Produce Safety in the United States: Epidemiological Trends and Risk Management Utilizing a Novel Screening Method for Shiga-Toxin Producing *E. coli* and *Salmonella* in Irrigation Water

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Dedication

I dedicate this dissertation to my parents and my late grandfather (whom I lost over that journey).

Abstract

Despite significantly improved technologies in food science and public health and tremendous efforts being put by governments to ensure food safety, foodborne outbreaks are still abundant worldwide. Produce products have been frequently implicated in foodborne illness outbreaks in recent years due to changes in consumer demands, consumption habits and production practices. A better understanding on epidemiology changes of produce outbreaks is needed to evaluate current risks associated with produce supply chain and to understand safety regulations regarding produce safety. In addition, it is evident that water used in produce production plays an important role in potentially introducing microbial contaminations. Therefore, its risk management is crucial for safety assurance of the produce supply chain.

The goal of this thesis research is to analyze the epidemiological trends of produce outbreaks and to improve the risk management of microbial quality of irrigation water. It summarizes the changing epidemiology of produce outbreaks in the United States from 1998-2007, establishes the baseline to further evaluate the potential impact from the recently implemented Food Safety Modernization Act (FSMA). The study also describes the development, optimization, and evaluation of a novel selective medium for sensitive enrichment and screening of *Shiga-toxin producing E. coli* and *Salmonella* in irrigation water. The developed enrichment-indicator system meets the increasing demand of method for multi-pathogen enrichment and detection in a single assay format allowing cost effective detection of STEC and *Salmonella* within 24 hours.

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Chapter 1: Literature Review

1.1 Foodborne Illness Outbreaks Overview

Foodborne illness has been a significant threat to us since the beginning of mankind. Despite significantly improved technologies in food science and public health and tremendous efforts being put by governments to ensure food safety, foodborne outbreaks are still abundant worldwide. The World Health Organization (WHO) identified thirty-one global foodborne hazards, which caused 600 million foodborne illnesses and 420,000 deaths in 2010 and reported that the global burden of foodborne diseases caused by these hazards was 33 million Disability Adjusted Life Years (DALYs) (WHO, 2015). At both the national and international levels, the foodborne outbreak scenario has been shifting from acute and highly local outbreaks from high levels of contaminations to relatively more outbreaks from low levels of contaminations of widely distributed commercial food products due to changes in food production and regulation and the more than ever widely distributed food supply chain (R. V. Tauxe, 1997). In the U.S., there were 5,760 foodborne outbreaks reported to the Foodborne Disease Outbreak Surveillance System during 2009-2015, resulted in 100,939 illnesses, 5,699 hospitalizations, and 145 deaths, with 82% of the hospitalizations and deaths attributed to *Listeria*, *Salmonella*, and *Shiga-toxin producing Escherichia coli* (STEC) (Dewey-Mattia, Manikonda, Hall, Wise, & Crowe, 2018). Multi-state outbreaks disproportionally contributed to large numbers of illnesses, hospitalizations and death despite its lower frequency (Dewey-Mattia et al., 2018), indicating increasing public health risks from the complicated food supply chain. It should

be noted that outbreak-associated foodborne illnesses are only a small subset of the estimated 9.4 million foodborne illnesses from known pathogens that occur annually in the United States potentially suggesting that the real public health risk is not properly recognized. (Scallan et al., 2011).

Furthermore, foodborne illnesses impose a substantial economic burden due to associated medical costs, productivity loss, and deaths. Hoffman et al. (2015) analyzed the economic burden of major foodborne illnesses acquired in the United States. The authors reported that the foodborne outbreaks with identified pathogens (20% of the total cases) impose over $15.5 billion in economic burden annually, which was primarily attributed to five specific pathogens (90%) including *Salmonella*, *Toxoplasma gondii*, *Listeria monocytogenes*, *Campylobacter*, and Norovirus (Hoffmann, Maculloch, & Batz, 2015). *Salmonella* species are the leading microbial cause of hospitalizations and deaths associated with foodborne illnesses imposing an estimated $3.7 billion in economic burden annually, which puts it at number one among all the pathogens in terms of economic burden (Hoffmann et al., 2015). On the other hand, STEC O157, which is a commonly identified *Shiga-toxin producing E. coli* serotype, caused less than 2% of the foodborne illnesses in 2013 in the U.S., half of these cases were hospitalized, and its per-case economic burden ranked fourth among all the pathogens (Hoffmann et al., 2015). Besides, STEC O157 is one of the leading causes of foodborne outbreaks in the U.S. and often associated with large outbreaks. For example, the multiple-state outbreak of fresh spinach in 2006, which resulted in 199 cases, 102 hospitalizations, and 3 deaths, was associated with STEC O157:H7 (CDC, 2006). On the other hand, there are other *Shiga-toxin producing E. coli*

serotypes that cause infections in addition to STEC O157, collectively referred to as STEC non-O157. Although STEC non-O157 infections are generally less severe than those caused by STEC O157, it is evident that these pathogens cause nearly twice the number of the illnesses resulted from STEC O157 and the number of STEC non-O157 infections has increased in recent years potentially due to changing laboratory practices (Gould et al., 2013; Hoffmann et al., 2015).

1.1.1 Foodborne Illness Outbreaks Associated with Produce Products

A large portion of produce is consumed raw, fresh-cut, or minimally processed, at which elimination of contamination is limited. Besides, some of the intrinsic factors of produce such as the high moisture content have been proven to promote microbial growth, which may increase risks of foodborne illnesses (FDA, 2003). According to the Center for Science in the Public Interest (CSPI), produce was among the most frequent sources of foodborne illness outbreaks in the U.S. from 1998-2007 accounting for 14.7% of the total outbreaks, only behind seafood (18.1%) (Center for Science in the Public Interest, 2009). Moreover, it is evident that produce has been an increasingly important source for foodborne illness outbreaks. It was reported that the number of foodborne illness outbreaks associated with produce has increased from 14.8% in 1998 to 22.8% in 2007 in the United States (Wadamori, Gooneratne, & Hussain, 2017). **Table 1** lists the major foodborne outbreaks associated with produce in the U.S. in the past two years. It is interesting to note that both *Salmonella* and *E. coli* O157:H7 have been frequently implicated in produce outbreaks in recent years often resulting in large scale infections (**Table 1**). Besides, both

imported and domestic produce products have been linked to foodborne outbreaks in recent years.

The increase in reported outbreaks related to produce may be the result of several trends. Consumption of produce is always associated with healthy eating patterns and believed to have health benefits such as reducing the risk for heart disease, lowering blood pressure, and protecting against certain types of cancers (Choosemyplate, 2019). The average Americans are recommended to consume 2.5 cup-equivalent of vegetables and 2 cup-equivalent of fruits per day at the 2,000-calorie level (US Department of Health Human Services, 2017). Despite the fact that the per capita consumption of produce has increased in the U.S. and perhaps in other industrialized countries (Regmi, 2001), the *Dietary Guide for Americans 2015-2020* highlights that about 80% of the U.S. population still has an eating pattern that is low in vegetables and fruits, and encourages the average Americans to increase their daily intake (US Department of Health Human Services, 2017), which could potentially help to maintain the momentum of the increasing produce consumptions. The U.S. Farm Bill has several programs that are designed to encourage fresh fruit and vegetable consumption and several programs are testing various ways to encourage women and children to eat more fresh fruits and vegetables (Çakır et al., 2018). Besides, there is an increasing consumer demand for fresh-cut fruit and vegetables, which are conveniently ready for consumption. However, fresh-cut produce deteriorates faster than corresponding intact produce and is more susceptible to pathogen contamination during its processing, storage, and distribution (Francis et al., 2012). For example, it was found that juices released from the cut ends of the salad leaves promoted the growth of

Salmonella and enhanced its motility and biofilm formation, potentially contributing to the persistence of *Salmonella* on salad leaves (Koukkidis, Haigh, Allcock, Jordan, & Freestone, 2017). Numerous foodborne outbreaks have been linked to the consumptions of fresh-cut fruits and vegetables (Harris et al., 2003). Furthermore, the high demand for produce leads to further expansion of the already spread out supply chains. It was estimated that the proportions of the imported fresh fruits and vegetables eaten in the U.S. increased from 23% to 53.1% and from 5.8% to 31.1%, respectively, between 1975-2016. Although the significance of imported produce relative to foodborne outbreaks associated with consumption of produce remains unclear, it is believed that produce products may suffer from transport and potential health risks exist due to variations in agricultural practices and food safety standards among different countries. On the other hand, the domestic produce is increasingly susceptible to microbial contaminations due to the increasingly centralized production of produce at agricultural facilities near animal production (Bennett, Littrell, Hill, Mahovic, & Behravesh, 2015). More than a third of U.S. vegetables and two-thirds of U.S. fruits and nuts are grown in California, which is also the home to 9% of the U.S. total cows (CDFA, 2018; Silverman, 2015). Produce can be easily contaminated in the field by direct and indirect contact with farm animal manure and wild animal feces. For example, the source of contamination for the romaine lettuce outbreak associated with *E. coli* O157:H7 in April 2018 was traced back to a canal near a concentrated animal feeding operation (CDC, 2018c). Furthermore, the FDA traceback information showed that clustering of other romaine lettuce farms also operated near the same canal (Meyer, 2018), which could impose high health risks to consumers if proper preventive measures are not taken.

Table 1: Major foodborne outbreaks associated with produce in the U.S. in the past two years (CDC, 2019a)

Year	Food Vehicles	Pathogens	Cases	Hospitalizations	Deaths	Country of Production
2019	Fresh basil	*Cyclospora*	241	6	0	Mexico
2019	Fresh papaya	*Salmonella Uganda*	81	27	0	Mexico
2019	Pre-cut melons	*Salmonella Carrau*	137	38	0	U.S.
2018	Romaine lettuce	*E. coli* O157:H7	62	25	0	U.S.
2018	Salad mix	*Cyclospora*	511	24	0	U.S.
2018	Pre-cut melons	*Salmonella Adelaide*	77	36	0	U.S.
2018	Romaine lettuce	*E. coli* O157:H7	210	96	5	U.S.
2018	Leafy greens	*E. coli* O157:H7	25	9	1	U.S.

1.1.2 Regulations for Safety of Produce Products

The Food and Drug Administration's (FDA) Food Safety Modernization Act (FSMA) was enacted in 2011 to strengthen the food system by shifting approaches from reactive to preventive. FSMA is constructed of seven major rules, including Preventive Controls Rules for Human and Animal Food, Produce Safety Rule, Foreign Supplier Verification Program (FSVP) Rule, Accredited Third-Party Certification Rule, Sanitary Transportation Rule, Mitigation Strategies to Protect Food Against Intentional Adulteration Rule (FDA, 2019b). Its enactment marked the first overhaul of the food safety regulation since the Food, Drug and Cosmetic Act (FDCA) was enacted in 1938.

