positive bacteria due to alterations in ribosomal structure and loss of macrolide affinity, genetic changes (erythromycin ribosome methylase genes) that cause modification of the 50S ribosomal subunit or methylates the 23S rRNA.	Genetic (*erm* genes), can be plasmid-borne, may be present in transposons, integrons, or in gene cassettes. Can be acquired via transduction and conjugation (HGT), and transformation. Resistance gene likely to be transferred in the human intestines from a food source. Development of resistance to erythromycin develops rapidly compared to the macrolide, tylosin. Very likely to be transferable.	Leclercq R. 2002. Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. Clinical Infectious Diseases 34:482-492. Weisblum B. 1995. Erythromycin resistance by ribosome modification. Antimicrobial Agents and Chemotherapy 39:577-585. Schlecht H, Bruno C. 2016. Macrolides. Merck Manual.

Costs Associated with Antibiotic-Resistant Infections

This is a problem because antibiotic resistant strains of bacteria are more difficult to treat, which means higher healthcare costs and greater overall health implications for those infected [130]. People infected with antibiotic-resistant strains of bacteria cost an additional ~$6,000-$30,000 dollars to treat than patients with antimicrobial-sensitive strains [131]. People with antibiotic-resistant infections usually require an increased recovery period, acquire greater

healthcare costs, and have greater healthcare complications, including death [132]. Carbapenem-resistant *Enterobacteriaceae* (CRE) are resistant to most antibiotics available today, and half of hospital patients with systemic infections die [133]. There are approximately 1,400 carbapenem-resistant *E. coli* infections per year with an estimate of 90 deaths, and while most are hospital-acquired it demonstrates the seriousness of antibiotic-resistant infections [133]. According to the Centers for Disease Control and Prevention, approximately 2 million people acquire antimicrobial-resistant infections per year, and 23,000 people die as a result of those infections [132]. In addition to the costs of human lives, economic costs are also of concern. Different studies suggest that antibiotic-resistance costs an additional $20 billion in extra direct healthcare costs annually in the United States [132]. Beyond this, there is the associated cost of the loss of productivity to the workforce, which is estimated at $35 billion per year [132].

Standard Industry Post-harvest Interventions for Fresh Produce

These increased costs demonstrate how important it is to reduce and prevent the spread of antibiotic-resistance. However, currently food industry regulations are not implementing any programs to reduce the prevalence of antimicrobial resistance in fresh produce. Standard post-harvest interventions for fresh produce include hydrocooling to remove field heat, washing with sanitizers such as a weak chlorine solution or peroxyacetic acid washes which are increasing in popularity and use, and ionizing irradiation [134].

Chemical Sanitizer Overview

The overall goal of chemical sanitizers is to decrease microbial load on produce and sanitize the wash water [135]. Unfortunately, chemical sanitizers are often ineffective at

sanitizing produce because they only have access to the surface of the produce, and do not reach the underlying stomata and junctions of leafy vegetables where microbes may be resident [136]. In addition, the use of sanitizers in re-circulating wash water may be contributing to expansion of the antibiotic-resistant bacterial (ARBs) populations, increasing the likelihood of the transfer of antibiotic-resistant genes (ARGs).

Mechanisms of Action for Chemical Sanitizers

The main two types of sanitizer utilized by fresh produce processors include variations of chlorine and peroxyacetic acid. Chlorine washes, such as sodium hypochlorite act as oxidizing agents and can have a variety of effects on microorganisms resulting in germicidal activity [137, 138]. One way in which chlorine can kill microbes is by creating disulfide bonds via the production of sulfenic acid and sulfinic acid that create crosslinks in microbial proteins, via the oxidation of sulfhydryl groups, rendering them nonfunctional [139]. Chlorine can also act as a disinfectant via reaction with protein amino groups by way of reaction with side chains of amino acids, DNA, or nucleotides which result in either cross-linking of bonds in proteins or interfering with base-pairing [140, 141]. Beyond this, it is thought that chlorine ‘s mechanism of action may also be somewhat dependent on inhibition of glucose oxidation, loss of adenine nucleotides, unfolding of proteins, and inhibition of DNA replication, however specific mechanisms related to these processes have yet to be elucidated [137, 142-145]. Chlorine can also act upon cells membranes and cell walls by destroying proteins present in the cell membrane and other enzymes, resulting in a leaky cell membrane [137]. This essentially disrupts all metabolic processes, effectively killing the microorganism [137, 138].

Sanitizer washes, such as Tsunami 100, are thought to utilize the same mechanisms as chlorine. The two active components in Tsunami 100 that result in germicidal activity are the peroxyacetic acid and the hydrogen peroxide [146]. Both peroxyacetic acid and hydrogen peroxide work as strong oxidizers that can remove electrons from various molecules, resulting in cell membrane and cell wall damage via hydroxyl radicals, similar to chlorine [147]. In addition, hydrogen peroxide has been demonstrated to denature proteins, and oxidize sulfhydryl bonds, similar to chlorine [147]. The mechanism of action of peroxyacetic acid is largely unknown, but it has been theorized that it denatures protein in a similar way as hydrogen peroxide, working on sulfhydryl bonds [148, 149].The oxidizing ability of both the peroxyacetic acid and hydrogen peroxide, combined with the low pH results in biocidal activity that is demonstrated effectiveness against some spores, yeast, and fungi in addition to bacteria [147].

Wash Water Disinfection

While chemical sanitizers are used to increase the safety of fresh produce, the main goal of the sanitizers is to prevent cross-contamination between individual pieces of produce. Wash water serves as a main vehicle for pathogen transfer in vegetable and fruit processing. It has been found that *E. coli* can be transferred in significant levels from inoculated fresh-cut escarole to both the wash water and non-inoculated escarole that was being washed in the same wash water as the inoculated escarole [150]. Tsunami at 500ppm and chlorine at 50ppm were effective at reducing inoculum levels to an undetectable level within the wash water while tap water was not [151]. Peroxyacetic acid and sodium hypochlorite washes were also shown to be effective at reducing *Murine Norovirus* 1 (MNV-1), *Bacteroides fragilis* HSP40–infecting phage B40-8, *Listeria monocytogenes*, and *E. coli* O157:H7 to undetectable levels in residual wash water

compared to a tap water control [152]. Tsunami and chlorine were also effective at preventing cross-contamination of *E. coli* between inoculated and non-inoculated lettuce [151]. These studies indicate that chemical sanitizers are useful for both sanitizing produce wash water and reducing cross-contamination between pieces of produce.

Chlorine Washes

Currently, commercial processors use a variety of sanitizers, varying concentrations, and varying contact times depending on a variety of factors. These factors include the type of crop, available equipment, and the target consumer market. At present, chlorine washes are the most widely used, however its efficacy can vary widely [134]. The type of crop can have a significant impact on the efficacy of chlorine washes. It has been shown that in a study where lettuce and coleslaw were exposed to the same wash treatment significantly different levels of reduction of both *Listeria innocua* and *E. coli* were achieved [153]. Washing of shredded lettuce resulted in a lower log-reduction of *Listeria monocytogenes* when compared to that of Romaine pieces, which can be attributed to an increased level of organic matter in the form of lettuce juice being released from the shredded lettuce pieces [154].

Bacterial inactivation via chlorine washes in the absence of organic matter was found to be dependent on the initial free chlorine concentration, contact time, and the pathogen itself, however when organic matter was present pathogen inactivation was dependent on both the initial free chlorine concentration and the amount of organic material present in the solution [155]. This suggests that decreasing the organic load prior to performing a chemical wash will increase the efficacy of the sanitizer and result in increased pathogen reduction and cross-contamination.

Chlorine wash efficacy is also dependent on temperature, contact time, and the amount of free chlorine available for antimicrobial action. Most sanitizers work best between 55-120°F [156]. *E. coli* O157:H7 was able to survive in wash water if the level of free chlorine was below 1ppm, and as a result there was an increase in cross-contamination from spinach to iceberg lettuce in the wash water system [157]. Chlorine washes with 50 parts per million (ppm) free chlorine are effective at reducing the presence of fecal coliforms on leafy greens [134]. Increasing the free chlorine concentration to 100 ppm decreases the total aerobic microbial CFU/g by 97.8% [134].

Beyond the chlorine washes, decreasing the pH from a standard 9.0 to 4.5 with different acids reduced microbial load 1.5-4 fold [134]. Acidified sodium chlorite (ASC) washes work in a similar way by decreasing pH and increasing the efficacy of the chlorine. Acidified sodium chlorite washes resulted in a 5.25 log-reduction of *E. coli* O157:H7 compared to an unwashed control on fresh-cut carrots [158]. Acidified sodium chlorite resulted in a 3-3.8 log-reduction compared to chlorinated water, which only resulted in a 2.1-2.8 log-reduction of *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* [159]. Acidified sodium hypochlorite washes also resulted in no re-growth of *E. coli* O157:H7 over a 14-day storage of the shredded carrots when stored at 5°C [158]. While decreasing pH and acidifying the wash water may help increase the log-reduction of pathogens in a chlorine wash system, chlorine contact time tends to hit a plateau. Increasing chlorine wash times from 5 to 30 minutes had little effect at decreasing microbial load because the reduction in microbes is dependent on the amount of available free chlorine [134].

The target organism also plays a key role in how effective a chemical sanitizer will be at pathogen reduction. *Salmonella* survives chlorine washes better than *E. coli* O157:H7, which in

turn has increased survival over a non-O157 Shiga-toxin *E. coli* strain [155]. However, other studies have indicated that there are few differences in bacterial reduction based on pathogen. In a study looking at the efficacy of acidified sodium chlorite and chlorinated water at reducing populations of *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* there were no significant differences in reduction between microbes enumerated from leafy greens [159]. In another study, sodium hypochlorite and acidified sodium chlorite have been shown to be equally effective at reducing aerobic mesophilic bacteria and *E. coli* O157:H7 populations, but that ASC washes were more effective at reducing yeast and mold [160]. *E. coli* populations are greatly reduced in the presence of sodium bicarbonate, peroxyacetic acid, and hydrogen peroxide, but are often susceptible to 50 ppm chlorine bleach, while viruses have been shown to survive all of the four chemical washes [161].

Peroxyacetic Acid Washes

Peroxyacetic acid (PAA) washes are increasing in popularity due to the minimal effect they have on health and quality of the product. In addition, sanitizers containing peroxyacetic acid are still effective at colder temperatures, meaning that PAA sanitizers can be used in hydrocooling systems [162]. Another benefit associated with peroxyacetic acid washes is that its efficacy is not greatly reduced in the presence of a higher organic load compared to sodium hypochlorite [152]. PAA sanitizers have also shown to have similar efficacy as chlorine washes. Peroxyacetic acid washes with a concentration of 100ppm used on lettuce are shown to decrease viral load, but have varying impact on different viruses; there was a 3.2 log reduction of *Feline Calicivirus*, a 2.3 log reduction of *Murine Norovirus*, and a 0.7 log reduction of *Hepatitis A virus* has been reported [163]. Tsunami 100 washes (80ppm) resulted in significant reduction of

Listeria monocytogenes on Iceberg lettuce from 5 log CFU/g to undetectable limits ($p<0.05$) [154]. PAA sanitizers have also demonstrated significant antimicrobial activity of 5-log reductions ($p< 0.05$) against bacterial strains isolated from water including *E. coli*, *P. aeruginosa*, and *Staphylococcus aureus* [164]. The efficacy of PAA, specifically against pathogens, has made PAA sanitizers a good choice for vegetable processors. In addition, shelf-life studies have shown that PAA can also be effective for preventing the re-growth of pathogenic bacteria [165].

Effect of Storage Times and Temperatures Post-Wash

Environmental conditions, such as the time and temperature during storage in the supermarket, are important considerations when evaluating populations of pathogenic bacteria and their subsequent safety. In addition, total bacterial counts are also often studied because initial microbial loads often translate into earlier spoilage of produce. Chemical sanitizer washes are often effective at decreasing initial total bacterial counts on produce, however it has been shown that after storage time of 8 days (3 days at 4°C + 5 days at 8°C) total bacterial counts on chemically-washed lettuce are often comparable to produce washed in tap water [166]. Tap water, sodium chlorite, and acidified sodium chlorite washes resulted in varying levels of initial reduction of total plate counts, yeasts, mold, and pathogenic bacteria on shredded carrots [167]. When these same shredded carrots were stored for 14 days at 5°C under passive modified atmosphere re-growth patterns varied greatly [167]. Total plate counts increased over the storage time period for carrots treated with tap water, sodium hypochlorite, and ASC (100-250ppm), while carrots washed with ASC (1000ppm) showed no increased in microbial growth at the 14 day sampling mark [167]. Apples, lettuce, strawberries, and cantaloupe washed with varying

sanitizers were stored at 4°C for 9 days and were enumerated for *E. coli* O157:H7, *L. monocytogenes*, mesophilic aerobic bacteria, yeasts, and molds [165]. While exposed to different sanitizers, produce from all treatment types resulted in unchanged pathogenic bacterial levels over storage, but an increase in mesophilic bacteria by 2-3 logs by the end of storage [165]. However, produce treated with chlorine dioxide (3 and 5ppm) and ozone (3ppm) both resulted in significantly higher final mold and yeast populations when compared with that of produce treated with chlorinated trisodium phosphate (100 and 200ppm) or peroxyacetic acid (80ppm) [165].

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CHAPTER 3: EFFECT OF STANDARD POST-HARVEST INTERVENTIONS ON THE SURVIVAL AND RE-GROWTH OF ANTIBIOTIC-RESISTANT BACTERIA ON FRESH PRODUCE

ABSTRACT

Raw vegetables are a source of food-borne outbreaks. The potential for fresh vegetables to serve as a vehicle for antibiotic -resistant bacteria is poorly understood. Antibiotics and antibiotic-resistant bacteria can persist in manure of animals administered antibiotics, and in compost made from this manure, where they may be transferred to produce via compost application. This study was performed to determine the survival of antibiotic-resistant bacteria, transferred from compost, on raw, peeled, carrots after washing with commonly used chemical sanitizers.

Multi-drug resistant *E. coli* O157:H7 and *Pseudomonas aeruginosa* were inoculated into a compost slurry in order to inoculate carrot surfaces with a background microbiota containing defined antibiotic-resistant bacteria relevant to produce. Carrots (n=3, 25g) were air-dried and stored at 4°C until washing with tap water, sodium hypochlorite/XY-12 (50 ppm free chlorine) or peroxyacetic acid/Tsunami 100 (40 ppm free paracetic acid), according to manufacturer's directions. Bacteria were then enumerated to determine the total aerobic bacteria, *E.coli* O157:H7, *Pseudomonas*, or antibiotic-resistant bacteria. Carrots from each compost treatment and subsequent wash were then individually packaged for storage at 2°C or 10°C for 7 days and were enumerated in the same manner on day 7. Additionally, the *tet*A gene was quantified to determine the effect of sanitizers and storage conditions on an antibiotic resistant gene carried by the inoculated bacteria.

Inclusion of sanitizer in the wash water significantly reduced the log CFU/g of inoculated *E.coli* and *Pseudomonas,* and bacterial populations resistant to cefotaxime (10µg/mL),

,sulfamethoxazole (100μg/mL), and tetracycline (3μg/mL). The most effective sanitizer for reducing the inoculated *E. coli* (EMB) and bacteria resistant to sulfamethoxazole (100μg/mL) was Tsunami 100 compared to washing with tap water or XY-12. For other groups of bacteria the log CFU/g reductions were comparable between Tsunami 100 and XY-12 sanitizers.

Re-growth of both the inoculated and native compost associated bacteria was prevented by storage at 2°C, as there were no significant differences in bacterial populations during the 14-day storage period. However, temperature abuse at 10°C resulted in significant re-growth of native *Pseudomonas*, compared to storage at 2°C ($p<0.05$). A sanitizer associated interaction between re-growth and temperature was also observed for bacteria resistant to clindamycin (25 μg/mL) and cefotaxime-resistant bacteria (10μg/mL) with substantial re-growth occurring only on carrots washed with Tsunami 100. There was no significant re-growth of the inoculated *E. coli* O157:H7 at either temperature. Results indicate that bacterial populations are reduced by post-harvest washes, but that antibiotic-resistant bacteria may increase if produce is temperature-abused during storage.

INTRODUCTION

Consumption of fresh-vegetables is an important part of a balanced diet. However, their consumption has been associated with food-borne illness outbreaks in part due to lack of any type of "kill" intervention step before consumption. Enterohemorrhagic *E. coli* (EHEC) is the most common implicated pathogen in produce-associated outbreaks-- accounting for 33% of outbreaks in Canada and 28% of multi-state outbreaks in the United States between 2001 and 2009 [1-3]. Outbreaks of food-borne illness are very costly to producers and consumers. These costs are amplified by an additional $6,000-$30,000 when the bacterial agents are resistant to

commonly used antibiotics as well as other costs in terms of increased recovery time, greater health complications, and increased loss of productivity in the workforce [4, 5].

Fresh fruits and vegetables are commonly inhabited by a variety of bacteria that are introduced from the soil, air, water and environment. Contamination sources of human pathogens or other types of antibiotic-resistant bacteria to raw vegetables include the application of improperly composted or raw manure to soil or plants, farm run-off, use of, contaminated water for irrigation or post-harvest washing [6].

To support the growing demand for fresh vegetables the soil must be amended with carbon sources to prevent soil subsidence. Cattle manure is frequently used for this purpose due the volume produced and need for environmentally responsible disposal practices. In the United States, good agricultural practices (GAPs) suggest that application of raw manure has a 120-day interval between application and harvest, while no time limit is required for application of compost as long as the composting method has been validated to reduce human pathogens including *E.coli* O157:H7 [7]. Composting may reduce antibiotic- bacterial populations that have been documented to increase in cattle fed both therapeutic and sub-therapeutic doses of antibiotics [8-11].

In an effort to improve quality and mitigate the risk of contamination with human pathogens, vegetable processors use chemical sanitizers in order to decrease microbial populations and prevent cross contamination wash water [12]. Free chlorine and peroxyacetic acid washes are effective at reducing the presence of pathogens such as *E. coli*, and *Listeria monocytogenes* on fresh produce [13] [14-16]. However, their effectiveness to reduce antibiotic resistant bacteria in wash water has not been examined.

The objective of these experiments was to quantify the survival and persistence of known inoculated pathogens and native antibiotic-resistant bacteria transferred from compost to carrots after washing the carrots with two food grade sanitizers. In addition, the survival and regrowth of the inoculated pathogenic and antibiotic-resistant bacterial populations will be monitored during storage at both optimal temperature conditions (2°C) and temperature-abuse conditions (10°C). Quantification of the *tet*A gene will be performed to determine how wash type and storage conditions affect this antibiotic resistance gene, which is present in our inoculum.

MATERIALS AND METHODS

Experimental design.

All carrots were subjected to a dip treatment prior to washing and storage. These included one of the four treatments: 1) Dipping in a compost slurry generated by composting manure from antibiotic dosed cows (antibiotic-compost), 2) Dipping in compost slurry generated by composting manure from non-dosed cows (control-compost), 3) Compost slurry generated by composting manure from cows administered antibiotics (dairy antibiotic compost) and a cocktail of *Pseudomonas aeruginosa* ATCC 47085 and *Escherichia coli* O157:H7 strain SMS-3-5 or 4) control compost and a cocktail of *Pseudomonas* and *E. coli*. Carrots from each treatment were washed with tap water, XY-12 (sodium hypochlorite), Tsunami 100 (peroxyacetic acid/hydrogen peroxide), or remained unwashed and then individually packaged for storage at 2°C or 10°C, allowing for 3 replicated enumerations on day 0, day 1, day 7 and day 14 (2°C only) to examine effect of sanitizer washes and/or compost type on the re-growth of antibiotic-resistant bacteria. Each experiment was replicated in triplicate using different lots of carrots, within each experiment a minimum of 3 samples were compared for each treatment combination (compost type/inoculation, sanitizer wash, storage temperature, storage time).