The Produce Safety (PS) Rule of FSMA focuses on setting the first-ever federal regulatory rules for the production, harvest, and handling of produce, aiming to prevent microbial contamination and foodborne illnesses associated with produce (Produce Safety Alliance, 2019a). It lays out key requirements covering agricultural water, biological soil amendments, sprouts, domesticated and wild animals, worker training and health and hygiene, and equipment, tools and buildings (FDA, 2019d). Specifically, the PS Rule established standards for all produce focusing on microbial hazards to reduce the risk of microorganisms that have been found to be of public health significance, such as *E. coli*, *Salmonella* and *Listeria*. However, its real impact on public health remains unclear (Collart, 2016). Besides, the PS Rule provides staggered sets of compliance dates based on business sizes ranging from January 2016 to January 2024 (**Table 2**). Although it was estimated that all farms with a size below $25,000 accounted for only 1.5% of covered fresh produce

acres (FDA, 2015), the limited oversight of the PS Rule over such farms is still a legitimate concern for the industry.

Bennett et al. (2018) analyzed produce-associated foodborne outbreaks in the U.S. between 1998-2013 to describe the baseline before the implementation of FSMA. The authors found that the number of raw produce outbreaks declined at 50% of the decline rate of the total number of foodborne outbreaks during the study period; moreover, the percentage of outbreaks attributed to raw produce among outbreaks with a food reported doubled during 1998-2013, suggesting the need to further strengthen safety measures in the production of raw produce (Bennett et al., 2018). However, the analysis was restricted to outbreaks associated with raw consumption of produce based on nonconclusive judgments, which does not necessarily cover the whole spectrum regarding produce safety. Besides, the analysis provided little information on the impact of the implementation of FSMA as the analyzed data was only obtained for 1998-2013 which was years ahead of the compliance dates for the PS Rule of FSMA. Therefore, a better understanding of the changes in the epidemiology of produce associated outbreaks based on the most up-to-date data is helpful to assess the impact of FSMA activities.

Table 2: Compliance dates of produce safety rule of FSMA (Produce Safety Alliance, 2019b)

Business Size	Compliance Dates for Sprouts	Compliance Dates for Most Produce	Water Related Compliance Dates	Compliance Dates for Qualified Exemption Labeling Requirement	Compliance Dates for Retention of Records Supporting a Qualified Exemption
All other businesses (>$500K)	1/26/2017	1/26/2018	1/26/2022	1/1/2020	1/26/2016
Small businesses (>$250K-500K）	1/26/2018	1/28/2019	1/26/2023	1/1/2020	1/26/2016
Very small businesses (>$25K-250K）	1/28/1029	1/27/2020	1/26/2024	1/1/2020	1/26/2016

1.2 Microbial Contamination of Produce from Irrigation Water

The microbial contamination of produce can occur at any stage in the supply chain, from pre-harvest production to post-harvest processing and distribution. Preharvest and postharvest sources of microbial contamination on produce are summarized in **Table 3**. Unfortunately, the importance of each of these elements along the supply chain relative to product contamination by pathogens remains unclear. However, it is evident that water used in production plays an important role in potentially introducing microbial contamination to the product. In fact, many foodborne illnesses associated with produce can be traced back to poor irrigation water quality. For example, the on-site pond used for irrigation water was found to be the source of contamination of tomatoes by *Salmonella Newport* in 2002, resulting in 510 illnesses (Greene et al., 2008). Jalapeno and serrano peppers contaminated with *Salmonella Saintpaul* caused 1500 illnesses in 2008, possibly due to agricultural water (Barton Behravesh et al., 2011). An *E. coli* O157:H7 outbreak linked to baby spinach in 2006 was attributed to the irrigation water contaminated by cattle feces (CDC, 2007). Most recently, the two multi-state outbreaks of *E. coli* O157:H7 infections associated with romaine lettuce in 2018 (April and December) have both been linked to agricultural water. The investigations identified the outbreak strains from the canal water for the April outbreak and from the farm agricultural water reservoir for the December outbreak (CDC, 2019c). Therefore, it is crucial to assess the risks of contamination from irrigation water and establish preventative measures to ensure the safe production of produce. The two significant factors affecting the risks of introducing pathogens from irrigation water to produce products include the source of water and

irrigation methods (Brackett, 1999; Leifert, Ball, Volakakis, & Cooper, 2008; Steele & Odumeru, 2004). Besides, the risk of product contamination from irrigation water with poor microbial quality is influenced by the characteristics of the microbial hazards and the produce products (Uyttendaele et al., 2015).

Table 3: Sources of microbial contamination of produce (Ijabadeniyi, 2011)

Supply Chain	Source of Contaminations
Preharvest	Feces Soil Irrigation water Water used to apply fungicides and insecticides Green or inadequately composted manure Air (dust) Wild and domestic animals Insects Human Handling
Postharvest	Feces Human handling Harvest equipment Transport containers and vehicles Wild and domestic animals Insects Air (dust) Wash and rinse water Processing equipment Improper storage Improper packaging Cross-contamination Improper handling after wholesale or retail purchase

1.2.1 Sources of Irrigation Water

Irrigation is one of the largest uses of water in the United States accounted for 38% of the total freshwater withdrawals in 2010 (Maupin et al., 2017). Common water sources for irrigation include municipal water, rainwater, groundwater, surface water, and treated wastewater. There are significant variations in the microbial quality of irrigation water from different sources. Zhu et al. (2019) evaluated the microbial quality of potential irrigation water sources in Arizona. The authors found that 7.1% and 64.3% of the collected samples were positive for STEC and *Salmonella*, respectively, which could potentially lead to bacterial contamination of produce grown in the region (Zhu et al., 2019). Therefore, the identification of a clean water source for irrigation is critical to produce production.

Municipal water is the water of potable quality offered by water companies (Codex Alimentarius Commission, 2003; Uyttendaele et al., 2015). It is generally of the best quality among other irrigation water sources imposing the lowest risk of microbial contamination. However, it is not always available in all regions and might not be an economically viable option for some growers due to its high cost. Moreover, it is evident that biofilms in municipal water distribution systems can serve as an environmental reservoir for pathogenic microorganisms and represent a potential source of product contamination from irrigation (Wingender & Flemming, 2011).

Another irrigation water source generally is considered of good quality is groundwater. It includes water seeped through from the surface and present in porous rocks below the surface, shallow wells, or deep aquifers (Codex Alimentarius

Commission, 2003; Uyttendaele et al., 2015). During the past couple of decades, groundwater has become increasingly important for irrigation accounting for 60% of the irrigation water supply in the United States (Siebert et al., 2010). However, the intensive exploitation of groundwater has severely depleted many of the world's major aquifers leading to concerns on the long-term sustainability of irrigations with groundwater (Foster, Brozović, & Butler, 2014). A study has suggested that 35% of the South High Plains in California will be unable to support irrigation within the next 30 years with the current groundwater depletion rate (Scanlon et al., 2012). Groundwater is generally less susceptible to microbial contamination when compared to surface water. However, it is believed that microbial contamination of groundwater can still occur when fecal material migrates into the subsurface through routes such as failed septic systems, leaking sewer lines, cesspools, and animal-related operations (Macler & Merkle, 2000). For example, massive groundwater contamination affected approximately 1,450 residents and visitors of South Bass Island, Ohio, in 2004 was attributed to the migration of microbial contaminants from wastewater treatment facilities and septic tanks to the lake and subsurface (Fong et al., 2007). Therefore, it is necessary to assess the risks of the microbial quality of groundwater used for irrigation.

Surface water includes water from surface that is exposed to the environment, such as rivers, canals, lakes, and open wells (Codex Alimentarius Commission, 2003; Uyttendaele et al., 2015). In addition to groundwater, surface water is one of the two primary water sources for irrigation worldwide (Gleick, 2000). In the United States, it was estimated that 57% of the total irrigation withdrawals of freshwater in 2010 were

from surface water (Maupin et al., 2017). In general, irrigation with surface water is believed to impose a higher risk to human health than irrigation with groundwater due to difficulties to prevent animal fecal contamination and runoff water from adjacent fields (Suslow et al., 2003). The microbial water quality of rivers, streams, and creeks is highly unpredictable due to the significant impact of activities upstream (Uyttendaele et al., 2015). Lakes generally are believed to have better microbial water qualities when compared to rivers, although lakes are also subject to surrounding sources of contamination from river inflow (Uyttendaele et al., 2015). Moreover, climate change and weather fluctuations tend to have a significant impact on the microbial quality of surface water. Paruch et al. (2015) found that an increase in microbial concentrations in surface water was immediately observed after the first rainfall events, and the contaminations with *E. coli* and intestinal parasitic protozoa were detected with concentrations up to three times higher during the wet/cool period than during the dry/warm period (Paruch, Mæhlum, & Robertson, 2015). The risk assessment of the microbial quality of surface water for irrigation tends to be more complicated, and a robust preventative plan is essential to ensure the safe production of produce irrigated with surface water.

1.2.2 Methods for Irrigation

Various irrigation methods have been developed over time to meet the irrigation needs of certain crops in specific areas (Bjorneberg, 2013). The most commonly used irrigation methods in the United States include flood system (surface irrigation), sprinkler system (overhead irrigation) and micro-irrigation system (subsurface irrigation), which

accounted for 54.6%, 36.7% and 8.6% of the irrigated acres respectively in 2015 (Dieter et al., 2018). And the method of irrigation can influence how effectively pathogens present in irrigation water are transmitted to plant surfaces (Steele & Odumeru, 2004).

Surface irrigation system leads water along the ground, either by flooding the whole area or leading the water along small furrows between the crop rows, using gravity as a force (Uyttendaele et al., 2015). It is typically used for field crops, pastures, and orchards (Bjorneberg, 2013). Surface irrigation can minimize contact of crops with contaminants in irrigation water, compared with overhead irrigation, because the edible portions of plants are not in direct contact with water (Steele & Odumeru, 2004). Solomon et al. (2001) compared the risk of transmission of *E. coli* O157:H7 on lettuce by different irrigation methods. The authors found that spray irrigation posted a substantially higher risk of contaminating the lettuce after a single exposure when compared to surface irrigation (Ethan B Solomon, Potenski, & Matthews, 2002). However, it should be mentioned that surface irrigation can also result in contaminations of root crops or vegetable crops growing near the ground and impose health risks to farmworkers as they are directly exposed to the irrigation water (Uyttendaele et al., 2015). Moreover, it is not uncommon that the surface water that leaves the field following application of irrigation water is diverted back to agricultural land to increase irrigation efficiency (Zhu et al., 2019), which might further spread contaminations and increase risks of recontaminations if pathogens are present in irrigation water.

Overhead irrigation system irrigates the plants by propelling water under high pressure as rain over the parcels (Uyttendaele et al., 2015). It is commonly used for a

wide variety of plants, including field crops, vegetables, orchards, turf and pastures (Bjorneberg, 2013). Overhead irrigation is believed to have the highest potential to transfer pathogens to crop surfaces, as water is directly applied to edible portions of most crops (Keraita, Konradsen, Drechsel, & Abaidoo, 2007). The splashing of sprayers can result in recontamination of the crop surface from the soil (Tiongco, Narrod, & Bidwell, 2009). Overhead irrigation resulted in a large number of lettuce plants tested positive for *E. coli* O157:H7 at harvest after a single exposure to the food pathogen (Ethan B Solomon et al., 2002). Besides, it was found that *E. coli* O157:H7 was harbored on the edible tissue of lettuce for more than 30 days following overhead irrigation (Ethan B Solomon, Pang, & Matthews, 2003). Furthermore, aerosol-borne pathogens might be carried further and create a health risk to nearby residents (Fattal, Margalith, Shuval, Wax, & Morag, 1987).

Subsurface irrigation system irrigates the plants by placing water low by the plants drop by drop or with micro-sprinklers by forming fog-like conditions (Uyttendaele et al., 2015). It is popular for permanently installed systems that irrigate trees, vineyards, orchards, and shrubs (Bjorneberg, 2013). In addition to increasing crop yields and water use efficiency (Camp, 1998; Schneider & Howell, 1998), subsurface irrigation is believed to reduce health risks by minimizing the exposure of the irrigation water to farmworkers and plants (Alum, 2001; Enriquez et al., 2003; G Oron, DeMalach, Hoffman, & Manor, 1992; Gideon Oron, Goemans, Manor, & Feyen, 1995). It was previously reported that the percentage of microorganisms transferred from irrigation water to surface of produce was up to three orders of magnitude higher for surface irrigation than subsurface

irrigation (Stine, Song, Choi, & Gerba, 2005). However, subsurface irrigation might create an area of permanently saturated or near-saturated soil, favoring the development of plant or animal pests, potentially causing product contaminations (Uyttendaele et al., 2015). Besides, *E. coli* O157:H7 was identified by Cooley et al. (2007) in the dust near produce production environments suggesting that subsurface irrigation using contaminated water could potentially contaminate the crops through dust or mud dispersal during field operations (M. Cooley et al., 2007).

It needs to be mentioned that although safety is always the primary concern in any food productions, a grower has to consider many other factors when choosing irrigation method such as types of water sources available, local cost of these water sources, cost of infrastructure, soil type and slope, and crop type or applicability of crop rotations (Mena, 2006). Therefore, risk assessments must be performed according to the actual production conditions.

1.2.3 Characteristics of Microbial Hazards and Produce Products

Once the microorganisms are introduced into irrigation water and via water to soil or plants, it is vital to understand the factors affecting their abilities to attach, survive, and even grow in the environments.

Bacteria are known to be able to attach to different surfaces, including surfaces of produce products, through a complex process facilitated by secretions of extracellular polymeric substances (Hassan & Frank, 2004). Intrinsic factors such as cell surface hydrophobicity and surface charge play an essential role in bacterial adhesion (Hassan &

Frank, 2004; Li & McLandsborough, 1999). Many recent studies have reported transmissions of food pathogens from irrigation water to the surface of produce products. For example, *E. coli* O157:H7 was able to transmit from contaminated irrigation water to lettuce surface and persist for up to 20 days (Ethan B. Solomon, Yaron, & Matthews, 2002). Besides, the transmission of microorganisms from irrigation water to plant is also affected by the physical properties of the edible portion of the plant, such as surface texture (Stine et al., 2005). Codex recommends paying special attention if the produce has physical characteristics such as leaves and rough surfaces that can trap water (Codex Alimentarius Commission, 2003). It was reported that the percentage of microorganisms transferred from irrigation water to surface of cantaloupe and lettuce via irrigation was significantly higher than to surface of bell pepper potentially due to their surface textures and locations of the edible portion of the plant in reference to irrigation water (Stine et al., 2005). Moreover, the infiltration of internal structures and tissues of plants by pathogenic bacteria may occur when produce surfaces are in direct contact with the cells in irrigation water. Burnett et al. (2000) observed infiltrations of *E. coli* O157:H7 into apples through the flora tube, attachment to seeds, cartilaginous pericarp, and internal trichomes which could potentially reduce the efficiency of the postharvest treatments (Burnett, Chen, & Beuchat, 2000). Besides, it is evident that the edible portions of a plant can become contaminated without direct exposure to a pathogen but rather through the transport of the pathogen into the plant by the root system (Ethan B. Solomon et al., 2002).

On the other hand, the survival of pathogens in the soil and on plants can also affect the risk of an outbreak. It has been previously reported that a 14 days gap between the last irrigation event with the contaminated water and the harvest significantly reduced the risks of product contamination (Stine et al., 2005). In fact, under the PS Rule of FSMA, if the water does not meet its criteria, the growers are allowed to use a specific time interval between the last irrigation and harvest to let potentially dangerous microbes to die off on the field (FDA, 2019d). However, it is not necessarily representative of industry practice for all crops. The survey data on United Kingdom (UK) irrigation practices found that half of the survey respondents growing salad onion, celery, and baby leaf salads harvesting their crops within 24 h of the last irrigation which would allow little opportunity for any natural die-off process to occur (Tyrrel, Knox, & Weatherhead, 2006). Moreover, some pathogens are known to be able to persist in soil or on crops for a long time after irrigation. For example, Islam et al. (2004) found that *Salmonella Typhimurium* was able to survive in soil samples for up to 231 days leading to contaminations of radishes and carrots detected even after seeds were sown for up to 203 days (Mahbub Islam et al., 2004). Similarly, it was found that *Salmonella Typhimurium* was able to persist on lettuce and parley for up to 63 days and 231 days, respectively (Islam, Doyle, Phatak, Millner, & Jiang, 2004). Solomon et al. (2002) reported that *E. coli* O157:H7 was able to persist on lettuce for 20 days following irrigation with contaminated water (Ethan B Solomon et al., 2002). Therefore, risks of product contamination of produce from irrigation water should be controlled through water quality management rather than relying on corrective actions.

1.2.4 Prevention and Control Measures for Microbial Quality of Irrigation Water

At the international level, the Codex Alimentarius (Codex) remains the most widely referenced international standards, guidelines, and recommendations related to food safety. The Code of Hygienic Practice for Fresh Fruits and Vegetables in the Codex provides a general framework of hygienic practices for the primary production and packing of produce products. It is recommended that the growers to identify the sources of water used on the farm, evaluate its quality and suitability for intended use, establish corrective actions to prevent or minimize contamination (Codex Alimentarius Commission, 2003). Besides, necessary microbial tests on irrigation water are recommended with a frequency depending on the water source and the risk of environmental contamination, but no specific microbiological standards are established (Codex Alimentarius Commission, 2003). It needs to be mentioned that the codes in Codex are flexible, of necessity, due to its intent to allow applications by different production systems for different groups of commodities, therefore often being criticized for being slow for adaptions and challenged by the emerging private standards (Codex Alimentarius Commission, 2003; Henson & Humphrey, 2011). Although not subject to the same legal enforcement of public regulations, market forces often make compliance with private standards mandatory in practice (Henson, 2008). In fact, many produce buyers now require that the growers to be audited annually against one or more specific buyer-imposed programs. One example is the SQF Food Safety Code for Primary Production from the Safe Quality Foods (SQF). It is a voluntary audit program recognized by the Global Food Safety Initiative (GFSI). The SQF code requires the

growers to assure the irrigation water is from a clean source or being treated to make it suitable for use (Food Marketing Institute, 2019). The code also includes requirements on microbiological testing and standards, which are based on the hazard analysis, best practices, and applicable legislation within the country of production, such as FSMA in the United States (Food Marketing Institute, 2019). And the SQF code requires that the water analysis must be conducted by an ISO17025 approved lab (Food Marketing Institute, 2019).

The production of produce in the United States is under the jurisdiction of the U.S. FDA and is subject to the PS Rule under FSMA. The PS Rule lays out key requirements covering agricultural water, biological soil amendments, sprouts, domesticated and wild animals, worker training and health and hygiene, and equipment, tools and buildings (FDA, 2019d). There are two sets of criteria established for the microbial water quality based on the presence of generic *E. coli*: a geometric mean (GM) of less than 126 CFU per 100 mL of water and a statistical threshold value (STV) of less than 410 CFU per 100 mL of water (FDA, 2019d). It is required to perform the water testing using methods equivalent to the U.S. EPA's method 1603 with the testing frequency based on the type of water source (**Table 4**). The criteria for the microbial quality of agricultural water in the PS Rule reply on the prevalent generic *E. coli* as an indicator of the presence of pathogens. However, some recent studies have identified a weak correlation between indicator bacteria and pathogenic bacteria (Benjamin et al., 2013; Dechesne & Soyeux, 2007; Jenkins, Fisher, Endale, & Adams, 2011). For example, Benjamin et al. (2013) investigated the occurrence of generic *E. coli*, *E. coli* O157 and

Salmonella spp. in water and sediment from leafy green produce farms and streams in central California. From an analysis of over 500 samples collected during two years, the authors found that the generic *E. coli concentration* was not significantly associated with the presence of either *E. coli* O157 or *Salmonella*, indicating its unreliability on predicting the presence of these pathogens (Benjamin et al., 2013). Similarly, Dechesne and Soyeux (2007) found no recurring evidence showing the correlation between fecal indicators and pathogen presence and highlighted the need to improve the suitability of pathogen detection methods for risk assessment of irrigation water (Dechesne & Soyeux, 2007).