Compost slurry preparation.

Compost generated from a prior experiment was used to prepare compost slurry [17]. In brief, manure from dairy cattle was collected from cows not currently receiving antibiotics (control), or dairy cattle administered therapeutic doses of Cephapirin and Pirlimycin (antibiotic). The manure was composted statically for 28 days, temperature was maintained at 55°C for 26 days. At the end of composting experiment the compost was stored at -20°C for 30 weeks until used. The compost was thawed, mixed by blending and stirring for homogeneity and stored at 4°C, for between 1-16 weeks, while the experiments were carried out. The compost slurry was prepared by blending 50g of compost and sterile DI water (450 ml for non-inoculated treatments or 440mL of sterile DI water and 10mL cocktail) for 45 seconds using an Oster® Beehive Blender (Oster®, Boca Raton, FL, USA). The compost mixture was then strained through a number 12 sieve into a sterile container to remove large wood chips. The compost slurry remained at room temperature (23-25°C) for 24 h prior to dipping of the carrots to acclimate the compost microbiota.

Bacterial strains and growth conditions for the inoculated compost trials.

Pseudomonas aeruginosa strain PAO1 with plasmid pUCP19 (ATCC 47085), a derivative of a *P. aeruginosa* strain PAO, and *Escherichia coli* O157:H7 strain SMS-3-5 (ATCC BAA-1743) were chosen due to resistance to multiple antibiotics and disinfectants. The *Pseudomonas aeruginosa* strain is resistant to the following antibiotics at the defined concentrations; tetracycline (4μg/mL), clindamycin (25μg/mL), sulfamethoxazole (100μg/mL), and vancomycin (11μg/mL). The *E. coli* O157:H7 strain is resistant to the following antibiotics at the defined concentrations; tetracycline (4μg/mL), cefotaxime (10μg/mL), clindamycin (25μg/mL), sulfamethoxazole (100μg/mL), and vancomycin (11μg/mL). Cultures from -80°C freezer stocks

were streaked onto Tryptic Soy Agar (TSA: Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 h at 37°C to obtain isolated colonies. An isolated colony of *Pseudomonas aeruginosa* was transferred to Pseudomonas Isolation Agar (PIA: Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 h at 37°C in its stationary phase. An isolated colony of *Escherichia coli* O157:H7 was transferred to Eosin Methylene Blue Agar (EMB: Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 h at 37°C in its stationary phase. Separate single colonies from PIA and EMB were transferred to separate tubes of Tryptic Soy Broth (TSB, Becton Dickinson) and incubated shaking (180 rpm) for 24 h at 37°C. Cells were washed two times in 9mL 0.1% (wt/vol) peptone (Sigma Aldrich, Co., MO) to remove any residual nutrients and media. Before suspension in 9mL 0.1% (wt/vol) peptone (Sigma-Aldrich, Co., MO) the compost slurry was inoculated with 5mL of each of the re-suspended *E. coli* and *P. aeruginosa* cells. Initial *E. coli* inoculum into the compost slurry was ~9.30 log CFU/mL . Initial *P. aeruginosa* inoculum into the compost slurry was ~9.00 log CFU/mL.

Carrot compost slurry dip/inoculation.

Organic petite baby carrots were obtained from a wholesale club in 3lb bags. Carrots were pre-washed and peeled. No additional treatments were applied. Carrots were stored at 4°C prior to use. Baby carrots, 800g, were quickly dipped in and out of the prepared compost slurry and allowed to dry for 1 h at room temperature (23-25°C) on wire racks prior to washing. In the inoculated trials an average transfer of 5.6 log CFU/g of *P. aeruginosa* and 6.0 log CFU/g of *E. coli* was achieved.

Carrot washing treatments

All compost/inoculated carrots were washed for 2 minutes with agitation achieved through a combination of using 2 stir bars at a speed setting of 125 placed evenly apart in the wash bin, and

back-and-forth stirring with a slotted spoon, in one of four solutions (200g each) **: 1).** Unwashed, **2).** Tap water wash (Blacksburg Municipal water, ~0.5ppm free chlorine, measured as 0ppm 3). Sodium hypochlorite (1563uL of XY-12 (EcoLab, St. Paul, MN) in 5L of tap water per manufacturers recommendation (50ppm free chlorine) with a 2 min contact time followed by a 30 sec rinse with tap water. 4). Peroxyacetic acid and Hydrogen peroxide, 977uL of Tsunami 100 (Ecolab, St. Paul, MN) in 5L of tap water mixed per manufacturers recommendation (40ppm free paracetic acid) with a 1.5 min contact time followed by a 30 sec rinse with tap water.

Wash water quality parameter measurements.

Temperature and pH of 20mL aliquots of the wash water were measured using the Fisher Scientific Accument® excel XL20 pH/conductivity meter. ORP measurements were taken for the wash water from the tap water and XY-12 washes using Fisher Scientific Accument® portable AP62 pH/mV meter. Free chlorine was measured for the tap water and XY-12 washes using free chlorine strips (Ecolab). Free paracetic acid was measured for the Tsunami 100 wash using free paracetic acid strips (Micro Essential Laboratories Hydrion® 0-160ppm Paracetic acid test strips). Samples of the wash water, 20mL, were taken both pre and post-wash of the carrots.

Media Preparation.

R2A, Eosin Methylene Blue, and Pseudomonas Isolation Agar were prepared according to manufacturer's direction (BD, Franklin Lakes, NJ). Antibiotic-supplemented media was prepared as follows. Stock solutions of antibiotics (ThermoFisher Scientific) were made by weighing dry, powdered antibiotics into sterile containers and mixing them with either 70% ethanol or sterile DI water according to solubility and manufacturer's direction. The stock solutions were mixed until the antibiotics were completely dissolved. The solutions were then syringe-filtered for sterilization. The stock solutions were stored at -20°C until the day of use. On the day media was

prepared the antibiotic stock solutions were thawed. Antibiotics were added to cooled (45-55°C) agar to achieve the following concentrations in R2A: tetracycline (3μg/mL), cefotaxime (10μg/mL), clindamycin (25μg/mL), vancomycin (11μg/mL), and sulfamethoxazole (100μg/mL). Concentrations were determined by plating of serial dilutions of dairy compost, and the desired concentration was determined to be where 50% reduction in bacterial populations were achieved on that media containing that specific antibiotic. The only antibiotic supplemented into PIA was tetracycline (4μg/mL) to select for the inoculated *Pseudomonas*, which expresses resistance to tetracycline at higher antibiotic concentrations.

Microbiological Detection.

Enumeration of total aerobic bacteria, antibiotic-tolerant bacteria, *E. coli*, and *P. aeruginosa* were performed according to the following method. Carrot samples (25 g) were stomached (Interscience BagMixer®, Guelph, Ontario) in 225 mL of sterile peptone tween 80 buffer (PT, both 0.1% w/v, BD, Franklin Lakes, NJ) for 2 minutes at a speed of 3 within a sterile blender bag (Fisher Scientific, Waltham, MA). The mixture was serial-diluted in sterile PT buffer and 100uL aliquots were spiral-plated (Spiral Biotech Autoplate 4000®, Bethesda, MD) onto the various media types (BD, Franklin Lakes, NJ) and were incubated for 18-24 h at 37°C.

DNA extraction

Bacteria were removed from the treated carrots using the following gentle physical and chemical methods designed to minimize the disruption of plant cells and release of plant DNA. Carrots were weighed into sterile filter bags and peptone buffer with Tween 80 (0.1% by volume each) was added in order to achieve a 1:10 dilution. The bag was then shaken at a speed of 220rpm for 5 minutes using a bench top rotator (Barnstead Multi-Purpose Rotator), subsequently hand-massaged for 2 minutes, and then shaken again using the bench top rotator for an additional 5

minutes. The liquid contents of the bag were vacuum filtered through a 0.22 um, 47-mm cellulose ester membrane (EMD Millipore) filter to collect bacterial cells. Filters were stored within sterile, DNase-free, O-ring screwcap tubes at -80°C until DNA extractions were performed. DNA extractions were performed from the filter using the FastDNA® Spin Kit for Soil (MP Biomedical™, Catalogue number: 116560000 Santa Ana, California, USA)) using a combination of physical and chemical disruption per manufacturers instructions. Briefly, the filter , sodium phosphate buffer and MT buffer (MP Biomedicals™) were shaken in the Lysing tube using the FastPrep® Instrument for 40 seconds at a speed of 6. The tubes were placed onto ice and incubated for 2 minutes, and shaken again. The samples were then incubated at room temperature (~25°C) for 2 hours to complete chemical lysis. The rest of the DNA extraction was performed according to the FastDNA® Spin Kit for Soil manufacturer directions. The DNA was eluted in 100 uL of DNase/pyrogen free water (manufacturer, origin) and the OneStep™ PCR Inhibitor Removal Kit (Zymo Research) used per manufacturer instructions. DNA was stored at -80°C until qPCR was performed.

Quantification of the antibiotic resistance gene tetA as effected by sanitizer wash and temperature of storage.

The numbers of copies of tetA and of 16S rDNA were determined from each treatment using quantitative real time PCR. Each .20 ml reaction contained 1ml of sample DNA, 5ml 2x EVAgreen Supermix (Bio-Rad), forward primer (400nM), reverse primer (400uM), and molecular grade water (Sigma-Aldrich). PCR amplification and detection was performed using the Bio-Rad CFX Connect™ Real Time System qPCR machine. PCR conditions consisted of: 1 cycle of 98°C for 2 minutes, 40 cycles of 98°C for 5 minutes, and then annealed for 5s at 55°C as described by Wang et. al 2012. qPCR analysis to determine the number of 16S rDNA copies was

performed as previously described. Standard curves were constructed by performing 10-fold serial dilutions of purified DNA from 0.1 to 100 ng/mL and the Ct values were extrapolated against the standard curve. Primer sets and their annealing temperatures are as follows: 16S rDNA, (55°C), and tetA (60°C). Ma et. al 2011. Ct values of *tetA* were normalized using the housekeeping gene 16S rDNA. Three technical replicates were performed.

Data and statistical analysis.

The proportion of antibiotic resistant bacteria was calculated by dividing the average total number of CFU of each separate media type by the average total number of CFU of total aerobic bacterial populations (R2A). The mean proportion for each of the three replicates and the mean tetA copies/16S rDNA were compared using the non-parametric multiple comparison Wilcoxon Each pair test. Additionally the bacterial CFU/g were log transformed. Two biological replicates were performed for the dairy control compost (both non-inoculated and inoculated). Three biological replicated were performed for the antibiotic dairy compost (both non-inoculated and inoculated). Statistical analyses were performed using JMP statistical software (version 10, SAS, Cary, NC). The effect of compost type (control or antibiotic) was compared using a one-way ANOVA to test for differences in the average log of antibiotic-tolerant bacteria of recovered CFU/g on carrots. The effect of storage temperature was compared using a one-way ANOVA to test for differences in the average log of average log of all bacteria types (antibiotic-tolerant, APC, *E. coli*, *P. aeruginosa*) of recovered CFU/g on carrots. The effect of day of storage and wash type were compared using an ANOVA analysis with a Tukey's post-hoc analysis to test for differences in the average log of all bacteria types (antibiotic-tolerant, APC, *E. coli*, *P. aeruginosa*) of recovered CFU/g on carrots. P-values of <0.05 were used to designate

significance. Independent variables included: Least squares means were obtained using Tukey adjustment.

RESULTS AND DISCUSSION

Effect of antibiotic administration to dairy cattle during manure collection influences the bacteria isolated on antibiotic containing media from carrots dipped in the resulting compost.

The bacterial populations (log CFU/g for any of the media types) recovered from compost alone were not significantly different between compost generated from the manure of cows that were administered antibiotics during collection and non-treated cows (Data not shown). This is consistent with a study performed by Ghosh et.al that found that the application of manure from cattle fed sub-therapeutic doses of antibiotics did not result in differences in antibiotic-resistant bacterial populations recovered from those soils compared to soils amended with manure from cattle with restarted antibiotic use, or from non-agricultural soils [18]. In addition, different studies have found that composting manure for 30 days decreased antibiotic concentrations by 85-99%, indicating that the bacteria in our compost may not have been exposed to antibiotics at a high enough level to perpetuate resistance [19, 20]. In this study, forced-temperature composting was used and antibiotic disappearance was almost complete, which is consistent with other literature, but may not accurately depict real world composting.

While administration of antibiotics during manure collection did not alter the antibiotic-resistant bacterial populations in the compost alone, after dipping the carrots in the different compost slurries, one change was observed. Carrots dipped in antibiotic-containing compost contained less bacteria capable of growth on media containing sulfamethoxazole compared to carrots that were dipped in compost generated from dairy cows not receiving antibiotics during

collection (control), when washed with tap water or Tsunami 100 (Table 3.1.1, Figure 3.1.1). Dipping carrots in compost generated from manure collected during antibiotic did not influence the recovery of bacteria on any other antibiotic-containing media compared to the control (Table 3.1.1, Table 3.1.2). Because antibiotic administration has been shown to alter the diversity of the gastrointestinal microbial community this suggests that the communities are different between the two composts. It was beyond the scope of this study to characterize theses changes, however examination of 16S rDNA amplicons in underway.

The main effect resulting from the different compost slurry dips was that when the compost slurry was co-inoculated with two ARB, there were increases in average log CFU/g *E. coli* recovered on EMB and *Pseudomonas* on PIA T, as expected (Table 3.2.1).

Determination of the effect of sanitizer wash on recovery of antibiotic resistant bacteria immediately after washing.

The effectiveness of washing with tap water, XY-12, or Tsunami 100 on recovery (log CFU/g) of inoculated *Pseudomonas* and *E. coli* bacteria, and native antibiotic resistant bacteria from compost-dipped carrots were compared. In general, washing resulted in significant reductions in some bacterial populations (log CFU/g); however the smallest reductions were seen when carrots were washed with tap water alone (Figure 3.2.1).

For the samples inoculated with two known ABR bacteria, the inclusion of Tsunami 100 resulted in the largest reductions of log CFU/g. *E. coli* was reduced by 0.61 log CFU/g after washing with tap water, 1.9 log CFU/g after washing with XY-12, and 2.61 log CFU/g after washing with Tsunami 100 (Table 3.1.2, Figure 3.2.1, Figure 3.2.2). Results indicate that the inclusions of chemical agents in wash water are an effective way at reducing this pathogenic *E. coli* strain. The peroxyacetic acid/hydrogen peroxide wash resulted in the greatest decrease of *E.*

coli O157:H7 populations. These results are consistent with other studies that have found that compared to water washes only, peroxyacetic acid sanitizers, like Tsunami 100, have demonstrated increased efficacy against pathogens, with the kill being significantly increased compared to chlorine washes, evidenced by 5-log reductions seen in bacterial strains including *E. coli*, *P. aeruginosa*, and *Staphylococcus aureus* [16]. Acidified chlorine treatments also significantly reduced both *E. coli* O157:H7 and *Listeria monocytogenes*, but to a lesser degree than peroxyacetic acid, which is what was consistently seen throughout our study [21]. Chlorine washes with free chlorine concentrations of 50ppm, which was achieved in our study, still resulted in significant reductions in the presence of fecal coliforms on leafy greens [22]. However in this study, peroxyacetic acid/hydrogen peroxide washes reduced *E. coli* O157:H7 the most compared to other washes, which indicates that peroxyacetic acid/hydrogen peroxide washes may be a better choice for vegetable processors to decrease their risk of cross-contamination of pathogens in their wash water and also on the produce itself (Figure 3.2.2). Tsunami 100 was the most effective at reducing this particular strain. However, reductions of pathogens using chemical sanitizers are multi-factorial and dependent on the specific bacteria, chemical concentrations, organic load, pH, contact time, etc. [23-29]. In addition, structure of the vegetable is also a critical factor, which was not tested in this study, but could be an area of future research.

In terms of other bacterial populations, inclusion of either sanitizer was effective at reducing bacterial populations (Figure 3.2.1, Figure 3.2.2). Average log CFU/g of *Pseudomonas* and *Pseudomonas* resistant to tetracycline (4 μg/mL) were not significantly different between unwashed and tap water washed carrots; however, washing with XY-12 or Tsunami 100 resulted in significant reductions compared to the unwashed control (Figure 3.2.1, Figure 3.2.2). Native

Pseudomonas resistant to tetracycline (4 μg/mL), the same as the marker strain was present on non-inoculated samples, however it was significantly less than on the inoculated carrots (Table 3.1.1). Tetracycline resistance is very widespread in environmental samples, and has been reported on foods including fresh produce, which indicates why we had recovery of *Pseudomonas* resistant to tetracycline from non-inoculated batches of carrots [30, 31]. In this study, we did not include a control of carrots that had not been dipped in the compost therefore we cannot definitely say the source of these tetracycline resistant *Pseudomonas*. However, it is not out of the realm of possibility that these carrots contained the tetracycline-resistant bacteria. In recent studies of grocery store produce, tetracycline-resistance and resistance to multiple antibiotics was present in up to 95% of *Pseudomonas* isolates from ready-to-eat produce possessed resistance to multiple classes of antibiotics [32].

While the source of these antibiotic-resistant bacteria may be multi-variable, washing with chemical sanitizers still demonstrated efficacy against these populations. Native, compost associated populations of bacteria resistant to cefotaxime (10 μg/mL), sulfamethoxazole (100μg/mL), and tetracycline (3μg/mL) were significantly reduced by washing, with the largest reductions in log CFU/g occurring after washing with Tsunami 100 (Figure 3.2.1). There have been no other published studies looking at the effect of sanitizer washes on antibiotic-resistant bacterial populations, however studies have shown that disinfectants are effective at reducing antibiotic-resistant bacterial populations on surfaces [33]. After washing, small statistically insignificant changes in log CFU/g were observed in total aerobic bacteria (TAB) on R2A, and log CFU/g of bacteria grown on R2A supplemented with clindamycin and vancomycin (Table 3.1.1, Table 3.1.2, Figure 3.2.1). Interestingly, changes in colony morphology were noted on both clindamycin and vancomycin-containing plates with changes to colony size and

morphology on more dilute plates. Perhaps increasing dilutions allowed for less competition, which allowed other bacteria to grow that were outcompeted by others. It is likely that these are different bacterial groups and future studies using Illumina sequencing of the 16S rDNA will be performed to better understand the changes to the bacterial community.

Bacterial survival and re-growth during storage.

Refrigerated storage at two different temperatures, optimal (2°C) and temperature abuse (10°C), resulted in different effects on the compost-associated bacteria and the inoculated ARB *E.coli* and *Pseudomonas* (Figure 3.3.1, Figure 3.3.2, Figure 3.3.3, Figure 3.3.4). Re-growth of both the inoculated and native compost-associated bacteria was prevented by storage at 2°C, as there were no significant differences between any log CFU/g values (TAB, antibiotic-tolerant, *E. coli* inoculum, *P. aeruginosa* inoculum) after the 14-day storage period at 2°C (results not shown). This is consistent with other literature in that *E. coli* O157:H7 did not have significant re-growth on any salad vegetables, but actually declined when the vegetables were stored at 5°C [34].

In addition, the proportion of bacteria that grew on the tetracycline (3μg/mL) containing media relative to the TAB –was reduced by storage at 2°C, with significant reductions for each of wash types after 7d of storage, except for unwashed carrots which experienced the same reduction but after 14d of storage (Figure 3.4.1). The proportion of sulfamethoxazole-resistant bacteria also experienced changes in populations when stored at 2°C over the 14d storage period, depending on the wash type (Figure 3.4.1). No current research exists on tetracycline or sulfa-resistant bacteria and temperature storage; however, these bacterial populations may just be more susceptible to the combination of sanitizer and subsequent temperature stress. The proportions of bacteria resistant to other antibiotics in the total bacterial population were not significantly affected by the 14-day storage time at 2°C (data not shown). This indicates the importance of

storing produce at the proper temperature, because it not only prevents re-growth of a pathogenic *E. coli* strain, but also prevented re-growth of antibiotic-resistant bacteria. This means pairing the use of chemical sanitizers with proper temperature storage may help mitigate the risk of antibiotic- resistant bacteria on fresh produce.

In terms of our inoculum, re-growth of the inoculated *E. coli* O157:H7 was not observed after 7 days of storage at either temperature. In fact statistically significant reductions occurred during storage compared to day 0 for unwashed and Tsunami 100 washed carrots at 2°C for 7 days (Figure 3.3.1, Figure 3.3.2). Our storage study indicated that once these sanitizers initially reduced these *E. coli* O157:H7 populations they did not re-grow under optimal temperature (2°C) or temperature-abuse (10°C) conditions, indicating that the inclusion of sanitizers are effective at initially reducing and preventing the re-growth of our particular *E. coli* O157:H7 strain. Interestingly, one study showed inhibition of *E. coli* at 5°C, but when the storage temperature was increased to either 12°C or 21°C and vegetables were stored for 14 days *E. coli* O157:H7 populations increased on all vegetables tested, with the exception of carrots [34]. This data suggests that carrots may not have enough surface-associated nutrients, or perhaps the right types of nutrients, to promote growth of *E. coli* O157:H7, which may explain why *E. coli* populations during our experiment did not increase even when stored at temperature abuse conditions [34]. The native microbiota of the carrots may have also outcompeted *E.coli* for nutrients.

While *E. coli* populations did not demonstrate re-growth at either temperature, temperature abuse at 10°C resulted in significant re-growth of native *Pseudomonas* on sanitizer-washed carrots when represented as average log CFU/g or as a proportion to total aerobic bacteria (TAB), over the 7d storage (Figure 3.3.3). This was not observed when stored at 2°C,

ultimately increasing the populations on average 1.4 log CFU/g for the Tsunami washed carrots and 0.8 log CFU for the XY-12 washed carrots compared to day 0 (Figure 3.3.3). These differences observed in the initial reduction and re-growth between *E. coli* O157:H7 and *Pseudomonas* could vary based on many factors. This may be due to differences in cell membrane permeability between the bacteria, with *Pseudomonas* having greater adaptability in controlling their cell wall permeability, evidenced by their ability to survive at a wider array of temperatures [35, 36]. However, the interaction between oxidizing sanitizers and cell wall permeability requires further research. After 7d of storage at 10°C the inoculated *P. aeruginosa* (PIA-Tet), increased to 4.5-5 log CFU/g, surpassing the original reduction due to washing (Figure 3.3.4). Increases in the relative proportions of *Pseudomonas* sp., *Pseudomonas* resistant to tetracycline (4μg/mL), and bacteria resistant to cefotaxime (10μg/mL) within the surviving TAB on carrots that were washed with Tsunami 100 occurred after storage at 10°C for 7d. In contrast, the relative proportions of vancomycin-resistant bacteria (11μg/mL) within the surviving TAB decreased (Figure 3.5.1). Average log data followed similar trends (Figure 3.5.2).

The proportion of bacteria that grew on the antibiotic containing media relative to the TAB was significantly reduced immediately after washing, with the exception of clindamycin-resistant bacteria (Figure 3.6.1). Significant re-growth of native *Pseudomonas,* both log CFU/g and the proportion of the TAB that were *Pseudomonas* increased, on sanitizer-washed carrots stored under temperature abuse conditions over the 7d storage (Figure 3.3.3, Figure 3.5.1). This was not observed when stored at 2°C ultimately increasing the populations on average 1.4 log CFU/g for the Tsunami washed carrots and 0.8 log CFU for the XY-12 washed carrots compared to day 0 (Figure. 3.3.3). After 7d of storage at 10°C the inoculated *P. aeruginosa* (PIA-Tet), increased to 4.5-5 log CFU/g, surpassing the original reduction due to washing (Figure 3.3.4,

Figure 3.5.1). This is expected and consistent with other literature because *Pseudomonads* are psychrophilic and are capable of growth within a wide range of temperature, including 10°C [37]. Interestingly, bacteria resistant to clindamycin demonstrated a significant increase in log CFU/g after 7 days of storage at 10°C compared to those stored at 2°C when carrots were washed with Tsunami 100 only (Figure 3.7.1). There was also a marked increase in log CFU/g on plates containing cefotaxime from day 0 to day 7 of storage at 10°C that was only observed in carrots washed with Tsunami 100 (Figure 3.5.1). There is little literature that focuses on the effect of storage temperature on antibiotic-resistant bacterial populations, so no inferences can be made as to why certain populations increased.

There was an association between carrots washed with Tsunami 100 and re-growth of some bacterial populations at 10°C, which is interesting and differs from the carrots washed with XY-12. One explanation for this re-growth pattern may be that the compost used contained trace amounts of a first-generation cephalosporin, cephapirin, which is an antibiotic in the same class as the human antibiotic cefotaxime. Perhaps the mechanism of action of the peroxyacetic acid/hydrogen peroxide wash interaction with the cephalosporin-containing compost type is what resulted in the significant re-growth of cefotaxime-resistant bacteria under specific temperature parameters; however because the mechanism of action of peroxyacetic acid is not well understood this is speculation. Perhaps this is due to a greater initial reduction that allows for greater subsequent re-growth, or an unknown interaction between the peroxyacetic acid/hydrogen peroxide combination, the temperature-abuse conditions during storage, and the subsequent exposure to antibiotics in the media type. This area requires further research because no studies have been performed looking at temperature abuse and antibiotic-resistance, but

emphasizes the fact that temperature-abuse may exacerbate antibiotic-resistance, as seen in this study.

***tet*A Quantification.**

Quantities of *tet*A copies, with respect to 16S rRNA copies, in samples varied greatly and did not follow a clear trend. On the initial wash day for the 2°C storage experiment, carrots washed in XY-12 had significantly greater amounts of *tet*A compared to any other wash type (Figure 3.8.1). The only significant difference seen between days of storage was observed in carrots washed with XY-12 and subsequently stored at 2°C for 7d, in which a significant decrease in *tet*A was observed (Figure 3.8.1). However, when carrots were washed on the initial day for the 10°C storage experiment this significance disappeared, with no significant differences being observed based on wash type (Figure 3.8.1). It should be noted that significant differences were seen between carrots washed in XY-12 and Tsunami 100 on different days of initial washing (Figure 3.8.1). After 7 days of storage at 2°C carrots washed with Tsunami 100 had significantly increased levels of *tet*A compared to any other wash type, while at 10°C unwashed samples became the wash type associated with the greatest amount of *tet*A (Figure 3.8.1).

The quantification of the antibiotic-resistance *tet*A gene suggests that storage of washed carrots under temperature-abuse conditions (10°C) actually decreases the quantity of *tet*A copy numbers compared to optimal temperature conditions (2°C); which this is consistent with current literature about temperature and prevalence of antibiotic-resistance genes [38] [39]. It is not known as to why there are less *tet*A gene copies at increased temperatures, but one possible explanation is that bacteria possessing the *tet*A gene were more likely to survive at lower temperatures because they are better adapted for unfavorable growth conditions. It is hypothesized that an increase in horizontal gene transfer occurs at colder temperatures in *P.*

aeruginosa, a phenomenon also seen with exposure to fluoroquinolones [39, 40]. Regardless, sanitizer washes can be helpful in mitigating the risk of ARG prevalence and transfer on fresh produces.

However, more research needs to be conducted, which may include increased storage time, and the transfer of antibiotic-resistance genes in wash water. In general, antibiotic resistance on fresh produce is a complex topic and requires further research.

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RESEARCH FIGURES

Table 3.1.1: Comparison of control versus antibiotic dairy compost-dipped non-inoculated carrots (compost type effect). Bacteria recovered (mean log CFU/g ± standard deviation) on different media types, after different wash types on day 0.

Standard deviation represents 1 standard deviation from the mean log CFU/g value. Capital letters and red coloring denote significant differences between treatment type (prior antibiotic administration or not) within the same wash type and media type ($p<0.05$). Lower-case letters and blue coloring denote significant differences between wash type within the same treatment type and media type ($p<0.05$). Purple coloring denotes significant differences were found between both wash type and treatment type ($p<0.05$). Absence of coloring denotes no significant differences were found.

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

Media Type	R2A	PIA	EMB	PIA T	R2A T	R2A CL	R2A CO	R2A V	R2A S
Treatment Type									
Carrots dipped in dairy compost produced from manure not containing antibiotics (DC-NON)									
Unwashed	8.40 ±3.43^{Aa}	5.09 ±1.03^{Aa}	0±0^{aB}	4.27 ±1.83^{Aa}	5.38 ±1.04 ^{Aa}	6.18 ±0.69^{Aa}	5.41±0.38^{Aa}	6.12 ±1.02^{Aa}	4.76 ±0.23^{B}
Tap Water	6.30 ±1.35^{Aa}	4.60 ±0.40^{Aa}	0±0^{aB}	3.48 ±1.67^{Aa}	4.62 ±1.96^{Aa}	5.63 ±1.26^{Aa}	4.85 ±0.08^{Aa}	6.02 ±1.45^{Aa}	4.76 ±0.12^{B}
XY-12	6.04 ±1.58^{Aa}	3.80 ±0.88^{Aa}	0±0^{aB}	2.09 ±2.95^{Aa}	3.94 ±2.74^{Aa}	5.28 ±1.73^{Aa}	4.51±0.30^{Aa}	5.50 ±2.15^{Aa}	4.33 ±0.10^{A}
Tsunami 100	5.97 ±1.52^{Aa}	3.27 ±0.22^{Aa}	0±0^{aB}	1.53 ±2.16^{Aa}	4.03 ±2.65^{Aa}	4.86 ±2.28^{Aa}	4.56 ±0.11^{Aa}	5.12 ±2.32^{a}	4.41± 0.10^{B}
Carrots dipped in dairy compost produced from manure from dairy cows receiving antibiotics (DCAB-NON)									
Unwashed	7.36 ±1.47^{Aa}	5.19 ±0.32^{Aa}	0±0^{aB}	3.98 ±0.30^{Aa}	4.55 ±1.13^{Aa}	5.67 ±1.41^{Aa}	5.31 ±0.26 ^{Aa}	5.69 ±1.45^{Aa}	4.49 ±0.06^{aB}
Tap Water	7.29 ±1.88^{Aa}	4.56 ±0.37^{Aa}	0±0^{aB}	3.57 ±0.38^{Aa}	4.85 ±1.26^{Aa}	5.54 ±1.21^{Aa}	4.86 ±0.35^{Aab}	6.29 ±1.55^{Aa}	3.89 ±0.19^{Ab}
XY-12	6.73 ±1.68^{Aa}	3.85 ±0.10^{Aa}	0±0^{aB}	2.30 ±2.00^{Aa}	4.23 ±1.19^{Aa}	5.40 ±0.88^{Aa}	4.32 ±0.02^{Abc}	6.08 ±1.42^{Aa}	3.81 ±0.33^{Ab}
Tsunami 100	7.05 ±2.02^{a}	2.66 ± 2.31^{Aa}	0±0^{aB}	0.92 ±1.60^{Aa}	4.31 ±1.19^{Aa}	5.31 ±1.59^{Aa}	4.20 ±0.17^{Ac}	5.54 ±2.50^{Aa}	3.72 ±0.22^{Ab}

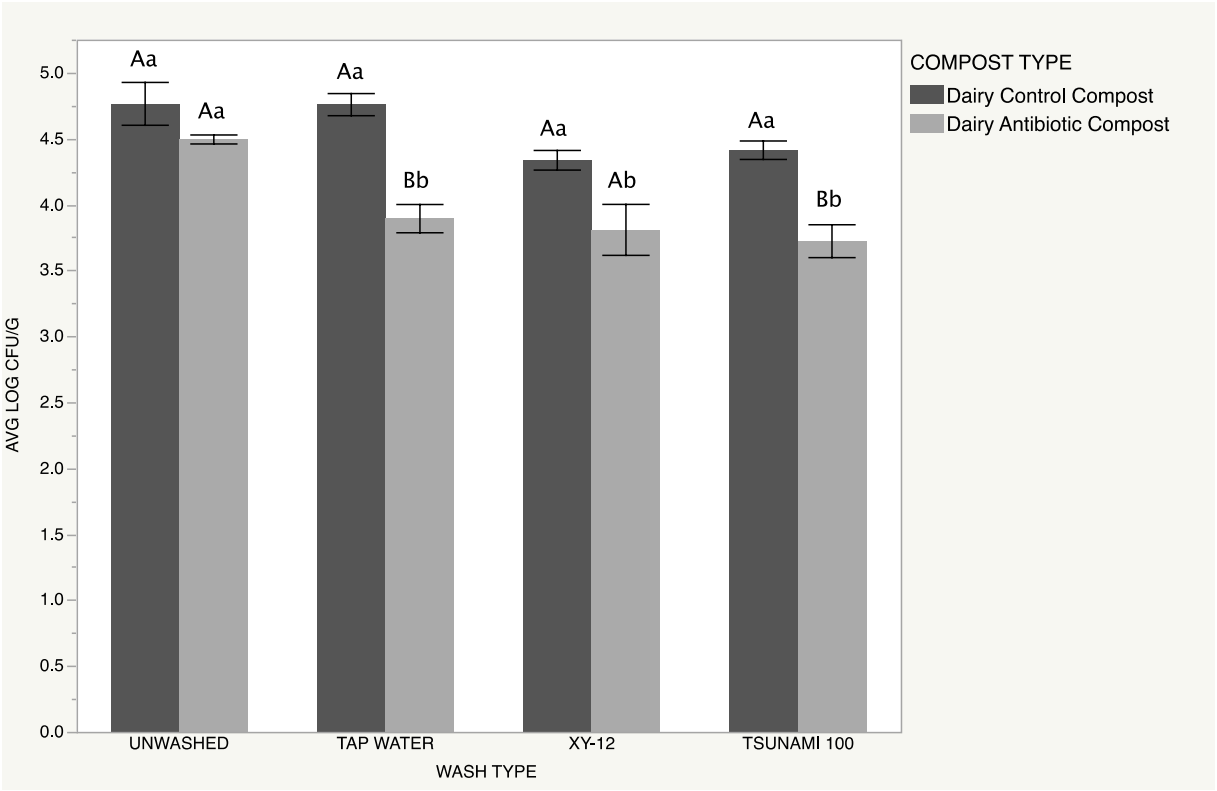

Figure 3.1.1: Antibiotic administration during manure collection for composting affects log CFU/g bacteria recovered on R2A media supplemented with sulfamethoxazole (100µg/mL) on Day 0 of washing (n=2).

Error bars represent 1 standard error from the mean.

Capital letters denote significant differences between compost type (dairy control versus dairy antibiotic compost) within the same wash type ($p<0.05$). Lower-case letters denote significant differences between wash type within the same compost type ($p<0.05$).

Table 3.1.2: Comparison of control versus antibiotic dairy compost-dipped inoculated carrots (compost type effect). Bacteria recovered (mean log CFU/g ± standard deviation) on different media types, after different wash types on day 0.

Standard deviation represents 1 standard deviation from the mean log CFU/g value. Lower-case letters and blue coloring denote significant differences between wash type within the same treatment type and media type ($p<0.05$). Capital letters denote significant differences between treatment type within the same wash type and media type ($p<0.05$). Absence of coloring denotes no significant differences were found.

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

Media Type	R2A	PIA	EMB	PIA T	R2A T	R2A CL	R2A CO	R2A V
Treatment Type								
Carrots dipped in dairy compost produced from manure not containing antibiotics and inoculated with MDR E.coli O157:H7 and Pseudomonas (DC-IN								
Unwashed	6.52 ±0.97^{Aa}	5.14 ±0.32^{Aa}	5.28 ±0.08^{Aa}	5.17 ±0.30^{Aa}	5.69 ±0.22 ^{Aa}	6.14 ±0.36^{Aa}	4.91 ±0.52^{Aa}	6.03 ±0.56^{Aa}
Tap Water	5.51±0.94^{Aa}	3.68 ±0.45^{Aab}	2.98 ±0.68^{Aab}	3.30 ±0.56^{Aab}	4.17 ±1.17^{Aa}	4.73 ±1.72^{Aa}	4.23 ±0.08^{Aa}	4.90 ±1.15^{Aa}
XY-12	7.29 ±1.23^{Aa}	5.66 ±0.33^{Ab}	5.87 ±0.15^{Abc}	5.64 ±0.28^{Abc}	6.25 ±0.26^{Aa}	6.52 ±0.48^{Aa}	5.28 ±0.61^{Aa}	6.30 ±0.36^{Aa}
Tsunami 100	6.16 ±0.66^{Aa}	3.92 ±0.40^{Ab}	3.98 ±0.05^{Ac}	3.94 ±0.12^{Ac}	4.48 ±0.12^{Aa}	5.01 ±0.86^{Aa}	4.32 ±0.02^{Aa}	5.30 ±0.92^{Aa}
Carrots dipped in dairy compost produced from manure from dairy cows receiving antibiotics and inoculated with MDR E.coli O157:H7 and Pseudom								
Unwashed	7.18 ±0.67^{Aa}	5.74 ±0.31^{Aa}	6.08 ±0.17^{Aa}	5.75 ±0.46^{Aa}	6.26 ±0.27^{Aa}	6.51 ±0.30^{Aa}	5.58 ±0.54^{Aa}	6.55 ±0.43^{Aa}
Tap Water	7.25 ±0.65^{Aa}	5.37 ±0.34^{Aa}	5.45 ±0.30^{Ab}	5.19 ±0.90^{Aa}	5.95 ±0.13^{Aa}	6.57 ±0.34^{Aa}	4.96 ±0.52^{Aab}	6.57 ±0.32^{Aa}
XY-12	6.92 ±1.10^{Aa}	4.45 ±0.32^{Ab}	4.17 ±0.20^{Ac}	4.13 ±0.52^{Ab}	4.70 ±0.35^{Ab}	6.06 ±0.53^{Aa}	4.55 ±0.29^{Abc}	5.94 ±0.65^{Aa}
Tsunami 100	7.17 ±0.37^{Aa}	4.23 ±0.89^{Ab}	3.55 ±0.17^{Ad}	3.55 ±0.49^{Ab}	4.19 ±0.59^{Ab}	6.04 ±0.25^{Aa}	4.20 ±0.31^{Ac}	6.10 ±0.16^{Aa}

Table 3.2.1: Comparison of non-inoculated versus inoculated antibiotic compost-dipped carrots (inoculation effect). Bacteria recovered (mean log CFU/g ± standard deviation) on different media types, after different wash types on day 0.

Standard deviation represents 1 standard deviation from the mean log CFU/g value. Capital letters denote significant differences between treatment type within the same wash type and media type ($p<0.05$). Lower-case letters and blue coloring denote significant differences between wash type within the same treatment type and media type ($p<0.05$). Absence of coloring denotes no significant differences were found.