Table 4: The requirements on microbial testing of irrigation water from different sources (FDA, 2019d)

Source of irrigation water	Initial Survey	Annual Survey
Untreated surface water	Microbial water quality profile based on minimum of 20 samples collected over the course of two to four years	A minimum of 1 sample per year to be added to the most recent 3 samples to establish the rolling dataset for the microbial water quality profile
Untreated ground water	Microbial water quality based on minimum of 4 samples collected during the growing season or over a period of one year	A minimum of 1 sample per year to determine the microbial water quality profile; if any annual survey fails, a minimum of 4 sample per growing season is required
Public water system	No requirements on testing	

1.3 Food Pathogen Detection Overview

The risk of foodborne illnesses can be managed by monitoring microbial contamination at points of potential contamination, such as irrigation for produce and its further processing. Therefore, rapid and reliable detection of food pathogens is essential to risk management. The global food pathogen testing market is projected to reach $10.79 billion per year by 2020 and expected to grow rapidly as legislation creates new standards for microbial monitoring (Alocilja & Radke, 2003; MarketsandMarkets, 2016). Among different food sectors, the produce sector accounted for about 10% of food pathogen testing in 1999 and was expected to substantially grow due to increasing focus by regulatory authorities (Alocilja & Radke, 2003). In general, food pathogen testing is performed in various locations such as government laboratories, reference laboratories, corporate laboratories, and on-site laboratories. And a considerable shift towards more on-site pathogen testing has been observed primarily due to better cost management and compliance with regulations (Brunelle, 2001).

Food pathogen detection can be categorized into conventional and rapid methods. Conventional methods depend on sample enrichment, followed by media-based metabolic tests (Alocilja & Radke, 2003). One example is the culture of STEC and *Salmonella* on CHROMagar™ media (CHROMagar Microbiology, Paris, France). Hirvonen et al. (2012) evaluated the performance of the CHROMagar™ STEC medium for routine detection of STEC strains. The authors found high detection sensitivities within major STEC serotypes and suggested it to be an interesting option for STEC screening. Similarly, detection of low levels of *Salmonella* was achieved when using the

CHROMagar™ Salmonella medium in conjunction with Immuno-Magnetic Separation (IMS) and found to be with high sensitivity and easy to perform (Taha, Mohamed, Srivastava, & Reddy, 2010). In general, the conventional method has a high success rate and is cost-effective thus remaining popular despite its slow turnaround time (Priyanka, Patil, & Dwarakanath, 2016).

Rapid methods for food pathogen detection are based on immunological or nucleic technologies. Immuno-assays can be used to detect substances with immunological properties or substances, which can be converted into immunologically active substances such as bacterial protein toxins and other metabolic products (Notermans & Wernars, 1991). Because immunological property based technologies are highly selective, specific, adaptable to portable devices, and often require less time to prepare the assay than conventional methods, immunoassays are widely used in detection systems (Alahi & Mukhopadhyay, 2017; McBride et al., 2003). For example, Chen et al. (2006) developed an array-based immunosorbent assay for simultaneous detection of *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (Chen & Durst, 2006). However, it should be noted that contaminants and food matrix debris in samples may interfere with antibody-target interaction hindering assay sensitivity; therefore, enrichment steps are essential for the detection of pathogens in food samples to overcome high detection limit (Gracias & McKillip, 2004).

Another popular rapid method for food pathogen detection is the polymerase chain reaction (PCR) analysis. The reliability of PCR-based detection methods depends on the concentration of the target cells and the complexity of the sample matrices. With

some complex matrices such as food and surface water when pathogen numbers are usually in the lower range, and the inhibitory compounds may present, sample preparation steps must be taken to limit the potential inhibitory effect and overcome problems of low pathogen numbers (Lampel, Orlandi, & Kornegay, 2000). Lantz et al. (1994) summarized some conventional sample preparation methods in PCR analysis for food pathogen, including physical methods such as dilution, enrichment, centrifugation, filtration, and some other DNA extraction methods and immunological methods (Lantz, Hahn-Hägerdal, & Rådström, 1994). With the advancements in PCR technology, multiple methods have been developed to rapidly detect genes specific to the target pathogens such as *stx1*, *stx2*, *eae* for STEC, and *invA* for *Salmonella*. Despite its many advantages such as speed, good detection limit, and potential for automation, PCR analysis is not always the first option for food pathogen testing due to high investment cost, intensive labor requirement and lack of standardized regulations (Malorny et al., 2003). It should also be noted that neither immunoassays nor PCR analysis allows pathogen strain isolation; therefore, further analysis of the strain is not available with these methods (Hirvonen, Siitonen, & Kaukoranta, 2012).

1.3.1 Culture Enrichment

Culture enrichment has been proven necessary and efficient in isolating foodborne pathogens from complex matrices for further detections (Hara-Kudo, Konuma, Nakagawa, & Kumagai, 2000). The use of culture enrichment before PCR analysis serves roles to (i) dilute PCR-inhibitory substance in samples, (ii) amplify the target microorganisms to reach detectable concentrations, (iii) dilute dead cells, (iv) possibly

isolate the target microorganisms for complementary tests (Sharma & Carlson, 2000). Vimont et al. (2005) reviewed the most commonly used enrichment protocols for isolation of STEC in different matrices. The authors found that Tryptic Soy Broth (TSB) is the most commonly used enrichment broth, and the incubation is commonly carried out at 35℃ or 37℃ for 16-24 hours for better performance and practical reasons (A Vimont, Vernozy-Rozand, & Delignette-Muller, 2006). Sampling for *Salmonella* often uses buffered peptone water (BPW) as a pre-enrichment medium (Taskila, Tuomola, & Ojamo, 2012). Non-selective enrichment has been advocated as offering advantages such as improved recovery of damaged cells and relatively short enrichment periods (Beumer, Te Giffel, Anthonie, & Cox, 1996; Petran & Swanson, 1993; Sheridan, Duffy, Buchanan, McDowell, & Blair, 1994; Walsh, Duffy, Sheridan, Blair, & McDowell, 1998). However, non-selective enrichment also promotes the growth of background microflora, which may outcompete the targeting microorganisms, potentially affecting detection sensitivities, particularly for samples with complex background microflora such as raw meat and surface water. For example, Oliveira et al. (2003) found that PCR procedures incorporating selective enrichment with Rappaport-Vassiliadis (RV) broth had greater sensitivity for the detection and identification of *Salmonella* when compared to non-selective enrichment with brain-heart infusion (BHI) broth (Oliveira, Rodenbusch, Cé, Rocha, & Canal, 2003).

The selectivity of the enrichment medium can be increased by adjusting growth conditions such as temperature. Most bacteria have optimal growth at the temperature of 37℃ or below. An elevated temperature might still allow the growth of particular

targeting microorganisms while suppressing the growth of some background microflora therefore potentially improving the selectivity of the enrichment. For example, it was reported that an incubation temperature of 42℃ improved selectivity of modified tryptic soy broth with novobiocin to isolate *E. coli* O157:H7 when compared to incubation at 37℃ (Raghubeer & Matches, 1990). Similarly, Spino (1966) found that the use of elevated temperature (41.5℃) improved the recovery of *Salmonella* from streams and yielded relatively low total coliform and fecal coliform densities (Spino, 1966). However, it should be noted that although the use of elevated temperature increases the selectivity of enrichment, it may also slow down the recovery of injured target microorganisms (Taskila et al., 2012).

The selectivity of enrichment media can be further enhanced by adding antibiotics to limit the growth of background microflora. One of the most commonly used antibiotics in selective enrichment media is novobiocin which can be found in many STEC and *Salmonella* enrichment media due to its toxicity towards most gram-positive bacteria while showing weak activity against gram-negative bacterial pathogens (Kirby, Hudson, & Noyes, 1956; Pao et al., 2005). For example, tryptic soy broth, modified with acid digest of casein, bile salts, and novobiocin (mTSB) for the selective enrichment of enterohemorrhagic *E. coli* in foods is commonly used in laboratory settings (USDA, 2017). However, poor enrichment performance of the mTSB for certain STEC and Salmonella serotypes was previously reported in ground beef (Eggers, Feirtag, Olstein, & Bosilevac, 2018). Moreover, the addition of novobiocin may inhibit the growth of some non-O157:H7 STEC strains potentially leading to false-negative results for detecting

STEC from food (A. Vimont, Delignette-Muller, & Vernozy-Rozand, 2007). Besides, many selective enrichment media are specifically formulated to exclusively promote the growth of a single targeting microorganism, such as Fraser broth (FB) for *Listeria monocytogenes* and RV broth for *Salmonella*. However, there is an increasing demand of media for multi-pathogen enrichment as it facilitates multi-pathogen detection in a single-assay format reducing the overall cost of testing per pathogen (Kim & Bhunia, 2008). Therefore, a selective enrichment medium with better selectivity for multiple food pathogens is needed.

1.3.2 Selective Enrichment Incorporated with Colorimetric Indicating System

It is common to incorporate indicating mechanism(s) into enrichment media to presumptively identify positive samples (Ushijima, Takahashi, Tatewaki, & Ozaki, 1983). One of the most commonly used indicating mechanisms is the colorimetric system. It is particularly attractive for on-site screening tests as color changes can be easily detected by the naked eyes (Tram et al., 2016). Carbohydrate metabolisms through bacterial fermentation pathways produce acids such as lactic acid, acetic acid and succinic acid which can lead to acidification of the growing medium triggering color change if a proper pH indicator is present. However, bacterial carbohydrate metabolism is extremely diverse. When incorporating an indicating system into enrichment media based on bacterial carbohydrate metabolism, it is important to carefully choose suitable carbohydrates based on properties of the enrichment media and carbohydrate fermenting abilities of the targeting microorganisms.

The Guidance for public health laboratories: isolation and characterization of Shiga toxin-producing Escherichia coli (STEC) from clinical specimens (2012) published by CDC and Association of Public Health Laboratories (CDC & PHL) listed fermentability of some common carbon sources by selected serotypes of STEC. It was indicated that glucose, lactose, mannitol, sorbitol, arabinose, raffinose, maltose, xylose, and trehalose were among the most fermentable carbon sources by the tested isolates of STEC (CDC & PHL, 2012). Glucose and maltose are widely fermentable by many bacteria thus offering no additional selectivity when used in pH based indicating systems. Moreover, it was reported that both glucose and maltose promoted the growth of lactic acid bacteria, which might interfere with the growth of STEC and *Salmonella* (Lawrence & Leedham, 1979). Mannitol is widely fermentable by *Staphylococcus aureus,* potentially leading to false-positive results. (Smyth & Kahlmeter, 2005). Although lactose is widely fermentable by *E. coli* strains, it is not fermentable by *Salmonella,* making it unsuitable to be used for pH based indicating system for *Salmonella*. On the other hand, the other listed carbohydrates seem to be good candidates to be used as the sole carbon source in pH based indicating systems for STEC and *Salmonella*. Both arabinose and xylose are one of the few pentoses used as sole carbon source by *Salmonella typhimurium* and several strains of *E. coli* (Brown, 1939). Sorbitol is known to be fermentable by many bacteria including most of *E. coli* and *Salmonella* strains. It should be noted that because typical *E. coli* O157: H7 does not ferment sorbitol at 24 hours (Farmer & Davis, 1985), it has been commonly used in media formulations to differentiate *E. coli* O157: H7 from other fecal *E. coli*, such as in the MacConkey Agar with Sorbitol (SMAC). However, sorbitol-fermenting *E. coli* O157 strains are not

uncommon. Many studies have reported isolations of sorbitol-fermenting *E. coli* O157 from clinical specimens (Gunzer et al., 1992; Karch et al., 1990). Trehalose is a disaccharide composed of two glucose molecules. It is an osmoregulatory solute in many bacteria, including *E. coli* and *Salmonella*, crucial for the prevention of osmotic dehydration of the cells under stressed growth environments (Dupray, Derrien, & Pichon, 1995; Giaever, Styrvold, Kaasen, & Strøm, 1988).