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

Media Type	R2A	PIA	EMB	PIA T	R2A T	R2A CL	R2A CO	R2A V	R2A S
Treatment Type									
Carrots dipped in dairy compost produced from manure from dairy cows receiving antibiotics (DCAB-NON)									
Unwashed	7.36 ±1.47^{Aa}	5.19 ±0.32^{Aa}	0±0^{aB}	3.98 ±0.30^{aB}	4.55 ±1.13^{aB}	5.67 ±1.41^{Aa}	5.31 ±0.26^{Aa}	5.69 ±1.45^{Aa}	4.49 ±0.06^{aB}
Tap Water	7.29 ±1.88^{Aa}	4.56 ±0.37^{aB}	0±0^{aB}	3.57 ±0.38^{Aa}	4.85 ±1.26^{Aa}	5.54 ±1.21^{Aa}	4.86 ±0.35^{Aab}	6.29 ±1.55^{Aa}	3.89 ±0.19^{Bb}
XY-12	6.73 ±1.68^{Aa}	3.85 ±0.10^{Aa}	0±0^{aB}	2.30 ±2.00^{Aa}	4.23 ±1.19^{Aa}	5.40 ±0.88^{Aa}	4.32 ±0.02^{Abc}	6.08 ±1.42^{Aa}	3.81 ±0.33^{Ab}
Tsunami 100	7.05 ±2.02^{a}	2.66 ± 2.31^{Aa}	0±0^{aB}	0.92 ±1.60^{aB}	4.31 ±1.19^{Aa}	5.31 ±1.59^{Aa}	4.20 ±0.17^{Ac}	5.54 ±2.50^{Aa}	3.72 ±0.22^{Bb}
Carrots dipped in dairy compost produced from manure from dairy cows receiving antibiotics and inoculated with MDR E.coli O157:H7 and Pseudomonas (DCAB-INOC).									
Unwashed	7.18 ±0.67^{Aa}	5.74 ±0.31^{Aa}	6.08 ±0.17^{Aa}	5.75 ±0.46^{Aa}	6.26 ±0.27^{Aa}	6.51 ±0.30^{Aa}	5.58 ±0.54^{Aa}	6.55 ±0.43^{Aa}	6.13 ±0.18^{Aa}
Tap Water	7.25 ±0.65^{Aa}	5.37 ±0.34^{Aa}	5.45 ±0.30^{Ab}	5.19 ±0.90^{Aa}	5.95 ±0.13^{Aa}	6.57 ±0.34^{Aa}	4.96 ±0.52^{Aab}	6.57 ±0.32^{Aa}	5.50 ±0.28^{Ab}
XY-12	6.92 ±1.10^{Aa}	4.45 ±0.32^{Ab}	4.17 ±0.20^{Ac}	4.13 ±0.52^{Ab}	4.70 ±0.35^{Ab}	6.06 ±0.53^{Aa}	4.55 ±0.29^{Abc}	5.94 ±0.65^{Aa}	4.36 ±0.32^{Ac}
Tsunami 100	7.17 ±0.37^{Aa}	4.23 ±0.89^{Ab}	3.55 ±0.17^{Ad}	3.55 ±0.49^{Ab}	4.19 ±0.59^{Ab}	6.04 ±0.25^{Aa}	4.20 ±0.31^{Ac}	6.10 ±0.16^{Aa}	3.89 ±0.20^{Ad}

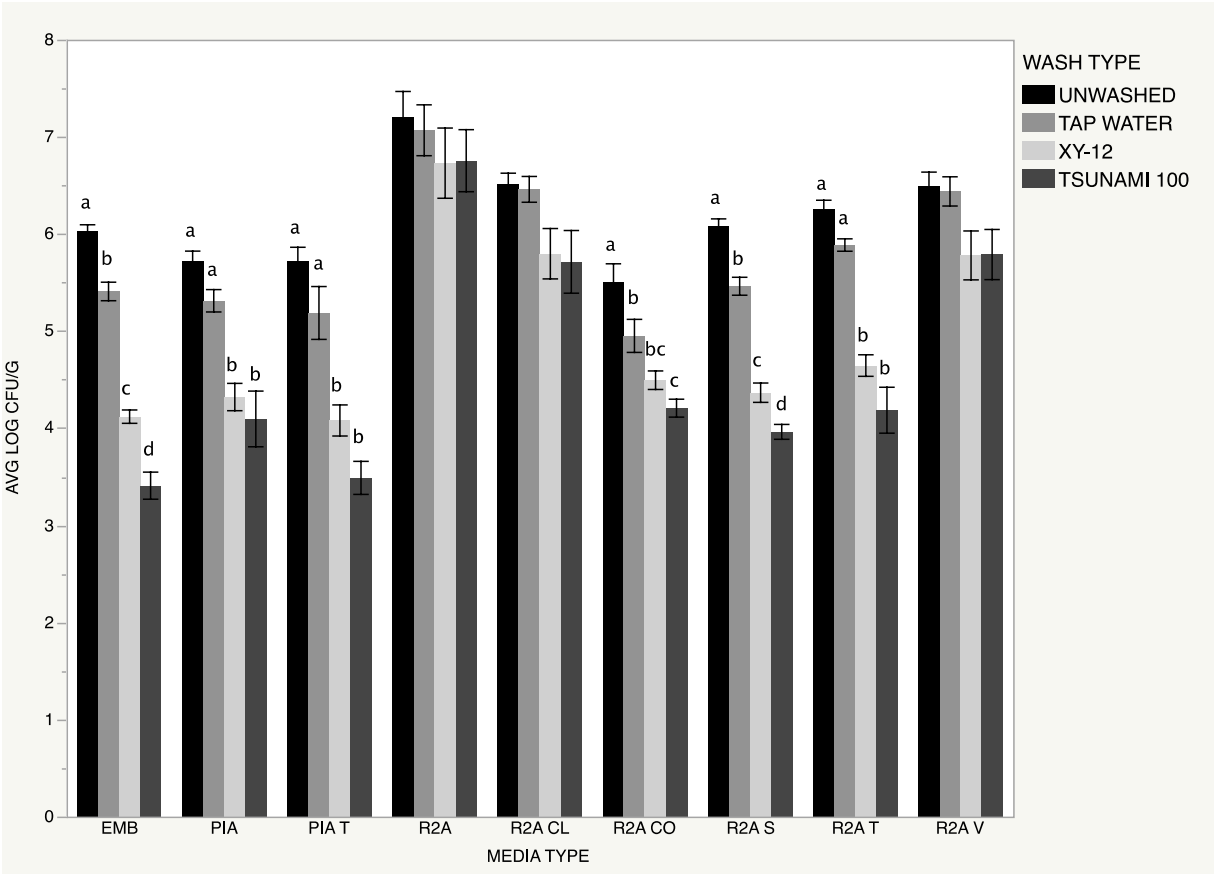

Figure 3.2.1: Effect of sanitizer wash on recovery of inoculated ABR bacteria, *E. coli* O157:H7 and *P. aeruginosa*, and native antibiotic resistant bacteria immediately after washing carrots dipped in inoculated compost (includes both dairy antibiotic and dairy control compost types) on different media types (n=8).

Error bars represent 1 standard error from the mean. Letters denote significant differences between wash types within media type ($p<0.05$). Absence of lettering denotes no significant differences.

EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

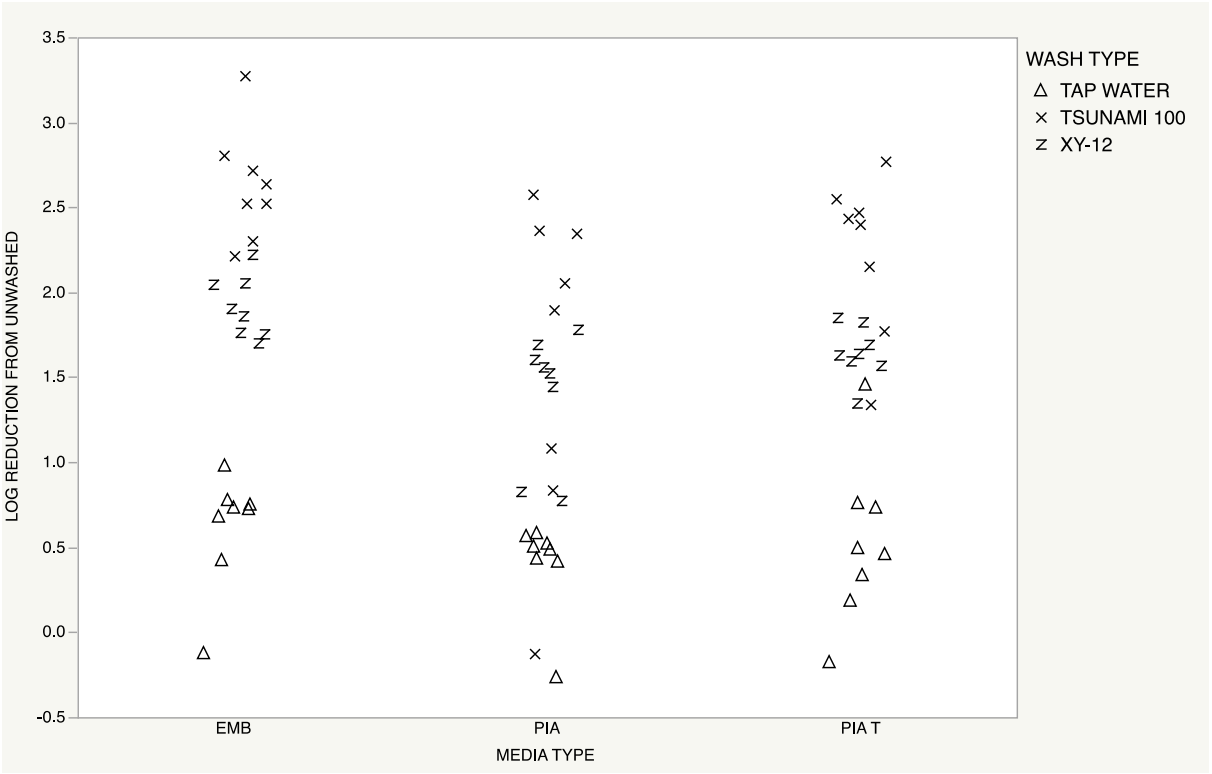

Figure 3.2.2: Log Reduction (relative to unwashed day 0 samples) of *E. coli* O157:H7 inoculum on EMB and *Pseudomonas aeruginosa* on PIA and PIA T recovered from carrots dipped in antibiotic dairy compost with inoculum based on wash type on day 0 (n=8).

EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline).

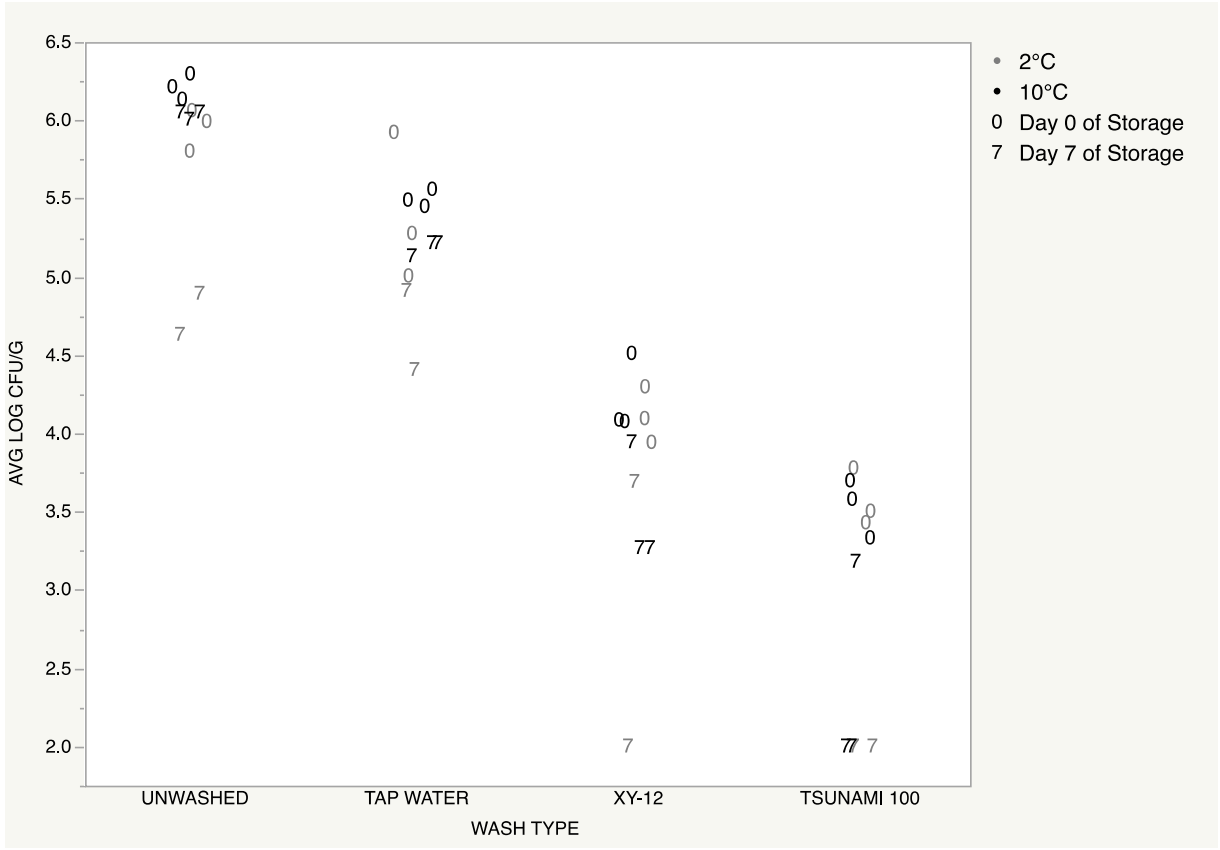

Figure 3.3.1: Comparison of the average log CFU/g of *E. coli* O157:H7 recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

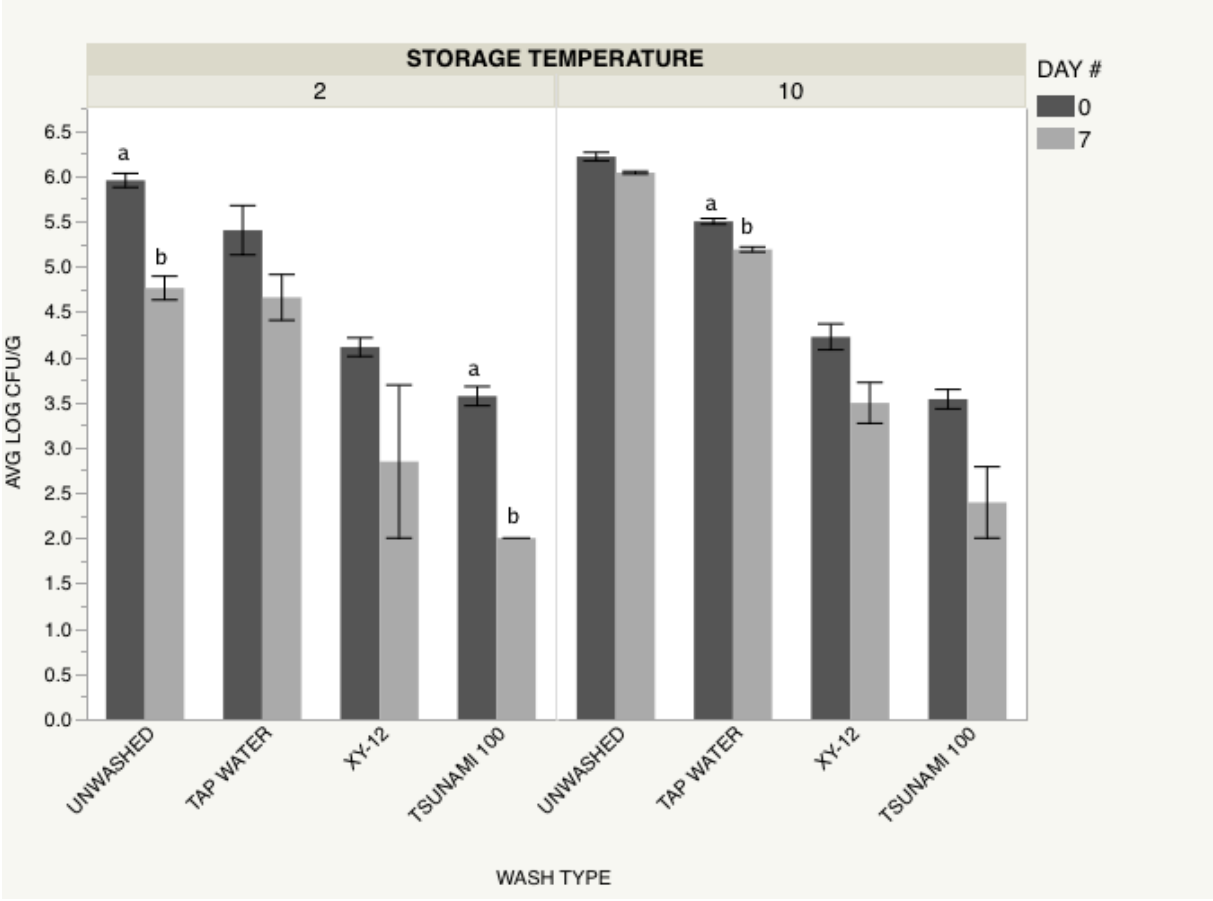

Figure 3.3.2: Comparison of the average log CFU/g of *E. coli* O157:H7 recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

Error bars represent 1 standard error from the mean.

Lower-case letters denote significant differences between day within wash type and storage temperature ($p<0.05$).

Absence of capital letters denote there were no significant differences between storage temperature within the same wash type and day ($p<0.05$). Absences of any letters denote there were no significant differences between storage temperature or day of storage within wash type.

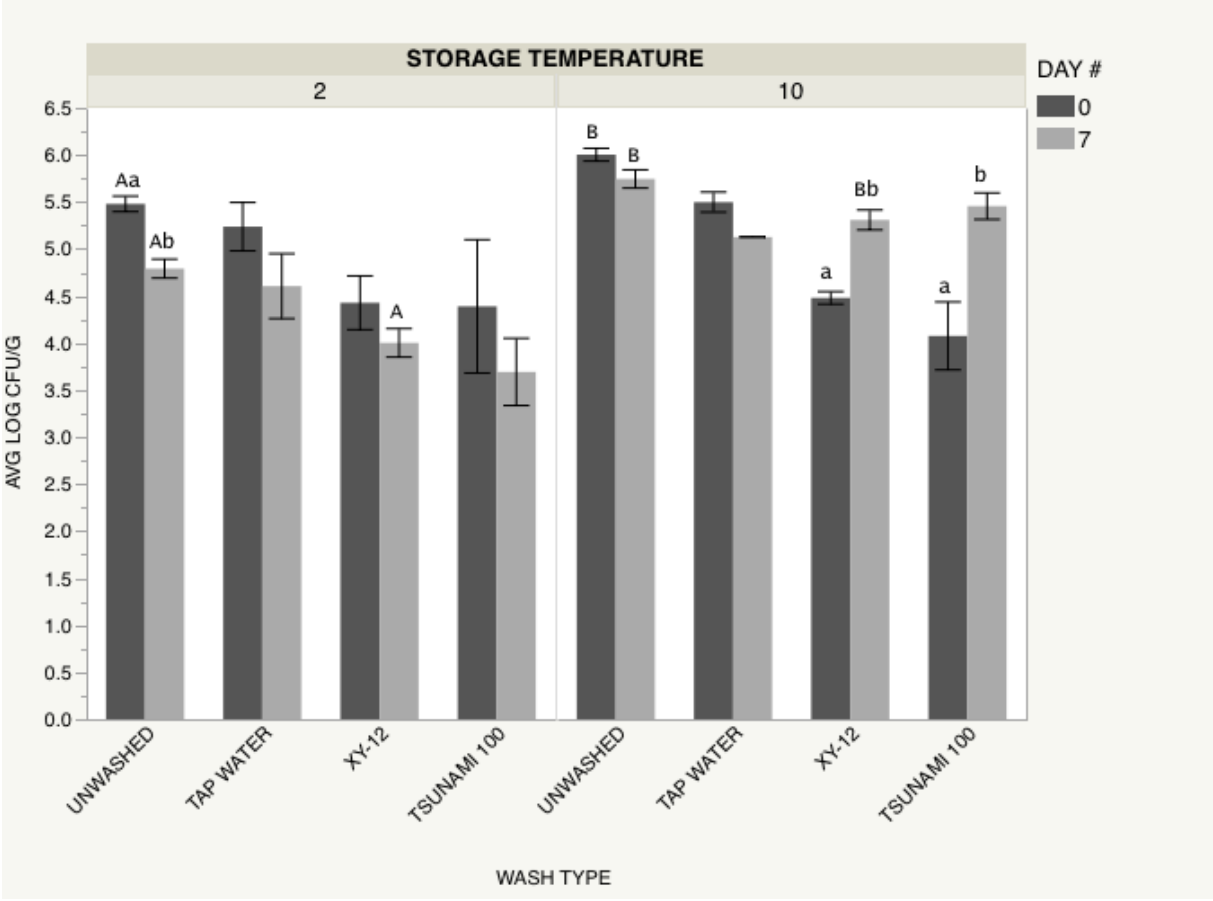

Figure 3.3.3: Comparison of the average log CFU/g of *Pseudomonas* sp. recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

Error bars represent 1 standard error from the mean.

Capital letters denote significant differences between storage temperature within the same wash type and day ($p<0.05$). Lower-case letters denote significant differences between day within wash type and storage temperature ($p<0.05$).

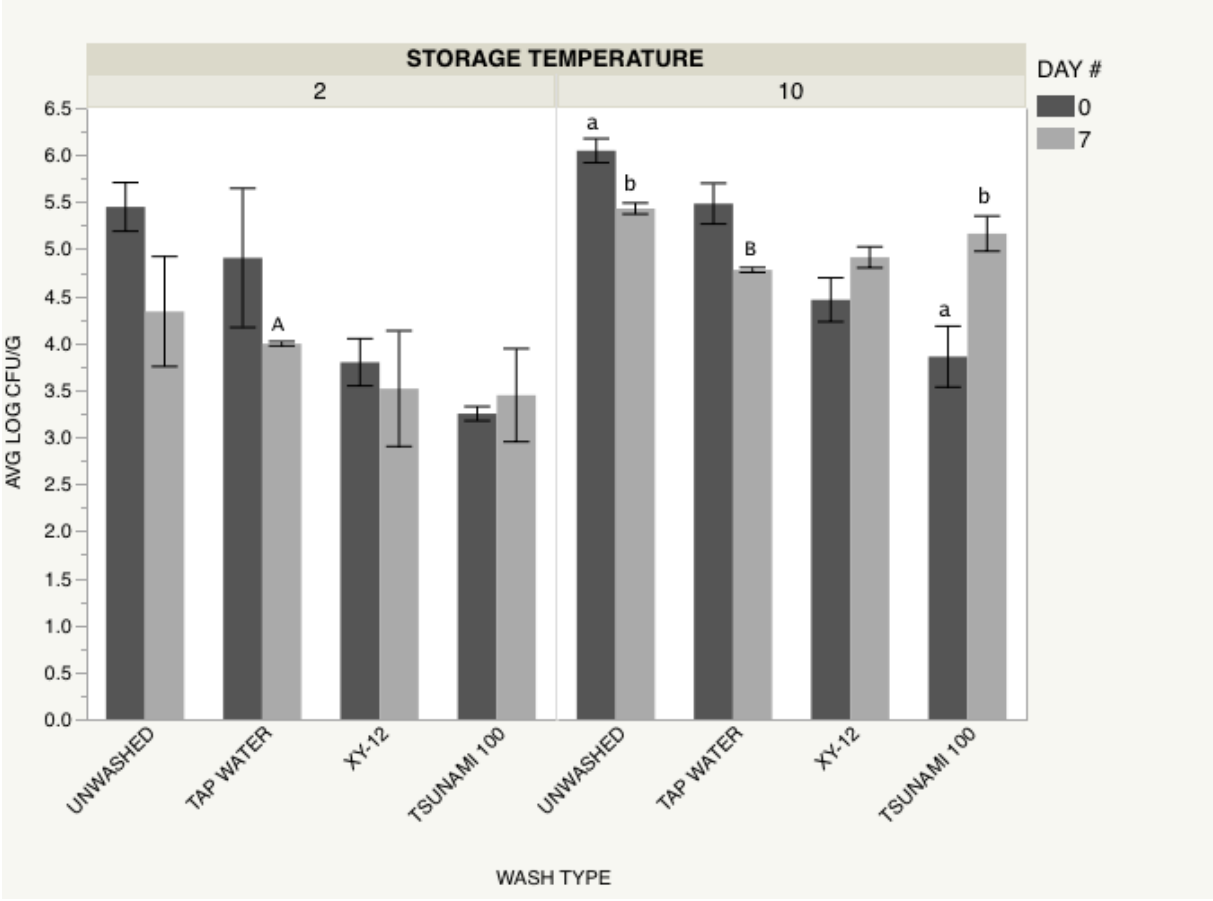

Figure 3.3.4: Comparison of the average log CFU/g of *Pseudomonas* sp. resistant to tetracycline (4µg/mL) recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

Error bars represent 1 standard error from the mean.

Capital letters denote significant differences between storage temperature within the same wash type and day ($p<0.05$). Lower-case letters denote significant differences between day within wash type and storage temperature ($p<0.05$).

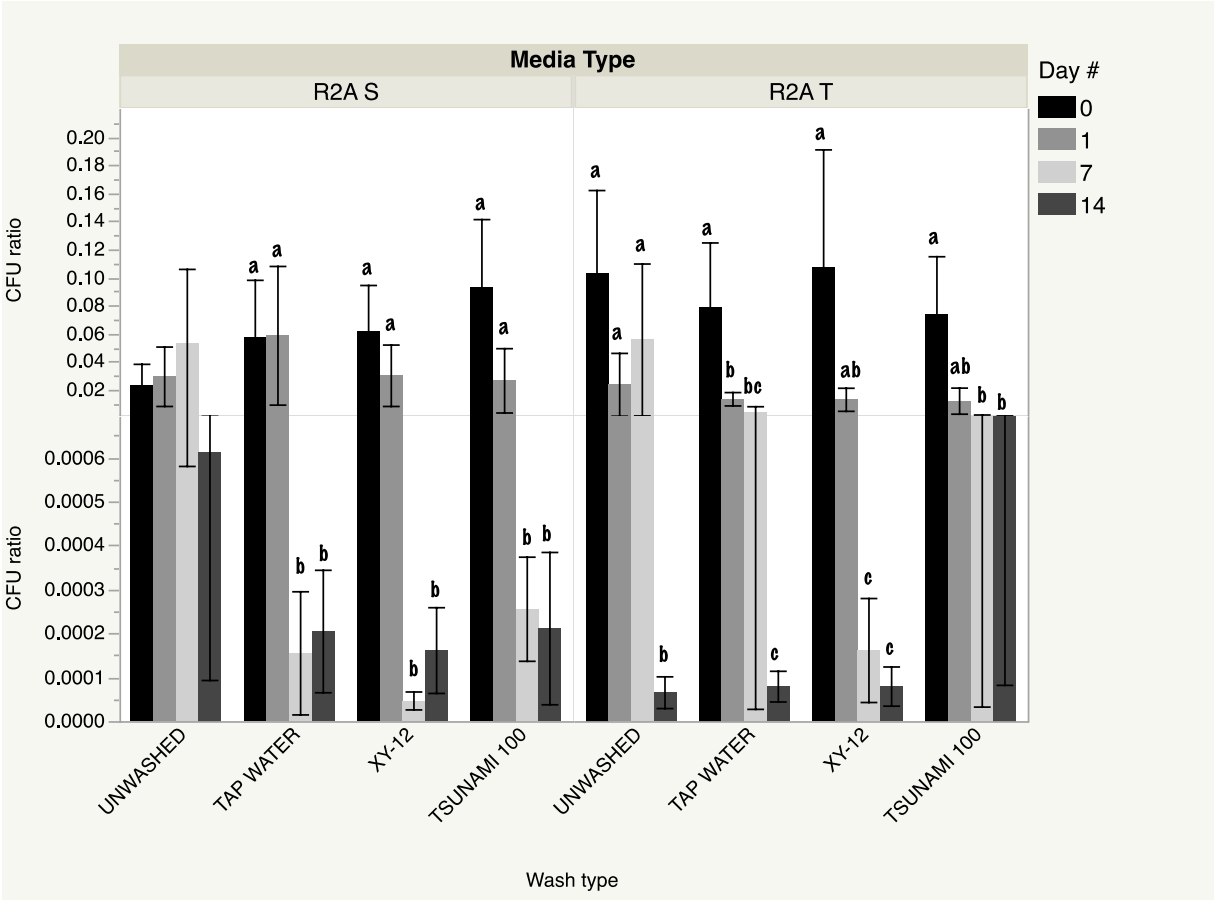

Figure 3.4.1: Length of storage changes the proportion of sulfamethoxazole-resistant and tetracycline-resistant bacterial populations with respect to total aerobic bacterial populations under optimal storage temperatures (2°C).

Note the split axis.

Error bars represent 1 standard error from the mean.

Lower-case letters denote significant differences between the day of storage within the same wash type and media type ($p<0.05$). Absences of lettering denote no significant differences.

Media types: R2A S=R2A supplemented with sulfamethoxazole (100µL/mL). R2A T= R2A supplemented with tetracycline (3µL/mL).

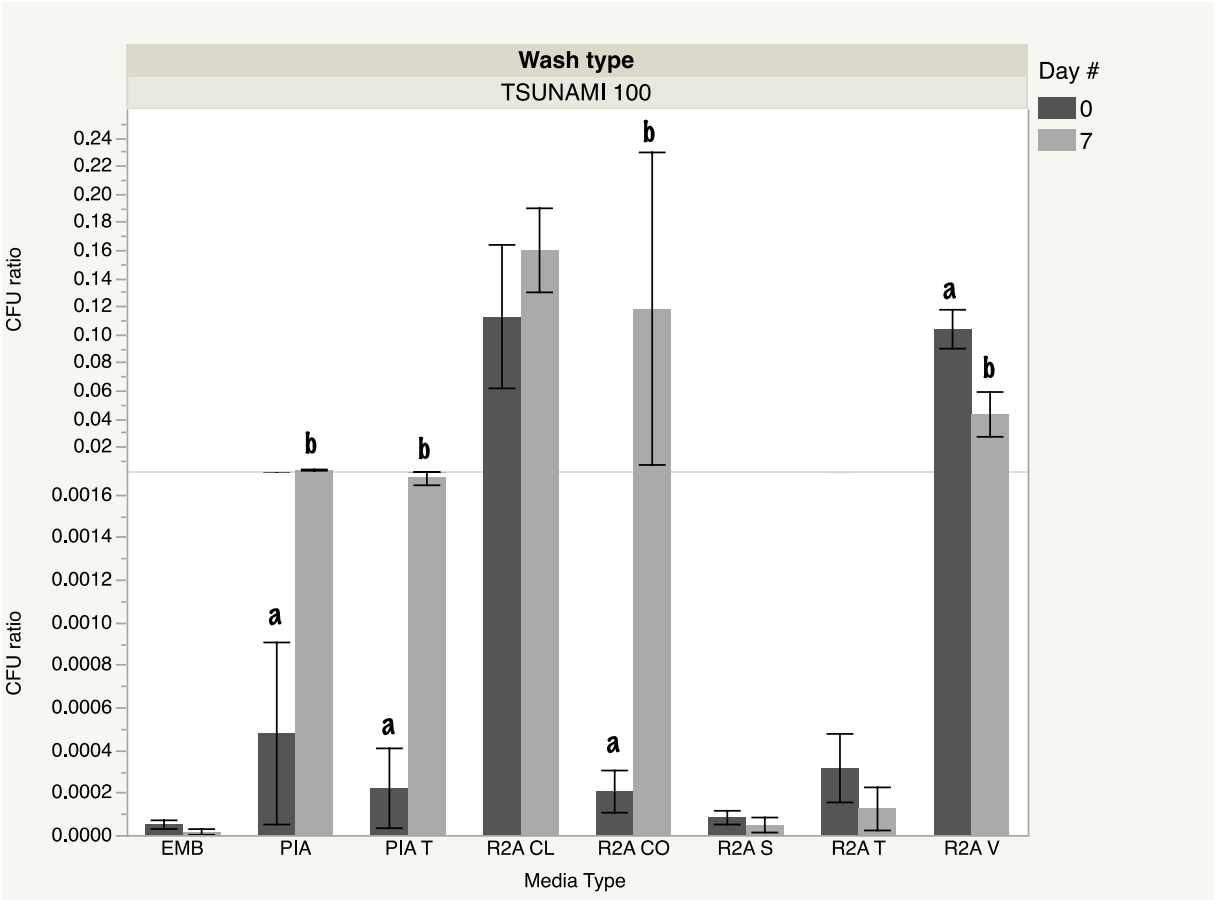

Figure 3.5.1: Tsunami 100 alters proportional *Pseudomonas* and antibiotic-resistant bacterial populations with respect to total aerobic bacterial populations under temperature abuse-conditions (10°C).

Note the split axis.

Error bars represent 1 standard error from the mean.

Lower-case letters denote significant differences between days within the same media type ($p<0.05$).

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

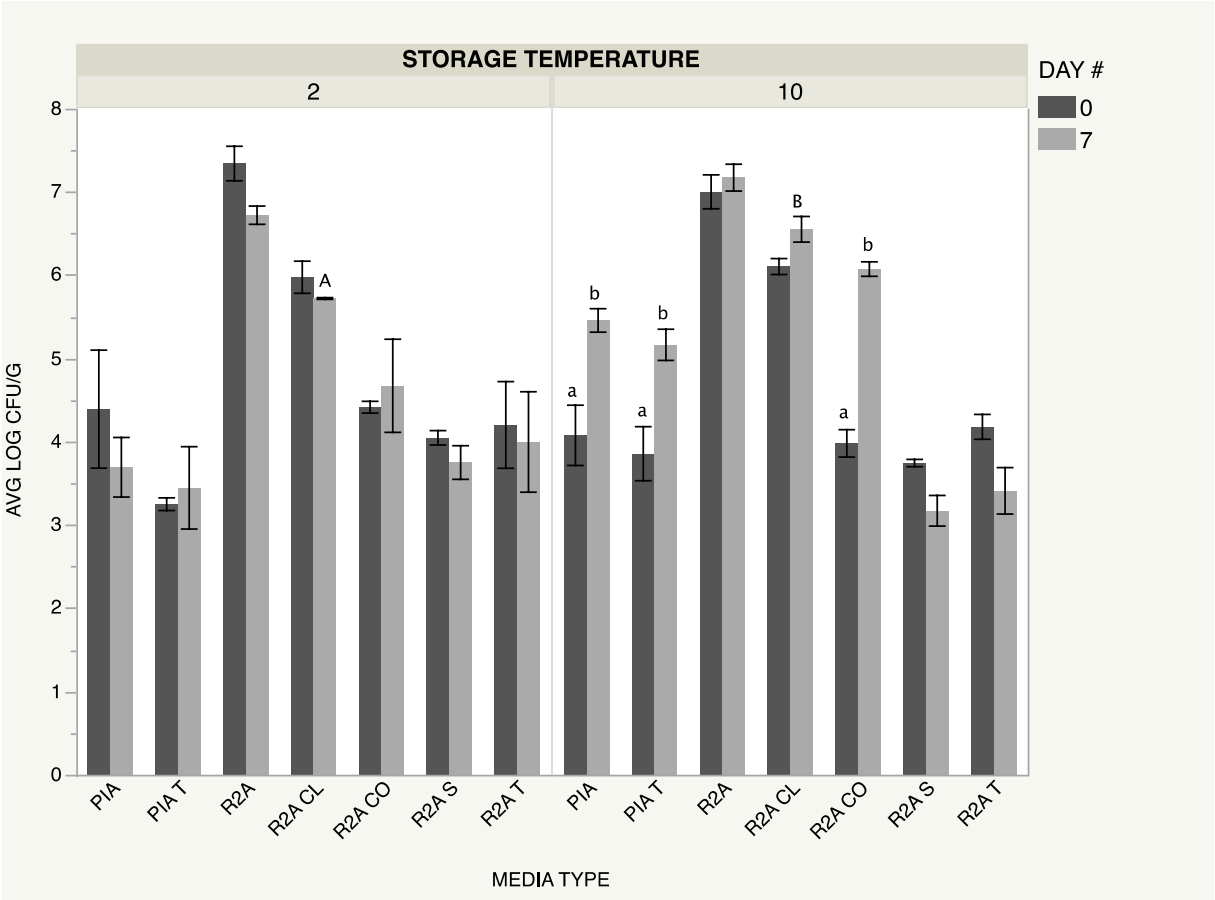

Figure 3.5.2: Comparison of the average log CFU/g recovered from Tsunami 100 (peroxyacetic acid/hydrogen peroxide) washed carrots dipped in antibiotic dairy compost with inoculum on different media types after different washes on Day 0 and Day 7 under both optimal temperature (2°C) and temperature-abuse conditions (10°C) (n=3).

Error bars represent 1 standard error from the mean.

Capital letters denote significant differences between storage temperatures on the same day (Day 7) of storage within the same media type ($p<0.05$). Lower-case letters denote significant differences between days within a set storage temperature and media type ($p<0.05$).

R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

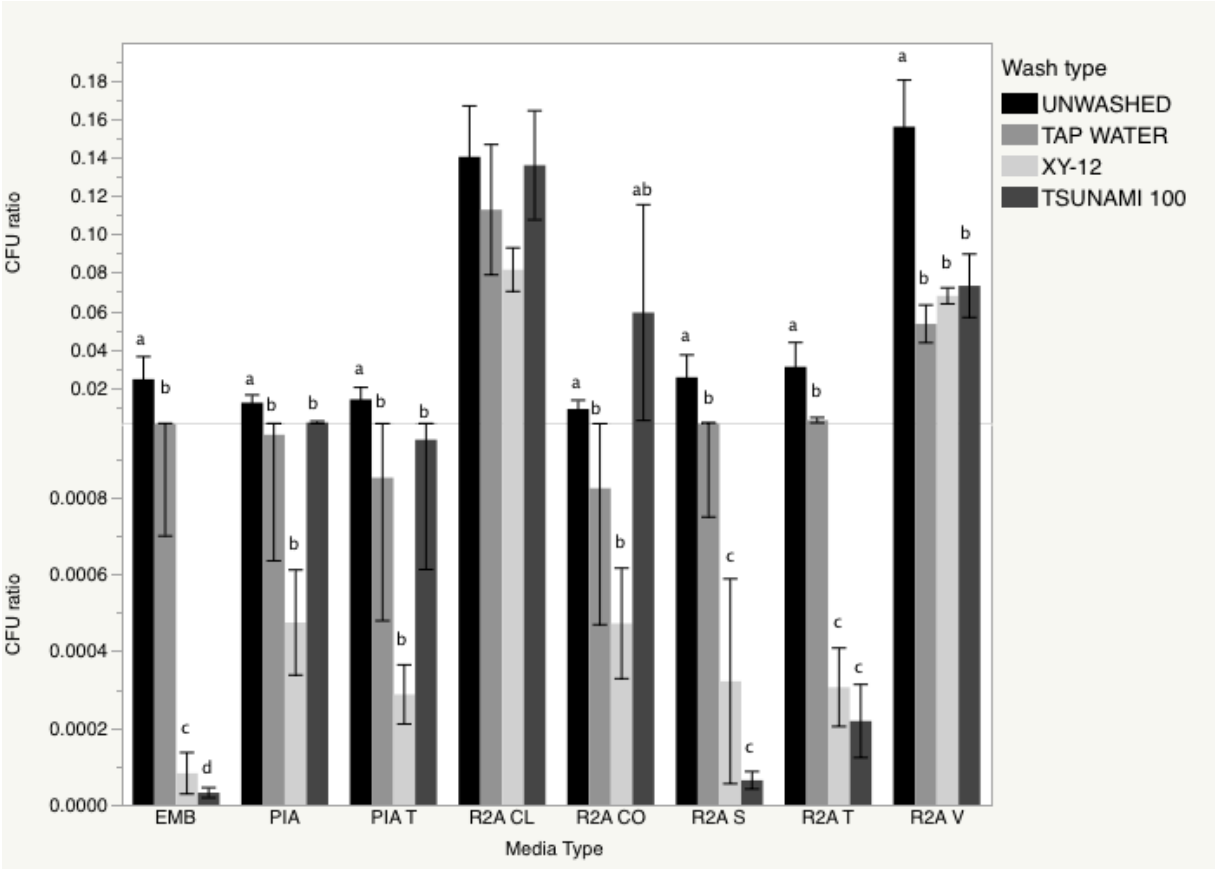

Figure 3.6.1: Wash type alters proportional *E. coli* and antibiotic-resistant bacterial populations with respect to total aerobic bacterial populations under temperature abuse-conditions (10°C).