1.3.3 PDX-STEC Selective Enrichment Medium

A highly selective enrichment media has been developed for selectively growing and detecting STEC and Salmonella (Olstein, 2016). The developed medium incorporates efflux pump inhibitors (EPIs) to suppress the growth of background microflora such as *Klebsiella*, *Enterobacter*, *Citrobacter*, and commensal *E. coli* species without compromising the growth of STEC and *Salmonella*. Efflux pumps (EPs) are transport proteins found in both Gram-positive and Gram-negative bacteria as well as eukaryotic organisms responsible for moving toxic substrates from within cells into the external environment (Webber & Piddock, 2003). In recent years, EPs have emerged as key drivers for antimicrobial resistance in bacteria, particularly among Gram-negative bacteria such as *E. coli* and *Salmonella* (Shriram, Khare, Bhagwat, Shukla, & Kumar, 2018). It is believed that Gram-negative bacteria can induce EPs for nonspecific outward transport of drug molecules resulting in resistance against multiple antibiotics (Masi, Refregiers, Pos, & Pages, 2017). The first EPI was reported by Lomovskaya et al. against RND-type EPs in *P. aeruginosa* and *E. coli* (Lomovskaya et al., 2001). Since then, many synthetic and natural EPIs have been discovered and tested. **Figure 1** illustrates the

inhibition of microbial efflux pumps via synthetic and natural EPIs. Askoura et al. (2011) summarized the different mechanisms of the inhibition of EPs: (1) disrupting the regulatory steps, (2) altering antibiotic structure, (3) inhibiting the assembly of EPs, (4) occupying the binding sites of EPs, (5) blocking outer membrane channel, (6) collapsing efflux energy (Askoura, Mottawea, Abujamel, & Taher, 2011). In the past few years, EPIs have been to exploited for potential use to enhance pathogen sensitivity to antibiotics against their developed antimicrobial resistance (Amaral, Martins, Spengler, & Molnar, 2014). The PDX-STEC medium is the first example, to the knowledge of us, of the incorporation of EPIs in an enrichment medium to enhance its selectivity for the target pathogen.

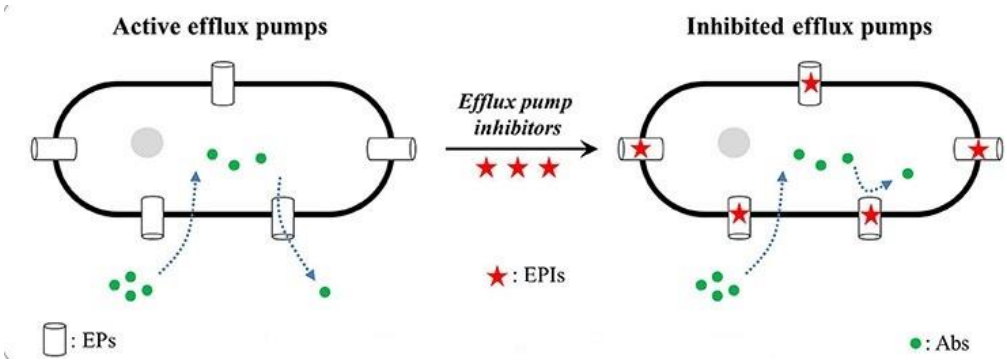

Figure 1: Diagram of a bacterial efflux pump and its inhibition by EPIs (Shriram et al., 2018)

In a previous study, Eggers et al. (2018) developed a method using the PDX-STEC medium to simultaneously enrich, isolate and detect STEC and *Salmonella* from ground beef. In conjunction with CHROMagar™ STEC and CHROMagar™ *Salmonella* Plus plates and real-time PCR analysis, the PDX-STEC medium allowed identification

of STEC and *Salmonella* in ground beef at low inoculation levels and showed greater sensitivity when compared to the use of mTSB as specified in official methods (Eggers et al., 2018).

Chapter 2: Research Objectives

The overall goal of this thesis is to understand the trends of epidemiology of produce safety and to improve the risk management of microbial quality of irrigation water. Specific research objectives include:

1. **Analysis of epidemiology of produce outbreaks in the United States**. As discussed earlier, foodborne illness outbreaks associated with produce products have been on the rise due to changing consumer eating habit, food supply chain, and agricultural practices. A better understanding on epidemiology changes of produce outbreaks is needed to evaluate current risks associated with produce supply chain. In addition, the Food Safety Modernization Act (FSMA) gives food safety regulators increased oversights on produce safety regulations. To evaluate the potential impact of FSMA on produce safety, a better understanding is needed regarding outbreaks attributed to the consumption of produce products. This objective is discussed in chapter 3.

2. **Development and adaptation of an enrichment-indicator system for screening of STEC and *Salmonella* from water samples**. Current risk assessments of microbial quality of irrigation water relies on the populations of generic *E. coli* which has been found to have little predicative power for the presence of pathogens. On the other hand, the direct detection and isolation of pathogens like STEC and *Salmonella* present a technical challenge necessitating time-consuming and costly laboratory procedures that often exceed the technical and financial capabilities of many small growers and reference laboratories. Therefore, a highly selective enrichment medium was developed and

adapted to an enrichment-indicator system which allows cost-effective enrichment and screening of STEC and *Salmonella* from water samples.

3. **Evaluation of the developed screening method and its application for real world water samples**. After development and optimization of the enrichment-indicator system, its efficacy was compared with a reference enrichment medium. In addition, an elevated incubation temperature was tested for improving screening selectivity. The developed system was also tested on water samples from different sources to evaluate its applicability for real world water samples. Objectives 2 and 3 are discussed in chapter 4.

Chapter 3: Epidemic Analysis of Foodborne Illness Outbreaks Attributed to Produce in the United States, 1998-2017

3.1 Introduction

Foodborne illness has been a significant threat to us since the beginning of mankind. Despite significantly improved technologies in food science and public health and tremendous efforts being put by governments to ensure food safety, foodborne outbreaks are still abundant worldwide, imposing significant public health risk and economic burden. Produce products have been frequently implicated in foodborne illness outbreaks in recent years due to changes in consumer demands, consumption habits and production practices. The Produce Safety Rule of the FDA Food Safety Modernization Act establishes the first federal regulatory standards for the production, harvest, and handling of produce, to prevent microbial contamination and reduce foodborne illness outbreaks associated with produce (Produce Safety Alliance, 2019a). However, its real impact remains unclear due to its staggered compliance dates and lack of up-to-date epidemic analysis to establish the baseline. Therefore, we analyzed produce-associated outbreaks reported to the CDC during 1998-2017 to understand the changes in the epidemiology of produce-associated outbreaks better.

3.2 Materials and Methods

3.2.1 Collection of Foodborne Illness Outbreaks Data

A foodborne illness outbreak is defined as when two or more people get the same illness from the same contaminated food or drink (FDA, 2019f). When exposure to a contaminated food source occurs in a single state, the outbreak is classified as a sing-state outbreak; when the exposure involves two or more states, the outbreaks are classified as multi-state outbreaks (Dewey-Mattia et al., 2018). Foodborne outbreaks are reported voluntarily by local, state and territorial health departments to CDC. Outbreak investigations are performed by the relevant health departments, and the relevant data is entered into the National Outbreak Reporting System (NORS) and further analyzed by CDC before publishing (CDC, 2018a)

The U.S. foodborne illness outbreaks data from 1998-2017 was retrieved from NORS (CDC, 2018d). The data includes information on the time of outbreaks (year and month), involved state(s), etiological agents, settings, numbers of illnesses, hospitalizations and deaths, implicated food vehicle, and/or ingredient. The etiologies include bacteria, viruses, chemicals and toxins, and parasites. Outbreak etiologies are classified as unknown, suspected and confirmed according to specific criteria (i.e., laboratory testing and clinical syndrome). Information on serotypes are only available to part of the outbreaks. An outbreak might involve multiple etiological agents. Food products and/or ingredients are identified as outbreak sources using laboratory, epidemic, trace back, environmental, and/or other evidence. When contaminated food ingredients are not identified for the outbreaks, the food product itself could provide the best information available. Some outbreaks investigations do not identify a source, and in these cases, the food is reported as unknown.

3.2.2 Epidemic Analysis of the Foodborne Illness Outbreaks Data

Foodborne illness outbreaks attributed to produce were identified based on the listed contaminated ingredients or food. Produce were categorized as vegetables or fruits and further classified into one of the twelve subcategories using the standard scheme developed by the Interagency Food Safety Analytics Collaboration (**Table 5**). When multiple etiological agents were implicated in the same outbreak, the associated data was included in the analysis of each etiological agents. Outbreaks that implicated multiple produce items were included in the analysis of each subcategory. Comparative analysis of the etiology of the outbreaks included only outbreaks with known etiology. Comparative analysis of the food category only included outbreaks with known food products or ingredients. Data analysis was conducted using RStudio version 1.2.1335 (RStudio Inc., Boston, MA, USA).