Note the split axis.

Error bars represent 1 standard error from the mean.

Lower-case letters denote significant differences between wash types within the same media type ($p<0.05$).

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

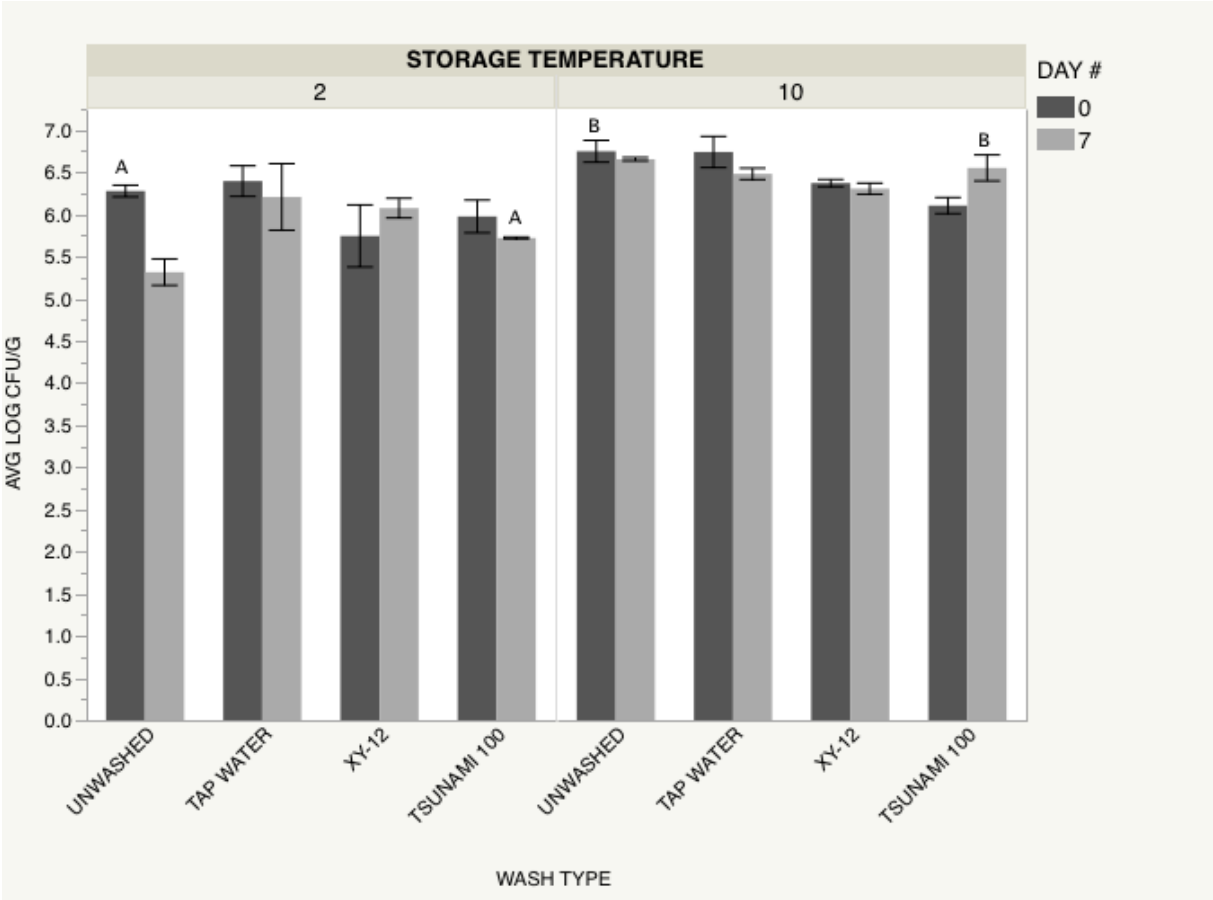

Figure 3.7.1: Comparison of the average log CFU/g bacteria resistant to clindamycin (25µg/mL) recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

Error bars represent 1 standard error from the mean.

Capital letters denote significant differences between storage temperature within the same wash type and day ($p<0.05$). Absences of lower-case letters denote there were no significant differences between day within wash type and storage temperature ($p<0.05$).

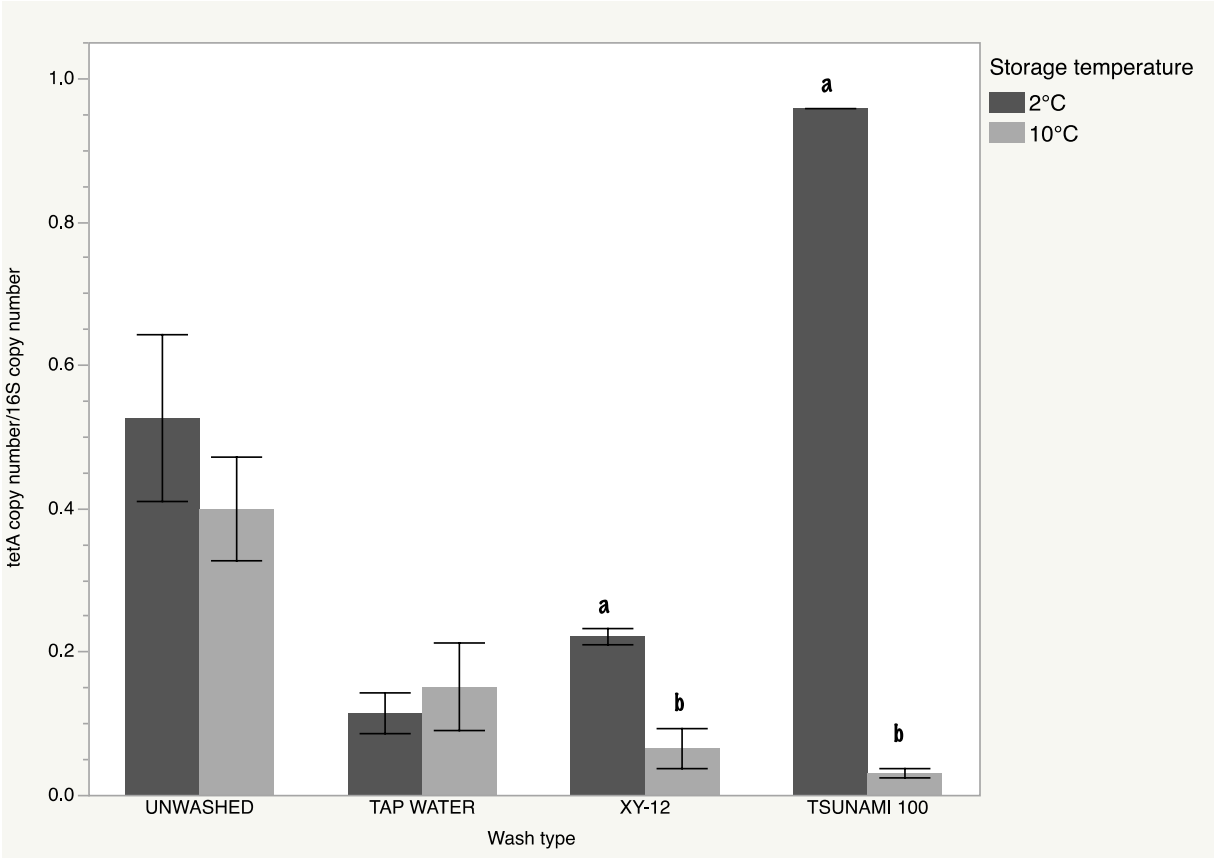

Figure 3.8.1 Effect of storage temperature and wash type on carrots dipped in inoculated dairy antibiotic compost after 7 days of storage on *tet*A copy number/16S rRNA copy number.

Error bars represent 1 standard error from the mean.

Lower-case letters denote significant differences between storage temperatures on day 7 of storage within the same wash type ($p<0.05$).

Absence of lettering denotes no significant differences.

CHAPTER 4: CONCLUSIONS AND FUTURE RESEARCH

The purpose of this research was to determine the effect of post-harvest interventions, specifically the use of chemical sanitizers, on the survival and re-growth of antibiotic-resistant bacteria. As part of this study inoculated strains of multi-drug resistant bacteria were included; *E. coli* O157:H7 to determine effectiveness of the sanitizers against a pathogen that is a possible contaminant of vegetables, and *Pseudomonas aeruginosa* because *Pseudomonads* are very prevalent in the plant phyllosphere and have a high capacity for mutation and horizontal gene transfer. As part of this study the effect of antibiotic administration during manure collection that is subsequently used for composting was evaluated for its effect on prevalence of antibiotic-resistant bacteria in conjunction with sanitizer washes. In addition, prevalence of the *tet*A gene was looked at in inoculated samples to determine if washing with sanitizers changed the prevalence of this antibiotic-resistant gene using qPCR technology.

The research concluded that the use of chemical sanitizers significantly reduced populations of our inoculated strains of *E. coli* O157:H7 and *Pseudomonas aeruginosa* in both the dairy control compost and the dairy antibiotic compost ($p<0.05$). Sanitizer washes on non-inoculated dairy antibiotic dipped carrots resulted in significant changes in bacteria resistant to cefotaxime (10μg/mL) and bacteria resistant to sulfamethoxazole (100μg/mL) based on wash type ($p<0.05$). In contrast, there were no significant differences based on wash type in non-inoculated dairy control compost. Carrots dipped in inoculated dairy control compost and subsequently washed in chemical sanitizers demonstrated significant changes in inoculated *E. coli* and *Pseudomonas* sp. as expected, as well as a change in bacteria resistant to sulfamethoxazole (100μg/mL) based on wash type alone ($p<0.05$). Similarly, the carrots dipped in inoculated dairy antibiotic compost showed similar significant changes in those same three

bacterial populations, although there were also significant changes in cefotaxime-resistant bacteria (10μg/mL), and total aerobic bacteria resistant to tetracycline (3μg/mL) based on wash type alone ($p<0.05$). These significant differences seen in cefotaxime-resistant bacteria (10μg/mL) based on wash type alone is only seen in dairy antibiotic compost dipped carrots not dairy control compost, whether they are inoculated or not. This may be due to the fact that one of the antibiotics administered to the dairy cattle was a 1st generation cephalosporin (cephapirin) and cefotaxime is a 3rd generation cephalosporin used in human medicine. This close relation of the two antibiotics by antibiotic class may be a factor in the cefotaxime-resistant (10μg/mL) bacterial population changes based on wash type in the dairy antibiotic compost.

There was also an interaction between compost type and wash type. Carrots dipped in inoculated dairy antibiotic compost showed changes in bacteria resistant to sulfamethoxazole (100μg/mL) and inoculated *E. coli* bacteria based on wash type compared to those dipped in a inoculated dairy control compost. There was less reduction in these bacteria on carrots dipped in inoculated dairy antibiotic compost when washed in tap water, XY-12, or Tsunami 100 compared to dairy control compost-dipped carrots. In addition, wash type was always significant in dairy antibiotic compost-dipped carrots, while it was only significant in a few cases in dairy control compost. Thus, washing with sanitizers can help reduce both bacterial pathogens and antibiotic-resistant bacteria compared to unwashed carrots or carrots washed in tap water. Carrots dipped in either non-inoculated compost showed native bacteria resistance to the different antibiotics to be in the range of 4-5.5 log CFU/g, however total aerobic bacteria populations from the different compost types were not different.

With respect to wash type, Tsunami 100 demonstrated the most significant differences in antibiotic-resistant bacterial populations immediately post-wash and also during the re-growth

stage at 10°C storage. In general, the use of sanitizers resulted in immediate decreases in antibiotic-resistant bacterial populations, and inoculated *Pseudomonas* sp. and pathogenic *E. coli* O157:H7. When the washed carrots were stored under optimal temperature conditions (2°C) no significant re-growth occurred in any bacterial populations tested, including pathogenic *E. coli*, indicating the importance of proper temperature control during storage. This emphasizes the importance of temperature-controlled storage. When carrots were stored under temperature-abuse conditions (10°C) there was still no significant re-growth of inoculated pathogenic *E. coli* O157:H7, indicating that use of chemical sanitizers are effective at reducing initial populations of pathogenic *E. coli* and that even under temperature-abused conditions re-growth of that pathogen does not occur. With respect to wash type, the only wash that resulted in re-growth changes of bacterial populations at 10°C was Tsunami 100. Carrots dipped in inoculated dairy antibiotic compost washed with Tsunami 100 and stored at 10°C demonstrated a significant increase in bacteria resistant to clindamycin at day 7 of storage compared to carrots stored at 2°C ($p<0.05$). Carrots washed with Tsunami 100 and stored at 10°C had significant increases in *Pseudomonas* sp, *Pseudomonas* sp. resistant to tetracycline (4μg/mL), and bacteria resistant to cefotaxime (10μg/mL) on day 7 of storage compared to the day they were initially washed ($p<0.05$).

With respect to *tet*A prevalence, unwashed samples demonstrated the highest amount of *tet*A with respect to 16S, and had significantly greater numbers of *tet*A genes than carrots washed with Tsunami 100 ($p<0.05$) on the initial wash day. After 7 days of storage at 2°C unwashed carrot samples had significantly greater amounts of *tet*A compared to carrots washed in tap water or XY-12 and stored under the same conditions ($p<0.05$). However, when carrots were stored at 10°C for 7 days carrots washed in tap water had significantly greater numbers of

*tet*A compared to those washed in Tsunami 100, but was not significantly greater than carrots washed in XY-12 ($p<0.05$). No significant correlations between normalized *tet*A log ratios and average log CFU/g of bacteria recovered on PIA supplemented with tetracycline were not found ($p<0.05$).

Future research regarding antibiotic-resistant bacteria, manure sources for compost, and sanitizer washes should grow the vegetables in the compost, rather than dip the carrots in compost slurry to better mimic real-world conditions. This will better illuminate how growing carrots or vegetables in compost generated from cows administered antibiotics during manure collection affects antibiotic-resistant bacteria. In addition, enumeration of antibiotic-resistant bacteria from carrots alone, if not grown in the compost, should be performed to be able to better distinguish the source of antibiotic-resistant bacteria, so it can be noted if the bacteria are from the carrots or the compost. Other studies should look at the internalization of antibiotic-resistant bacteria, rather than surface bacteria only to better quantify the entire carrot that will be ingested.

Future studies should also address microbial communities via metagenomic analysis in order to better understand which bacteria are present and which bacterial genera are most responsible for the antibiotic-resistance. Beyond this, more quantification of antibiotic-resistance genes using qPCR should be performed. Transfer experiments should also be conducted in order to better determine the transfer of genes in processing wash water between bacterium. In general, more replicates should be performed to account for different prevalence of antibiotic-resistance on grocery-store carrots and increase statistical power. It would be interesting to expand this study to other vegetables, especially vegetables with different textures and surfaces, as well as vegetables that grow differently (ex: root vegetables versus vegetables that grow above-ground).

Beyond this, the prevalence of antibiotic-resistant bacteria could be looked at in conjunction with other post-harvest treatments, such as irradiation.

SUPPLEMENTARY FIGURES AND APPENDICES

List of Supplementary Figures and Tables

List of Appendices

SUPPLEMENTARY FIGURES

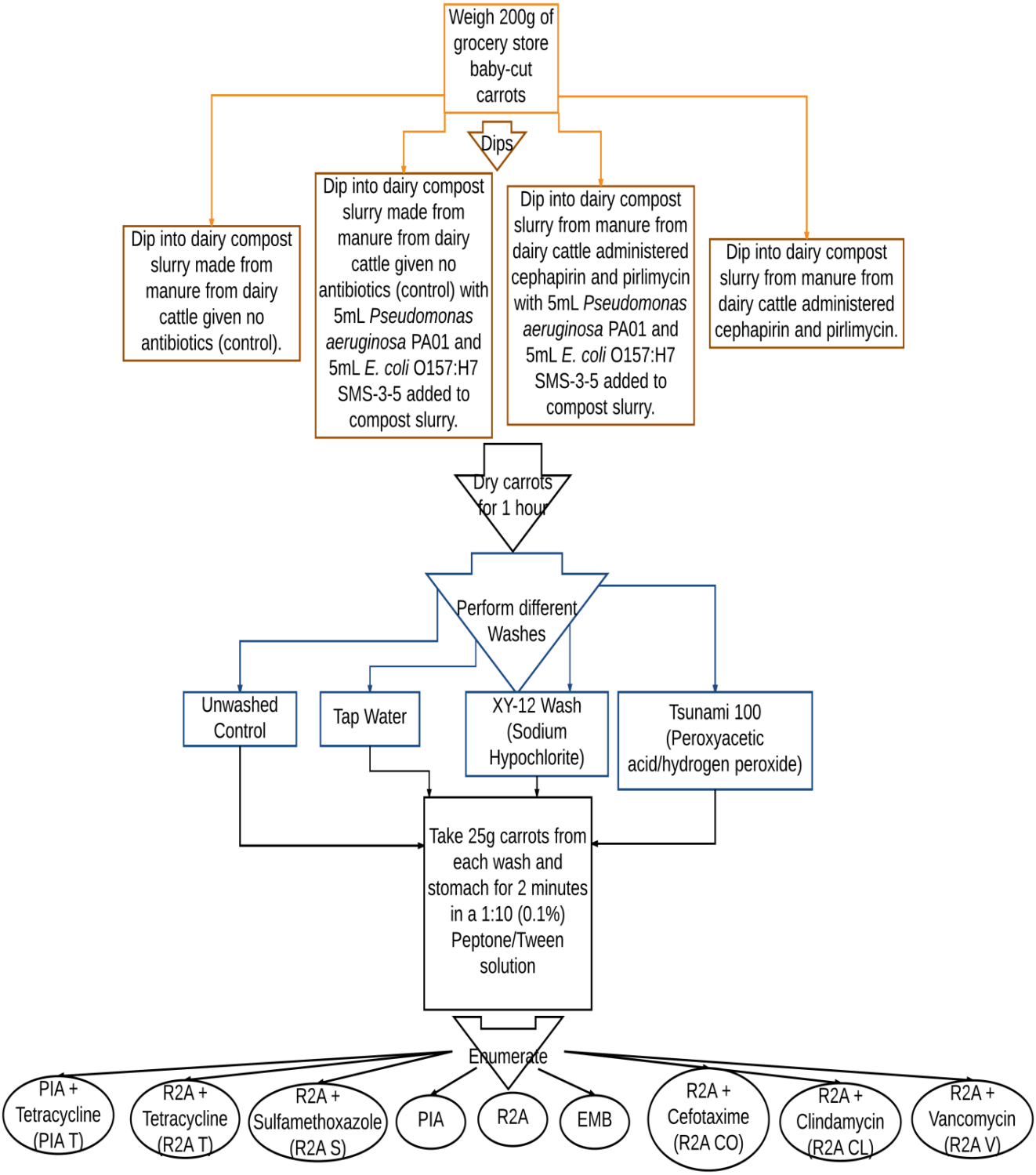

Figure S1. Methods Diagram.

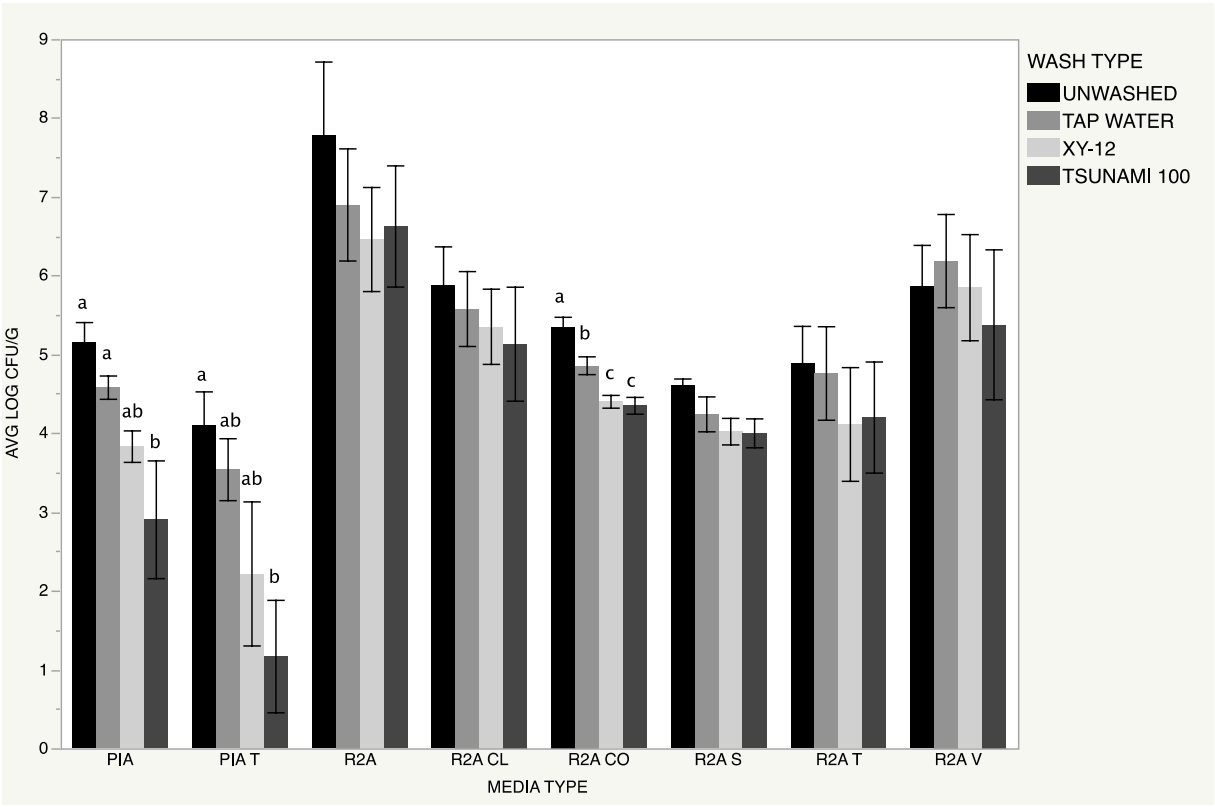

Figure S2: Effect of sanitizer wash on recovery of antibiotic resistant bacteria immediately after washing carrots dipped in non-inoculated compost (includes both dairy antibiotic and dairy control compost types) on different media types (n=5).

Error bars represent 1 standard error from the mean.

Letters denote significant differences between wash types within media type ($p<0.05$). Absence of letters within media type indicates no statistical difference based on wash type ($p>0.05$).

Media types: PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

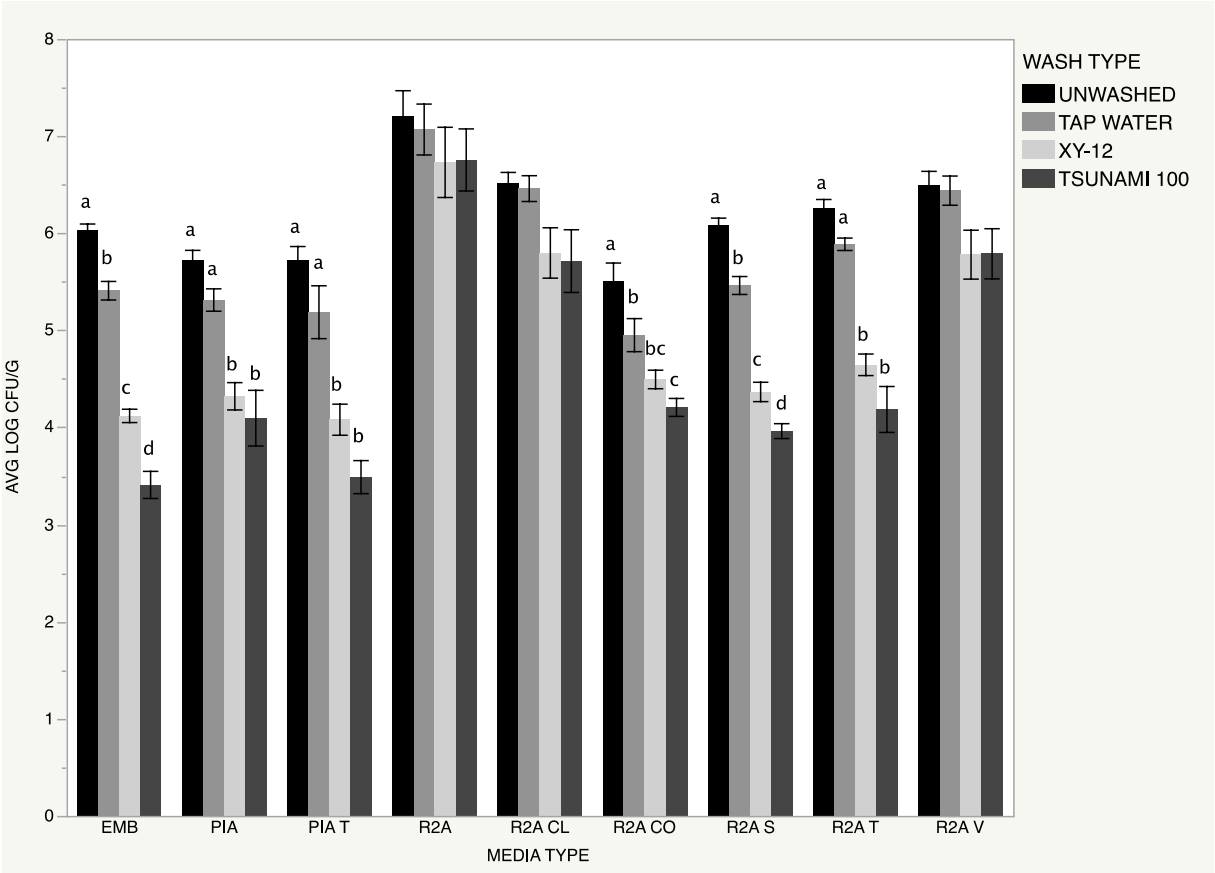

Figure S3: Effect of sanitizer wash on recovery of inoculated ABR bacteria, *E. coli* O157:H7 and *P. aeruginosa*, and native antibiotic resistant bacteria immediately after washing carrots dipped in inoculated compost (includes both dairy antibiotic and dairy control compost types) on different media types (n=4).

Error bars represent 1 standard error from the mean.

Letters denote significant differences between wash types within media type ($p<0.05$).

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

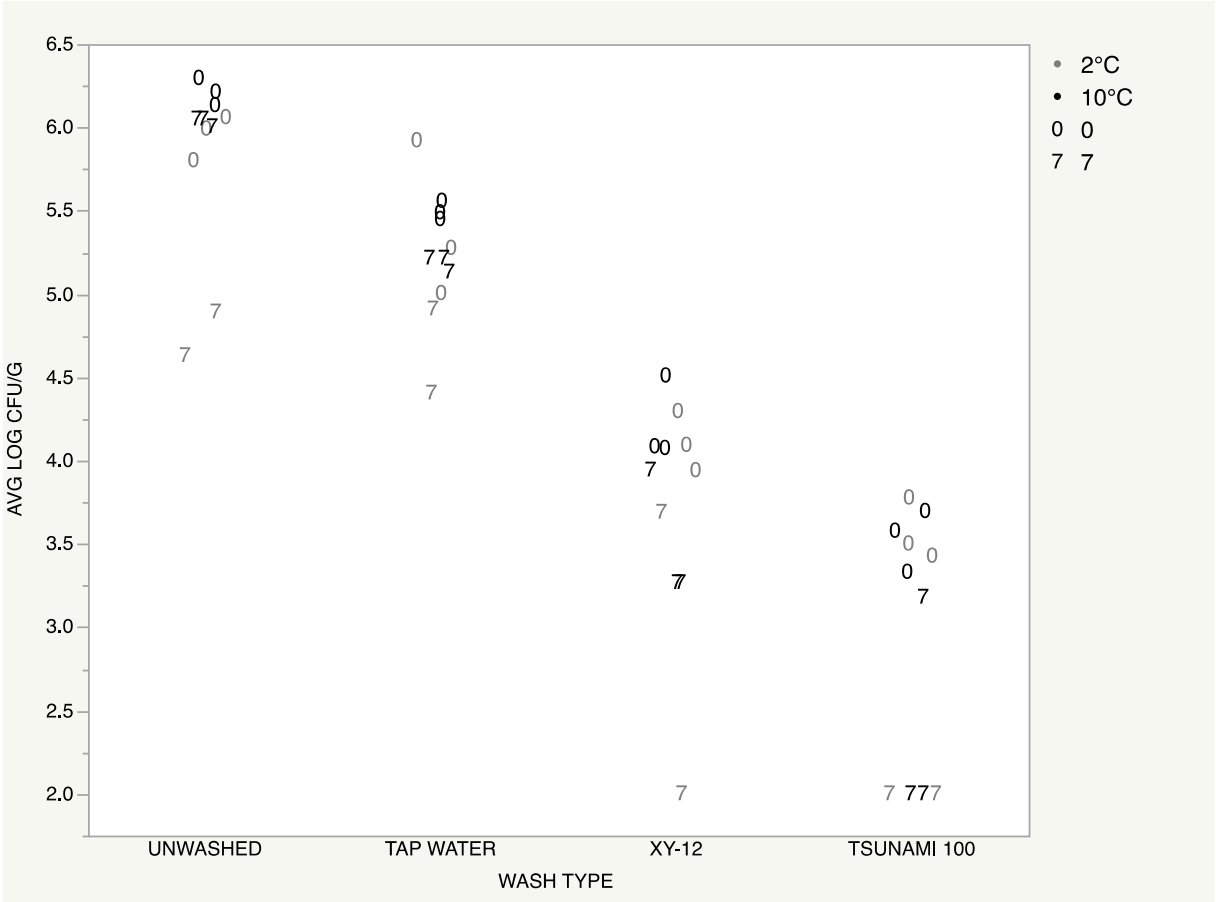

Figure S4: Comparison of the average log CFU/g of *E. coli* O157:H7 recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

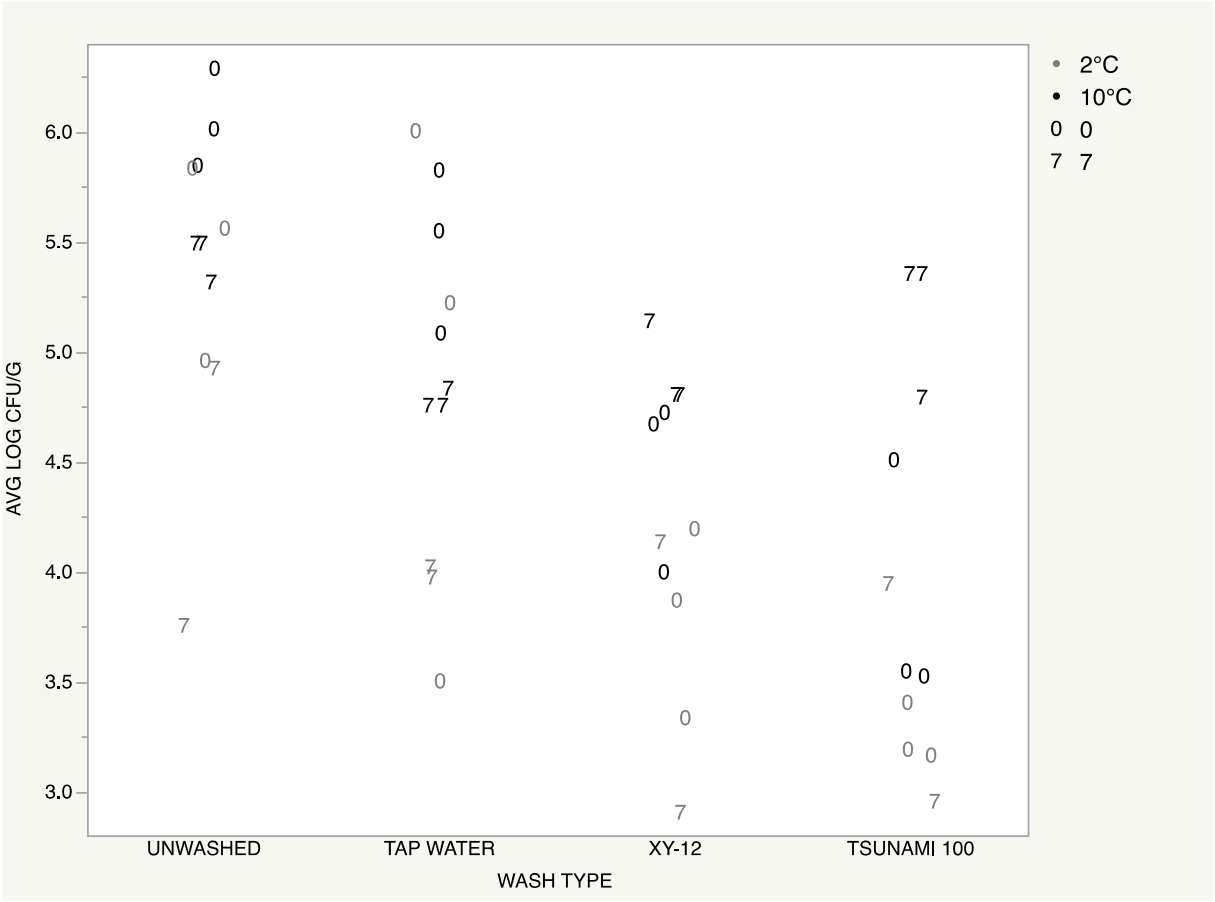

Figure S5: Comparison of the average log CFU/g of *Pseudomonas* sp. resistant to tetracycline (4µg/mL) recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

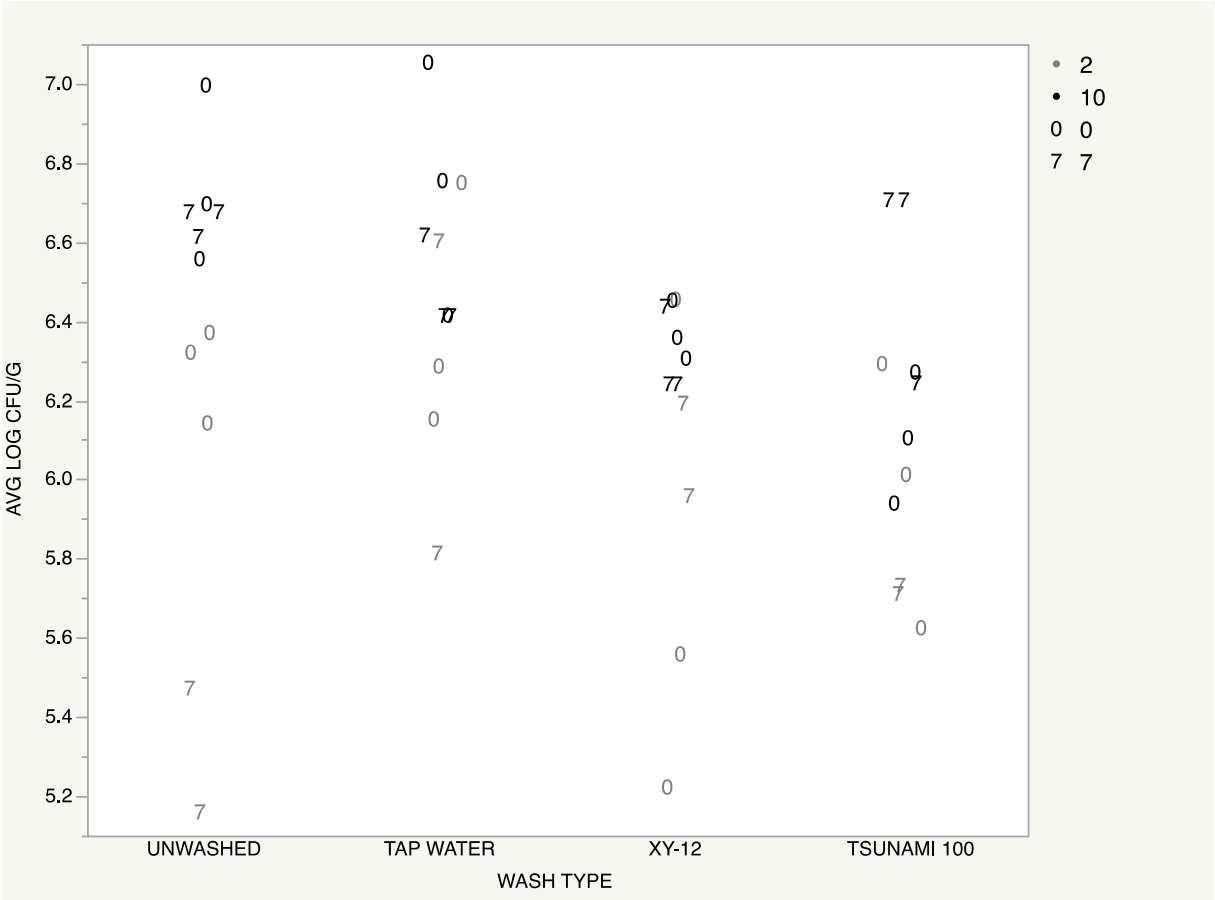

Figure S6: Comparison of the average log CFU/g of total aerobic bacteria resistant to clindamycin (25µg/mL) recovered from inoculated dairy antibiotic compost-dipped carrots at Day 0 and Day 7 of storage under both optimal temperature (2°C) and temperature-abuse conditions (10°C).