Table 5: Interagency Food Safety Analytics Collaboration Food Categories with Examples [a]

Produce category	Produce subcategory (examples)
Vegetables	Fungi (portabella and button mushrooms)
	Sprouts (alfalfa, mung bean and soybean sprouts)
	Root and underground vegetables (carrots, beets, potatoes, garlic, onions)
	Seeded vegetables (squashes, cucumbers, tomatoes, peppers, sweet corns)
	Herbs (basil, cilantro)
	Vegetable raw crops (broccoli, asparagus, celeries, lettuce, spinach)
Fruits	Melons (cantaloupes, watermelons)
	Pome fruits (apples, pears)
	Stone fruits (apricots, cherries)
	Small fruits (blueberries, strawberries)
	Tropical fruit (bananas, mangoes)
	Sub-tropical fruit (avocadoes, oranges)

[a] Adapted from the Interagency Food Safety Analytics Collaboration Food Categories with Examples retrieved from https://www.cdc.gov/foodsafety/ifsac/projects/food-categorization-scheme.html (accessed July 28th, 2019)

3.3 Results and Discussion

3.3.1 Epidemic Analysis of Foodborne Illness Outbreaks: Overview

During 1998-2017, there were 20,854 foodborne illness outbreaks resulting in 403,110 outbreak-associated illnesses, 16,517 hospitalizations, and 392 deaths reported to CDC (**Table 6**). Of these, etiological agents were known for 13,890 (67%) outbreaks, with 671 (3%) of them linked to two or more etiological agents. Of the total foodborne illness outbreaks with known etiological agents, 48% were caused by bacteria, 44% by viruses, 7% by chemicals and toxins, and 1% by parasites. Among all the etiological agents, norovirus was the leading cause for foodborne illness outbreaks contributing to 42% of the total foodborne illness outbreaks, followed by *Salmonella* (19%), *Clostridium* (8%), *Staphylococcus* (5%), *Bacillus* (5%), *E. coli* (4%) and *Campylobacter* (3%). It is interesting to note that 54% and 14% of the total hospitalizations, 27% and 11% of the total deaths were attributed to *Salmonella* and *E. coli* respectively (**Table 6**). It can be explained by the higher hospitalization rates associated with *Salmonella* and *E. coli* previously reported by Scallan et al. (2011). The authors estimated that the hospitalization rates for *Salmonella* and STEC O157 were 27.2% and 46.2%, respectively, which were significantly higher than some other primary etiological agents such as norovirus, which had a hospitalization rate at 0.03% (Scallan et al., 2011). During 1998-2017, the relative importance of the primary etiological agents to the total foodborne illness outbreaks with known etiological agents remained consistent with some fluctuations.

Among the total foodborne illness outbreaks, there were 414 outbreaks involved two or more states, leading to 26,357 multi-state-outbreak-associated illnesses, 4,640 hospitalizations, and 167 deaths. Although the multi-state outbreaks only accounted for less than 2% of the total outbreaks, significantly larger public health impact was associated with them including 7% of the total illnesses, 28% of the total hospitalizations and 42 % of the total deaths suggesting significantly larger health risks imposed by multi-state foodborne illness outbreaks. The number of single-state illness outbreaks started to increase after 2014, following a continuous decrease since 2006 (**Figure 2**). In comparison, the number of multi-state foodborne illness outbreaks was continuously on the rise during the study period, increased from 65 during 1998-2001 to 128 during 2014-2017 (**Figure 3**). It should be mentioned that the increase of foodborne illness outbreaks does not necessarily suggest increasingly higher health risks from the food supply chain. It is believed that the dramatic improvements in recent years on pathogen detection and outbreak surveillance have resulted in more reports of foodborne illnesses (Scutti, 2018). The median numbers of illnesses caused by single-state outbreaks remained consistent during the study period ranging from 8-11 (**Figure 2**), while the median numbers of illnesses caused by multi-state outbreaks fluctuated and decreased to 17 during 2014-2017 which was still more than twice of the median number of single-state outbreaks during the same period (**Figure 3**). The decrease was partially due to the improving public alert system of major foodborne illness outbreaks through increased media coverage, government websites, and social media.

Most multi-state foodborne illness outbreaks were caused by bacterial pathogens, with 59% (245) of these outbreaks attributed to *Salmonella* and 24% (101) attributed to *E. coli* suggesting that they may impose higher risks of contaminating food production in early farm to table chain. It is interesting to note that norovirus was not among the leading pathogens for multi-state foodborne illness outbreaks despite its dominating significance in the total foodborne illness outbreaks. This can be explained by the fact that most foodborne transmission of norovirus occurs by contaminations from infected food handlers during preparation and service, which normally leads to outbreaks that are geographically centralized (Hall et al., 2011). In comparison, bacterial pathogens such as *Salmonella* and *E. coli* are believed to be the leading sources of contamination of many common food commodities such as eggs, meat, and dairy products. In fact, some of the worst foodborne illness outbreaks in the U.S. were attributed to bacterial pathogens such as the 2006 spinach outbreak linked to *E. coli* O157:H7 which resulted in 205 illnesses and 3 deaths, the 2009 peanut butter outbreaks linked to *Salmonella* which resulted in 714 illnesses and 9 deaths, and the 2011 cantaloupe outbreak attributed to *Listeria* which resulted in 147 illnesses and 33 deaths (Healthline, n.d.).

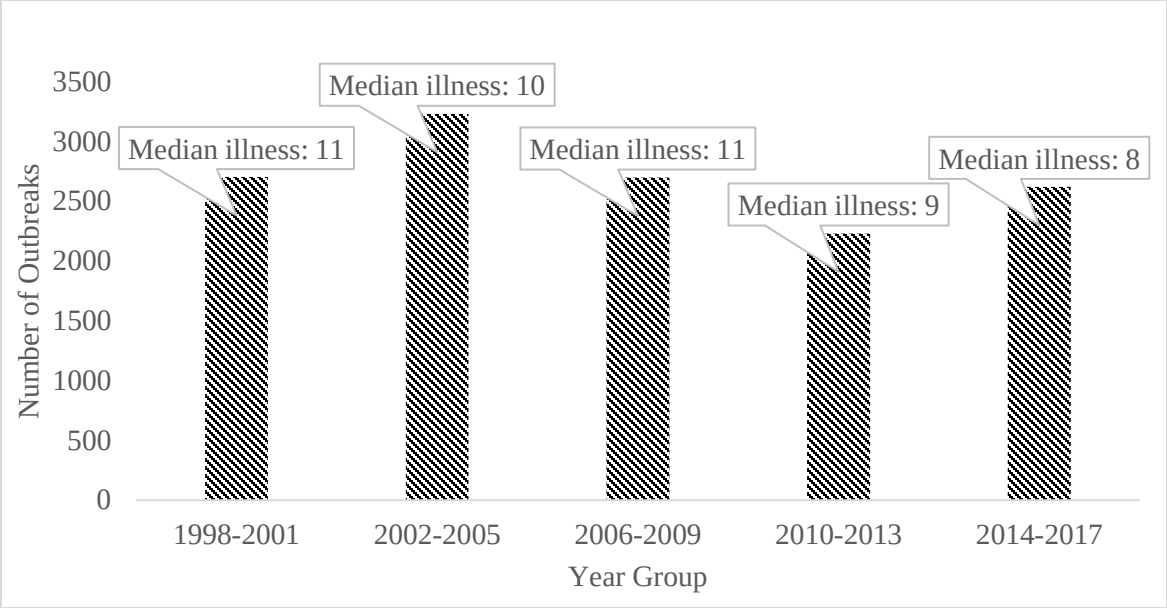

Figure 2: Number of single-state foodborne illness outbreaks and median number of the associated illnesses – National Outbreak Reporting System, U.S., 1998-2017

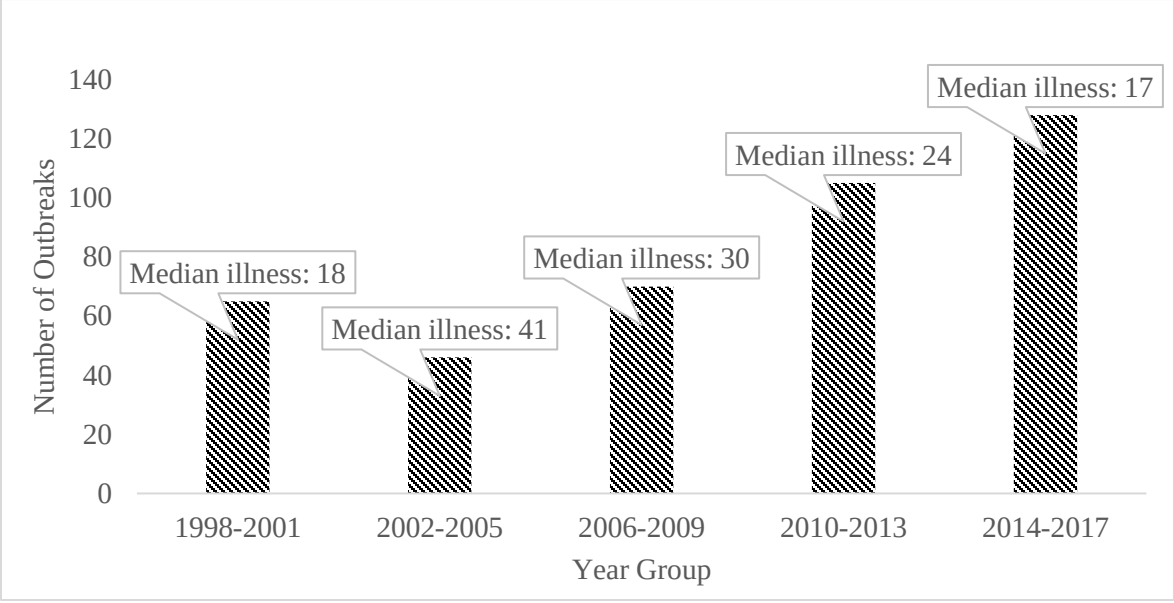

Figure 3: Number of multi-state foodborne illness outbreaks and median number of the associated illnesses – National Outbreak Reporting System, U.S., 1998-2017

Table 6: Number and percentage of reported foodborne outbreaks and associated illnesses, hospitalizations, deaths by etiology - National Outbreak Reporting System, U.S., 1998-2017 [c]

Etiology	No. of outbreaks				No. of illnesses				No. of hospitalizations				No. of deaths			
	CE	SE	Total	%	CE	SE	Total	%	CE	SE	Total	%	CE	SE	Total	%
Bacteria																
Salmonella	2477	234	2711	19	69967	2425	72412	22	8424	130	8554	54	98	2	100	27
Clostridium	488	648	1136	8	21182	14659	35841	11	336	75	411	3	27	7	34	9
Staphylococcus	242	453	695	5	7651	3332	10983	3	498	85	583	4	5	1	6	2
Escherichia coli	549	66	615	4	12018	1702	13730	4	2177	79	2256	14	37	2	39	11
Campylobacter	384	115	499	3	7941	661	8602	3	318	56	374	2	1	0	1	0
Bacillus spp.	121	552	673	5	2720	6342	9062	3	72	61	133	1	2	3	5	1
Shigella spp.	159	30	189	1	7146	557	7703	2	300	10	310	2	2	0	2	1
Vibrio spp.	103	75	178	1	1518	338	1856	1	64	10	74	0	2	1	3	1
Listeria spp.	76	2	78	1	881	59	940	0	685	1	686	4	140	0	140	38
Other bacteria	32	131	163	1	573	1419	1992	1	31	5	36	0	1	0	1	0
Subtotal [a]	**4631**	**2306**	**6937**	**48**	**131597**	**31494**	**163121**	**49**	**12905**	**512**	**13417**	**84**	**315**	**16**	**331**	**89**
Chemical and Toxin																
Mycotoxins	28	6	34	0	125	62	187	0	61	11	72	0	6	1	7	2
Ciguatoxin	260	39	299	2	1019	144	1163	0	126	12	138	1	1	0	1	0
Scombroid toxins	349	70	419	3	1211	344	1555	0	37	12	49	0	0	0	0	0
Other toxins	116	147	263	2	1164	1022	2186	1	116	27	143	1	1	0	1	0
Subtotal [a]	**753**	**262**	**1015**	**7**	**3519**	**1572**	**5091**	**2**	**340**	**62**	**402**	**3**	**8**	**1**	**9**	**2**
Parasites																
Giardia	20	3	23	0	381	67	448	0	2	10	12	0	0	0	0	0
Cyclospora	36	4	40	0	1776	47	1823	1	36	1	37	0	0	0	0	0
Cryptosporidium	34	6	40	0	819	68	887	0	30	3	33	0	0	0	0	0
Trichinella	22	1	23	0	126	3	129	0	26	1	27	0	0	0	0	0
Other parasites	3	1	4	0	41	3	44	0	2	1	3	0	0	0	0	0
Subtotal [a]	**115**	**15**	**130**	**1**	**3143**	**188**	**3331**	**1**	**96**	**16**	**112**	**1**	**0**	**0**	**0**	**0**
Viruses																

Noroviruses	3430	2598	6028	42	109613	43253	152866	46	1162	387	1549	10	13	3	16	4
Hepatitis virus	95	1	96	1	2890	4	2894	1	399	0	399	3	8	0	8	2
Other viruses	25	108	133	1	985	2712	3697	1	8	24	32	0	6	1	7	2
Subtotal [a]	**3550**	**2707**	**6257**	**44**	**113488**	**45969**	**159457**	**48**	**1569**	**411**	**1980**	**12**	**27**	**4**	**31**	**8**
Single Etiology [b]	8644	4575	13219	63	234837	68329	303166	75	13566	825	14391	87	338	15	353	90
Multiple Etiology [b]	322	349	671	3	13545	9104	18693	5	1076	203	1141	7	9	3	12	3
Unknown Etiology [b]	NA	NA	6964	33	NA	NA	81251	20	NA	NA	985	6	NA	NA	27	7
Total	**8966**	**4924**	**20854**	**100**	**248382**	**77433**	**403110**	**100**	**14642**	**1028**	**16517**	**100**	**347**	**18**	**392**	**100**

No., number; CE, confirmed etiology; SE, suspected etiology.

[a], the subtotal of each etiological category reflects their frequencies of being implicated in outbreaks, they might not add up to the number of total outbreaks due to outbreaks that attributed to multiple etiological agents; the denominator for the percentages is the sum of subtotals of each etiological category; because of rounding, percentage numbers might not add up to 100

[b], the denominator for the percentages is the total outbreak numbers; because of rounding, percentage numbers might not add up to 100

[c], the structure of the table was adapted upon the work of Bennett et al. (2018)

3.3.2 Epidemic Analysis of Produce Outbreaks in the U.S.

During 1998-2017, there were 3,032 foodborne illness outbreaks attributed to produce products, resulted in 92,009 outbreak-associated illnesses, 4,729 hospitalizations, and 141 deaths reported to CDC (**Table 7**). Of these, etiological agents were known for 2,257 (75%) outbreaks, with 108 (4%) of them linked to two or more pathogens. Of the total foodborne illness outbreaks with known etiological agents, 50% were caused by viruses, 46% by bacteria, 3% by chemicals and toxins, and 1% by parasites, which was generally in parallel with observations for the overall foodborne illness outbreaks. Similarities on the relative importance of the leading etiological agents for outbreaks associated with produce and all food were observed. Among all the etiological agents, norovirus was still the leading cause for foodborne illness outbreaks associated with produce contributing to 48% of the total foodborne illness outbreaks, followed by *Salmonella* (18%), *Clostridium* (8%), *E. coli* (6%), *Bacillus* (5%), and *Staphylococcus* (4%). It was noted that the outbreaks associated with produce were more frequently linked to *E. coli* when compared to the overall foodborne illness outbreaks. While efforts to reduce contaminations in meat and shell eggs have led to reductions in the contamination of these food types with *Salmonella* and *E. coli* (Braden, 2006; Naugle, Holt, Levine, & Eckel, 2005), the contaminations of produce with these pathogens have

caused a number of outbreaks each year. The most recent ones include the fresh papaya outbreak in 2019 attributed to *Salmonella Uganda* which resulted in 81 illnesses and 27 hospitalizations, the pre-cute melons outbreak in 2019 attributed to *Salmonella Carrau* which resulted in 137 illnesses and 38 hospitalizations, and the two romaine lettuce outbreaks in 2018 both attributed to *E. coli* O157:H7 which resulted in 272 illnesses, 120 hospitalizations and 5 deaths in total (CDC, 2019a).

There were 178 (6% of all produce outbreaks) multi-state outbreaks attributed to produce resulted in 15,297 illnesses (17% of all illnesses associated with produce outbreaks), 2664 hospitalizations (56% of all hospitalizations associated with produce outbreaks), and 92 death (65% of all deaths associated with produce outbreaks). It generally aligns with attributions of the overall multi-state foodborne illness outbreaks. The number of reported sing-state produce outbreaks decreased by more than 55% from 1998-2001 to 2014-2017 (**Figure 4**). The decrease was primarily attributed to the decreasing produce outbreaks linked to norovirus (**Figure 8**) as it was the leading cause for total produce outbreaks during the study period. It is believed that the continuous efforts on compliance with requirements in the FDA Food Code (FDA, 2019a) had some positive impact, especially those that exclude symptomatic and post-symptomatic workers, prohibit bare-hand contact with ready-to-eat foods, and ensure appropriate hand

washing (Dewey-Mattia et al., 2018). Further improvements are expected as the FDA continues to strengthen regulations through the fundamental requirements in PS Rule of FSMA on worker training and health and hygiene. On the other hand, it was noted that the number of reported multi-state outbreaks associated with produce had been continuously increasing during the study period and nearly tripled from 22 outbreaks during 1998-2001 to 60 during 2014-2017 (**Figure 5**). Moreover, in recent years (2013-2017), more than 50% of the total multi-state foodborne illness outbreaks were attributed to produce indicating significantly higher risks of contaminations early in the farm to table chain during produce production (**Figure 6**). The overall trend of the median numbers of illnesses associated with single-state and multi-state produce outbreaks were in alignment with the median numbers of illnesses associated with the overall foodborne illness outbreaks. However, produce outbreaks had larger median numbers of illnesses than the overall foodborne outbreaks suggesting a more significant public health impact from produce outbreaks.

Most multi-state produce outbreaks were caused by bacterial pathogens, with 68% (212) of these outbreaks attributed to *Salmonella* and 23% (41) attributed to *E. coli*. It was noted that *Salmonella* contributed to larger portion of produce multi-state outbreaks than it did to the total multi-state outbreaks suggesting that the upstream produce

production is especially susceptible to *Salmonella* contaminations. In fact, many multi-state produce outbreaks were traced back to *Salmonella* contaminations early in the farm to table chain. For example, the on-site pond used for irrigation water was found to be the source of contamination of tomatoes by *Salmonella Newport* in 2002 resulting in 510 illnesses (Greene et al., 2008). Jalapeno and serrano peppers contaminated with *Salmonella Saintpaul* caused 1500 illnesses in 2008, possibly due to agricultural water (Barton Behravesh et al., 2011).

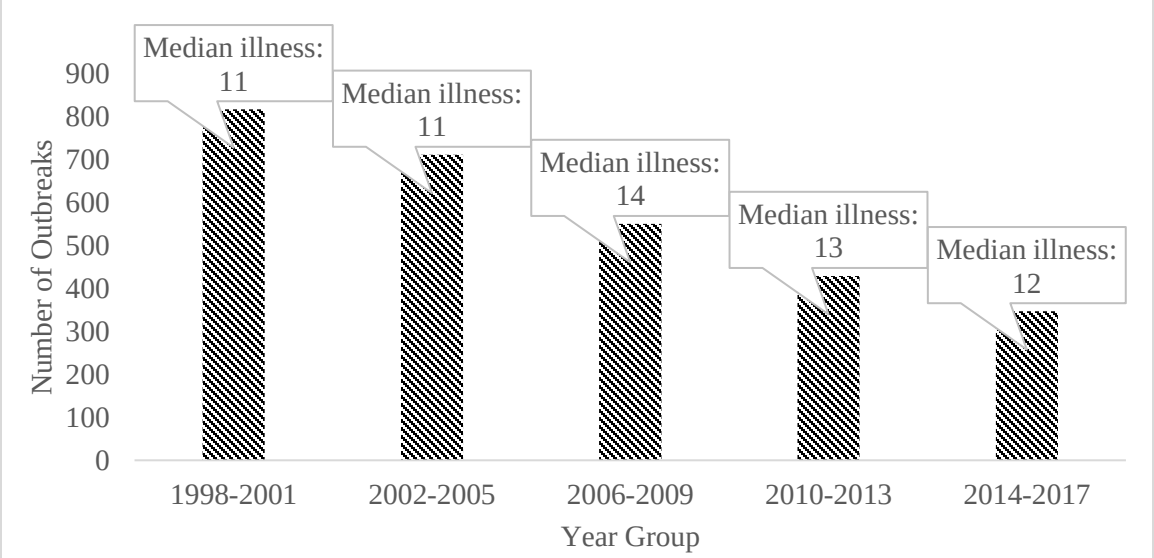

Figure 4: Number of single-state produce outbreaks and median number of the associated illnesses – Foodborne Disease Outbreak Surveillance System, U.S., 1998-2017