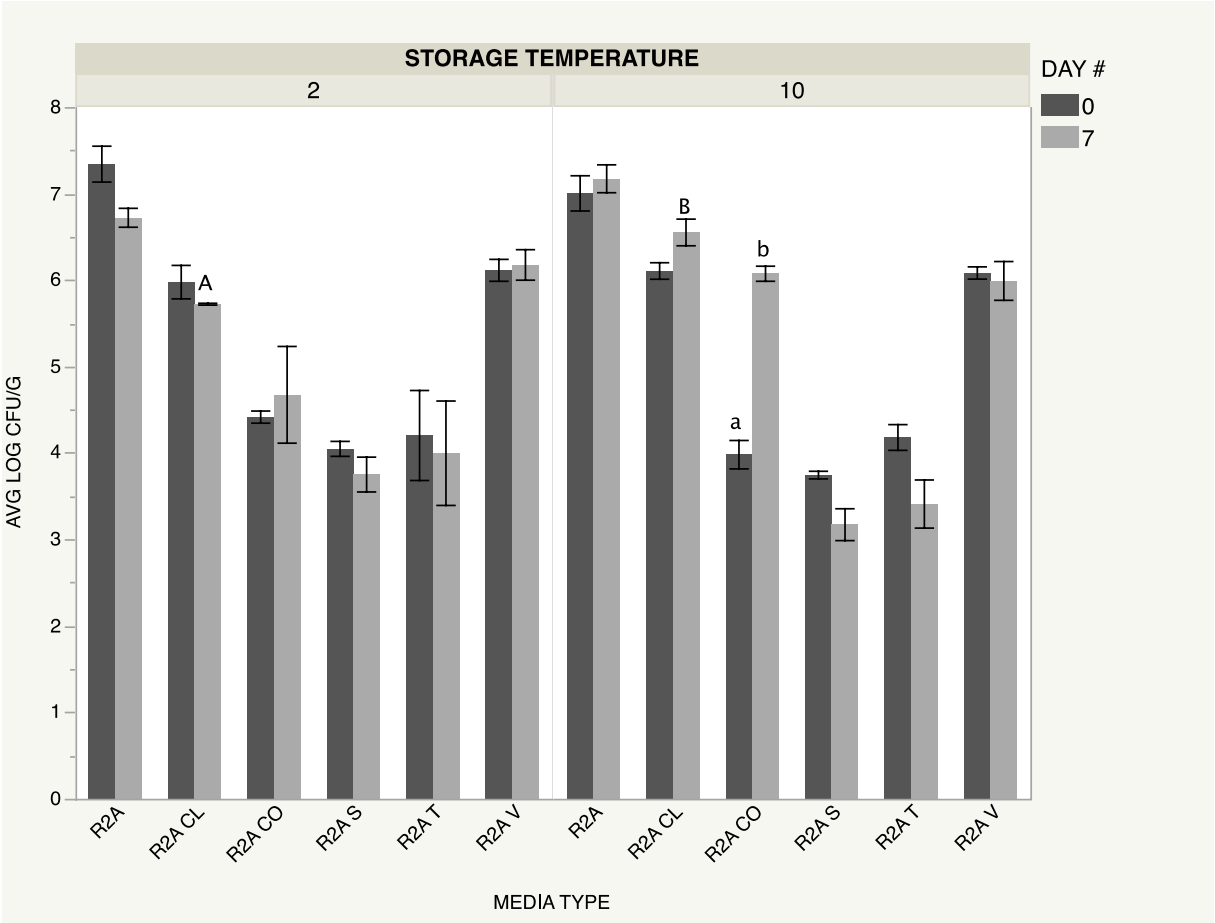

Figure S7: Comparison of the average log CFU/g recovered from Tsunami 100 (peroxyacetic acid/hydrogen peroxide) washed carrots dipped in antibiotic dairy compost with inoculum on different media types after different washes on Day 0 and Day 7 under both optimal temperature (2°C) and temperature-abuse conditions (10°C) (n=3).

Error bars represent 1 standard error from the mean.

Capital letters denote significant differences between days within a set storage temperature and media type ($p<0.05$). Lower-case letters denote significant differences between temperature storage on the same day within the same media type ($p<0.05$).

Media types: R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

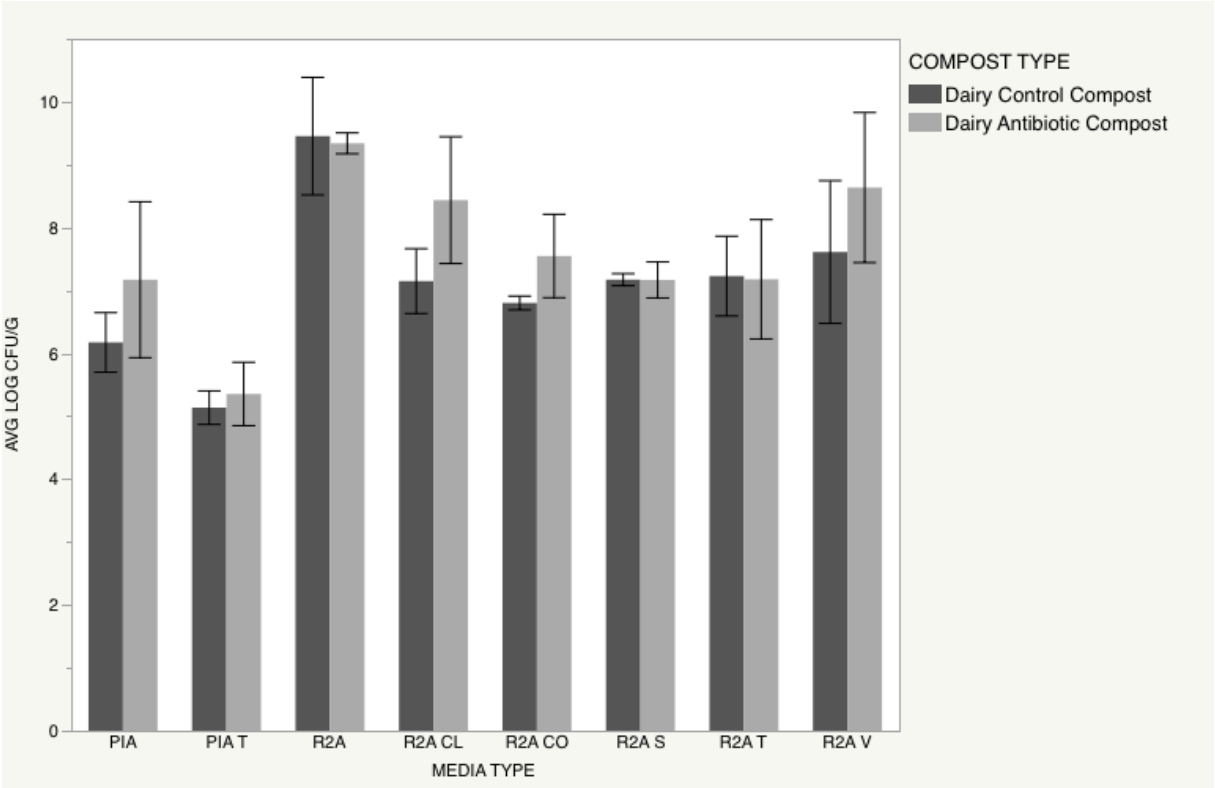

Figure S8. Comparison of bacterial populations recovered from non-inoculated dairy control and dairy antibiotic compost slurry (n=2).

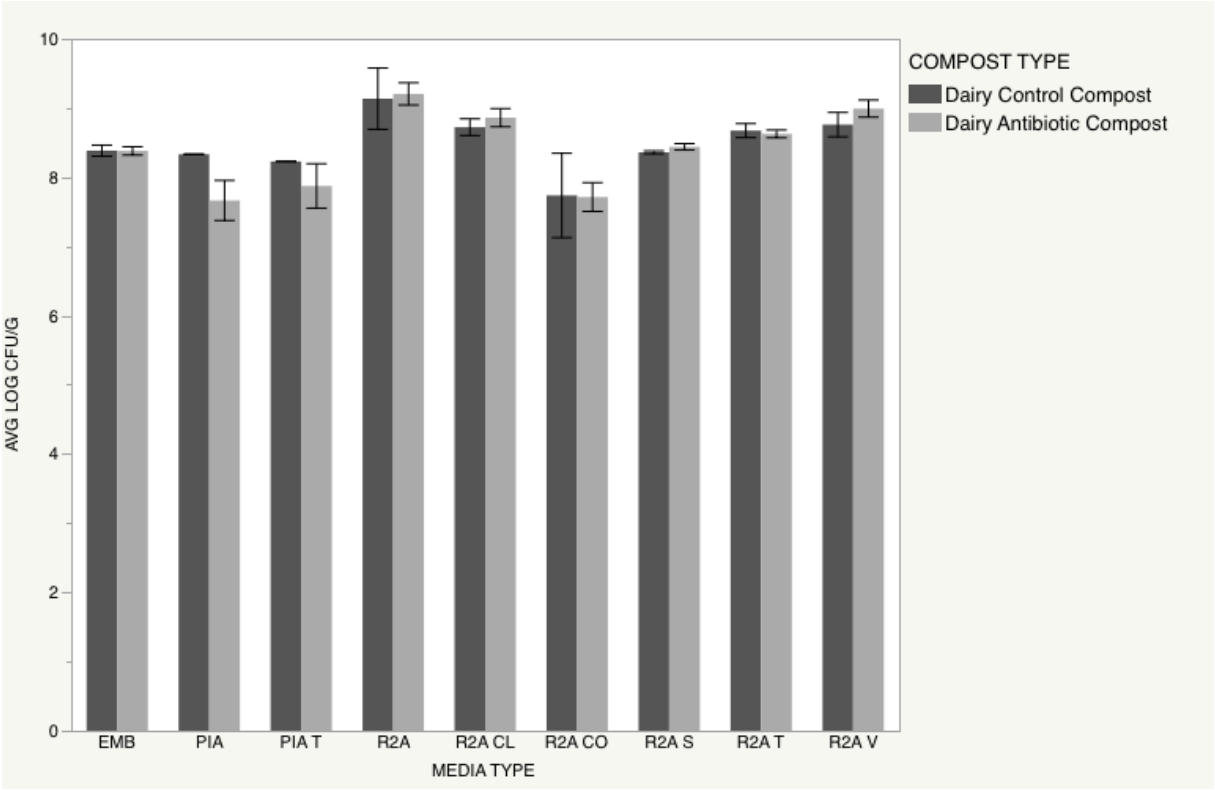

Figure S9. Comparison of bacterial populations recovered from inoculated dairy control and dairy antibiotic compost slurry (n=2).

Error bars represent 1 standard error from the mean.

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

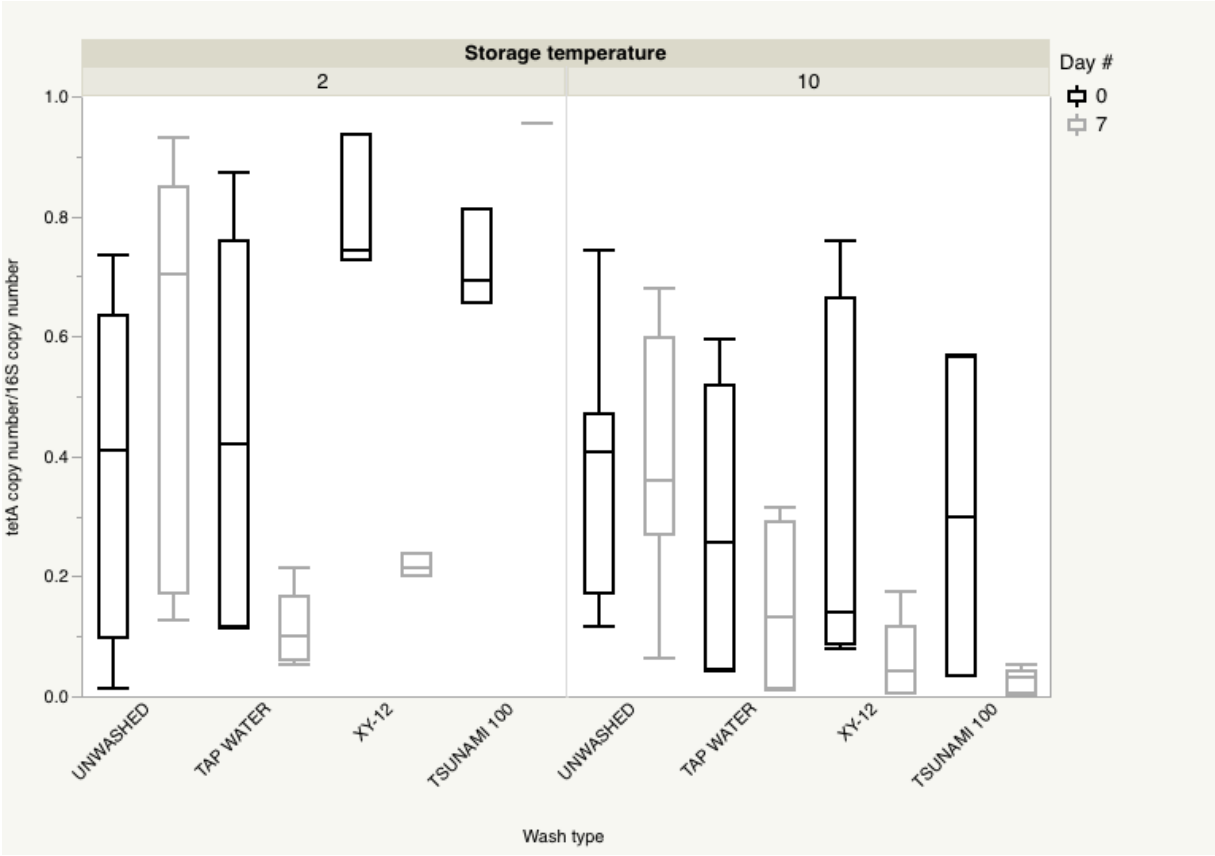

Figure S10. Comparison of copy numbers of *tet*A/ 16S rRNA copy numbers (n=3).

Boxes represent quantiles.

Table S1. Comparison of the average proportion of different bacterial and antibiotic-resistant bacterial populations to total aerobic bacteria on day 14 of storage at optimal storage temperature (2°) (n=3).

Values listed in the table represent the average value ± 1 standard deviation from the mean.

Capital letters denote significant difference between media types within the same wash type ($p<0.05$). Absence of lower-case lettering means there were no differences between wash types within the same media type ($p<0.05$).

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

	Wash Type			
CFU Proportion	Unwashed	Tap Water	XY-12	Tsunami 100
EMB/R2A	0.0000405±0.0001^{A}	0.0000512±0.0001^{A}	0.00000488±0.00001^{A}	0.000000314±4.761e-7^{A}
PIA/R2A	0.00104±0.0024^{B}	0.0000455±0.0010^{B}	0.000388±0.0006^{B}	0.000201±0.0002^{BD}
PIA T/R2A	0.000158±0.0003^{B}	0.0000416±0.00006^{AB}	0.000139±0.00019^{B}	0.0000572±0.00007^{B}
R2A CL/R2A	0.0412±0.0386^{C}	0.0991±0.191^{C}	0.1000±0.1712^{C}	0.0696±0.0567^{C}
R2A CO/R2A	0.00123±0.0034^{BD}	0.000257±0.0004^{B}	0.000793±0.0017^{B}	0.00124±0.0021^{D}
R2A S/R2A	0.000613±0.0016^{B}	0.000204±0.0004^{AB}	0.00016±0.0003^{B}	0.00021±0.0005^{BD}
R2A T/R2A	0.0000646±0.0001^{AB}	0.0000783±0.0001^{AB}	0.0000785±0.0001^{B}	0.000749±0.0021^{BD}
R2A V/R2A	0.126±0.234^{C}	0.0686±0.0971^{C}	0.0791±0.1332^{C}	0.0898±0.1073^{C}

Table S2. Comparison of the average proportion of different bacterial and antibiotic-resistant bacterial populations to total aerobic bacteria based on wash type at temperature abuse conditions (10°C) for combined day 0 and day 7 of storage (n=3).

Values listed in the table represent the average value ± 1 standard deviation from the mean.

Capital letters denote significant difference between media types within the same wash type ($p<0.05$). Lower-case lettering means there were no differences between wash types within the same media type ($p<0.05$).

Media types: EMB= *E. coli* O157:H7 inoculum. PIA= *Pseudomonas* sp. PIA T= *Pseudomonas* sp. resistant to tetracycline (4µg/mL tetracycline). R2A= total aerobic bacteria. R2A CL= bacteria resistant to clindamycin (25µg/mL). R2A CO=bacteria resistant to cefotaxime (10µg/mL). R2A S= bacteria resistant to sulfamethoxazole (100µg/mL). R2A T= bacteria resistant to tetracycline (3µg/mL). R2A V= bacteria resistant to vancomycin (11µg/mL).

	Wash Type			
CFU Proportion	Unwashed	Tap Water	XY-12	Tsunami 10
EMB/R2A	0.0244 ± 0.0288^{AaB}	0.00102 ± 0.0008^{Ab}	0.000082 ± 0.0001^{Ac}	0.0000314 ± 0.00
PIA/R2A	0.0121 ± 0.0099^{AaB}	0.000963 ± 0.0008^{Ab}	0.000474 ± 0.0003^{Bb}	0.00182 ± 0.001
PIA T/R2A	0.0139 ± 0.0153^{AaB}	0.000852 ± 0.0009^{Ab}	0.000287 ± 0.0002^{Bb}	0.00095 ± 0.000
R2A CL/R2A	0.14 ± 0.0650^{aC}	0.113 ± 0.0833^{aB}	0.0816 ± 0.0279^{aC}	0.136 ± 0.069
R2A CO/R2A	0.00884 ± 0.0112^{Aa}	0.000824 ± 0.0009^{Ab}	0.000472 ± 0.0004^{Bb}	0.0591 ± 0.137
R2A S/R2A	0.0254 ± 0.0286^{AaB}	0.00123 ± 0.0012^{Ab}	0.000322 ± 0.0007^{ABc}	0.0000638 ± 0.00
R2A T/R2A	0.0309 ± 0.0311^{aB}	0.00299 ± 0.0034^{Ab}	0.000306 ± 0.0002^{Bc}	0.000218 ± 0.000
R2A V/R2A	0.156 ± 0.0596^{aC}	0.0533 ± 0.0241^{Bb}	0.0679 ± 0.0103^{bC}	0.0731 ± 0.040

APPENDICES

Appendix A: Carrot source information

Table A. Use-by dates and lot codes of carrots from Bolthouse Farms Organic Petite Baby-cut carrots purchased from Sam's Club used in this experiment.

Use-by Date	Lot Code
02/14/16	26R A0904 SC116B17
02/14/16	22R AO902 SC116C11
03/18/16	18R AO906 SC1168617
04/10/16	22R AO902 SC116C11
04/28/16	28R A1016 SC116C30
04/29/16	27R A1015 SC116C30
06/06/16	21R B0826 SC11EE06
06/06/16	24R B0826 SC116E06
06/06/16	28R B1025 SC116E10

Appendix B: Wash water information

Table B. Averages of wash water parameter values between all washes during the duration of the experiment.

Pre or Post Carrot Wash	Wash Type	pH	Temperature (°Celsius)	Free Chlorine (parts per million)	Free Paracetic Acid (parts per million)	ORP (milliVolts)	Turbidity (Abs at 540nm)
Pre	Tap Water	6.59	22.17	~0ppm	N/A	339	0.002
Post	Tap Water	6.71	22.03	~0ppm	N/A	395.7	0.016
Pre	XY-12	7.33	22.50	~50ppm	N/A	715.7	0.000
Post	XY-12	7.47	22.33	~31.7ppm	N/A	723.0	0.013
Pre	Tsunami 100	4.40	22.73	N/A	~40ppm	N/A	0.001
Post	Tsunami 100	4.47	22.47	N/A	~35ppm	N/A	0.015

Appendix C: Preliminary experiment: Baseline levels of antibiotic resistance of grocery store carrots

Table C. Baseline antibiotic-resistance ± standard deviation recovered from grocery store carrots.

R2A V (11μg/mL)	R2A CO (10μg/mL)	R2A CL (25μg/mL)	R2A T (3μg/mL)	PIA T (4μg/mL)
5.70 ± 0.38	5.44 ± 0.72	5.24 ± 0.54	5.45 ± 0.05	3.04 ± 0.09

Appendix D: Inoculum resistance information

Table D. Antibiotic resistance of our inoculum.

Inoculated Organism	R2A V (11μg/mL)	R2A CO (10μg/mL)	R2A CL (25μg/mL)	R2A T (3μg/mL)	R2A S (100μg/mL)	PIA T (4μg/mL)
E. coli SMS-3-5	YES	YES	YES	YES	YES	N/A
Pseudomonas aeruginosa PA01	YES	NO	YES	YES	YES	YES