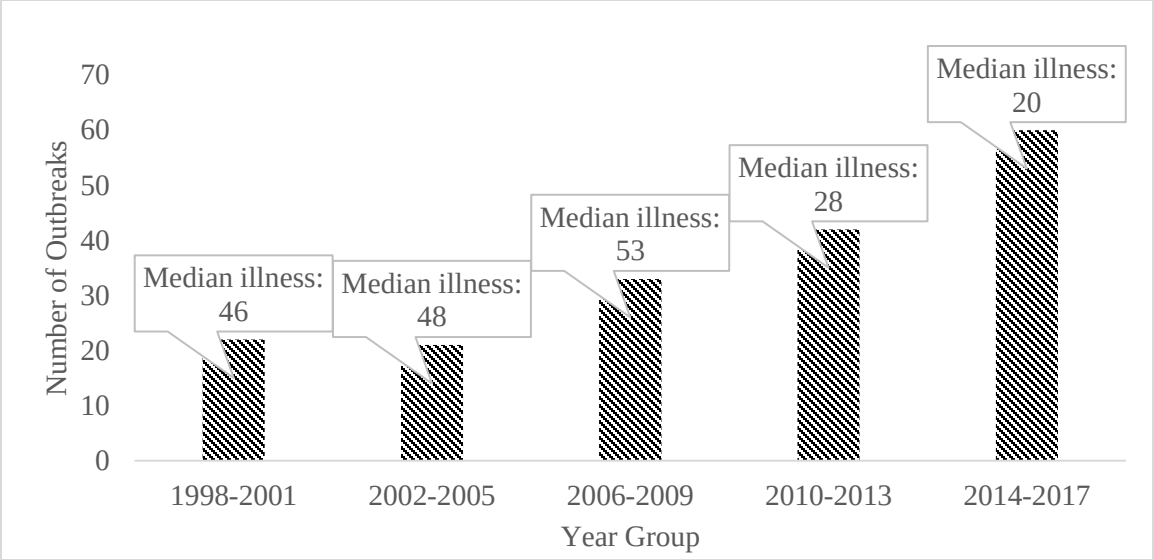

Figure 5: Number of multi-state produce outbreaks and median number of the associated illnesses – Foodborne Disease Outbreak Surveillance System, U.S., 1998-2017

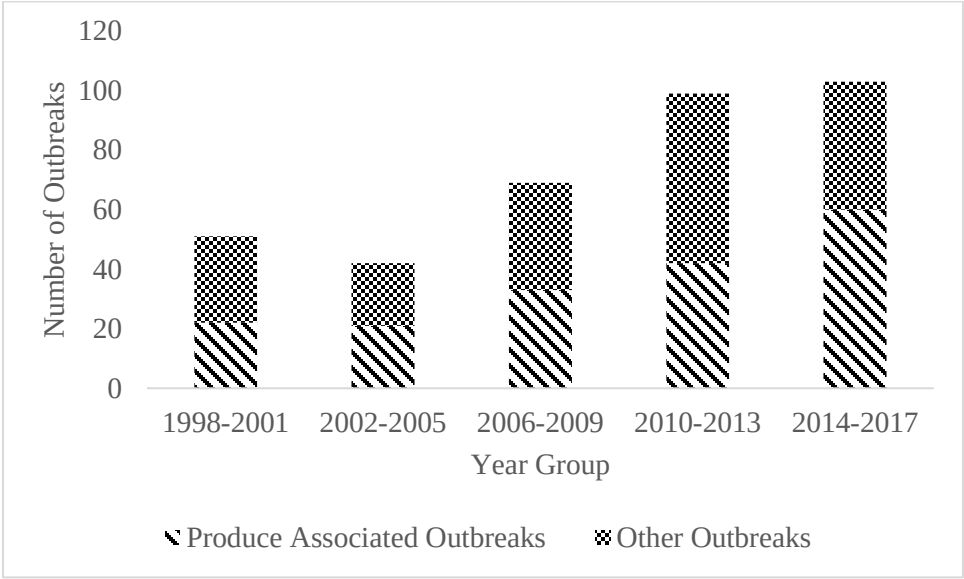

Figure 6: Number of multi-state foodborne illness outbreaks attributed to produce and other foods, – National Outbreak Reporting System, U.S., 1998-2017

Table 7: Number and percentage of reported foodborne outbreaks and associated illnesses, hospitalizations, deaths, attributed to produce, by etiology – National Outbreak Reporting System, U.S., 1998-2017 [c]

Etiology	No. of outbreaks				No. of illnesses				No. of hospitalizations				No. of deaths			
	CE	SE	Total	%	CE	SE	Total	%	CE	SE	Total	%	CE	SE	Total	%
Bacteria																
Salmonella	381	30	411	18	20731	317	21048	26	2716	19	2735	59	41	1	42	31
Clostridium	81	97	178	8	5561	1333	6894	8	97	6	103	2	7	1	8	6
Staphylococcus	36	55	91	4	1606	748	2313	3	39	6	45	1	2	0	2	1
Escherichia coli	130	13	143	6	4684	1046	5730	7	785	28	813	17	14	0	14	10
Campylobacter	33	9	42	2	1139	45	1184	1	27	4	31	1	0	0	0	0
Bacillus	31	90	121	5	892	1209	2101	3	6	5	11	0	0	0	0	0
Shigella	37	4	41	2	3324	218	3542	4	107	1	108	2	2	0	2	1
Vibrio	0	3	3	0	0	12	12	0	0	0	0	0	0	0	0	0
Listeria	12	1	13	1	260	56	316	0	246	1	247	5	55	0	55	41
Other bacteria	4	13	17	1	168	158	326	0	0	0	0	0	0	0	0	0
Subtotal [a]	**745**	**315**	**1060**	**46**	**38365**	**5142**	**43466**	**53**	**4023**	**70**	**4093**	**88**	**121**	**2**	**123**	**92**
Chemical and Toxin																
Mycotoxins	27	6	33	1	123	62	185	0	61	11	72	2	6	1	7	5
Scombroid toxins	1	0	1	0	5	0	5	0	0	0	0	0	0	0	0	0
Other toxins	16	19	35	2	248	103	351	0	18	0	18	0	0	0	0	0
Subtotal [a]	**44**	**25**	**69**	**3**	**376**	**165**	**541**	**1**	**79**	**11**	**90**	**2**	**6**	**1**	**7**	**5**
Parasites																
Giardia	3	1	4	0	71	25	96	0	0	0	0	0	0	0	0	0
Cyclospora	19	2	21	1	853	21	874	1	22	0	22	0	0	0	0	0
Cryptosporidium	8	1	9	0	405	6	411	1	20	1	21	0	0	0	0	0
Subtotal [a]	**30**	**4**	**34**	**1**	**1329**	**52**	**1381**	**2**	**42**	**1**	**43**	**1**	**0**	**0**	**0**	**0**
Viruses																
Norovirus	663	459	1122	48	24838	10111	34949	43	210	107	317	7	3	1	4	3
Hepatitis virus	17	0	17	1	1309	0	1309	2	109	0	109	2	0	0	0	0

Other virus	4	22	26	1	159	330	489	1	0	1	1	0	0	0	0	0
Subtotal [a]	**684**	**481**	**1165**	**50**	**26306**	**10441**	**36747**	**45**	**319**	**108**	**427**	**9**	**3**	**1**	**4**	**3**
Single Etiology [b]	1431	718	2149	71	62008	14051	76059	83	4026	175	4201	89	125	4	129	91
Multiple Etiology [b]	56	52	108	4	3427	2035	4566	5	417	30	424	9	5	0	5	4
Unknown Etiology [b]	NA	NA	775	26	NA	NA	111384	121	NA	NA	104	2	NA	NA	7	5
Total [a]	**1487**	**770**	**3032**	**100**	**65435**	**16086**	**92009**	**209**	**4443**	**205**	**4729**	**100**	**130**	**4**	**141**	**100**

No., number; CE, confirmed etiology; SE, suspected etiology.

[a], the subtotal of each etiological category reflects their frequencies of being implicated in outbreaks, they might not add up to the number of total outbreaks due to outbreaks that attributed to multiple etiological agents; the denominator for the percentages is the sum of subtotals of each etiological category; because of rounding, percentage numbers might not add up to 100

[b], the denominator for the percentages is the total outbreak numbers; because of rounding, percentage numbers might not add up to 100

[c], the structure of the table was adapted upon the work of Bennett et al. (2018)

3.3.2.1 Epidemic Analysis of Produce Outbreaks by Subcategories

Among all the produce outbreaks with subcategories specified, 1,744 (85%) were attributed to vegetables, and 301 (15%) were attributed to fruits (**Table 8**). There were 1,228 produce outbreaks without specified subcategories primarily due to difficulties to further classify outbreaks attributed to salads. The subcategories most commonly implicated in produce outbreaks were seeded vegetables (30%), vegetable row crops (26%), root and underground vegetables (21%), and melons (4%). These four subcategories also resulted in the largest number of outbreak-associated illnesses, hospitalizations, and deaths. It needs to be mentioned that the total outbreaks of each subcategory combined (**Table 8**) are more than the total foodborne outbreaks associated with produce (**Table 7**) due to outbreaks that were attributed to multiple produce products. Outbreaks attributed to seeded vegetables, vegetable row crops, root, and underground vegetables, and melons decreased during the study period (**Table 9**). The numbers of outbreaks attributed to fungi, herbs, and tropical fruits increased by 100%, 280%, and 320%, respectively, from 1998-2001 to 2014-2017. It was partially attributed to the change of the U.S. consumer's eating habits which include more healthy ingredients such as fungi and fresh herbs and the increasing consumer demand on tropical fruits (Cook, 2011) increasingly. On average, outbreaks attributed to melons (median = 23), herbs (median = 21), and sprouts (median = 20) resulted in the highest number of illnesses, while those attributed to fungi were the smallest (median = 4).

The microbial contamination of produce can occur at any stage in the supply chain, from pre-harvest production to post-harvest processing and distribution. Among all

the elements along the supply chain relevant to produce product contamination, some of the pre-harvest elements such as irrigation water play important roles in introducing microbial contamination to the produce product in early farm to table chain potentially causing large scale multi-state foodborne illness outbreaks. Seeded vegetables (48), vegetable row crops (40), sprouts (31), melons (19), and tropical fruits (13) were the most frequently implicated produce among all the multi-state produce outbreaks with specified subcategories. Furthermore, the number of multi-state produce outbreaks attributed to seeded vegetables, vegetable row crops, sprouts increased during the study period (**Figure 7**), which consequently led to the increase of overall multi-state produce outbreaks (**Figure 6**). It is evident that tomatoes and leafy vegetables are among the leading causes of recurrent multi-state produce outbreaks accounting for most of the multi-state outbreaks associated with seeded vegetables and vegetable row crops respectively (Bennett et al., 2018; Cummings et al., 2001; Greene et al., 2008). Besides, sprouts associated foodborne infections have been a recurrent problem in the U.S. (P. J. Taormina, Beuchat, & Slutsker, 1999). Pathogenic bacteria can internalize or infiltrate into sprout seeds to evade chemical treatment and remain dormant during storage for years (Ferguson et al., 2005; Holliday, Scouten, & Beuchat, 2001; Inami, Lee, Hogue, & BRENDEN, 2001). One contaminated lot of sprout seeds might be distributed widely leading to contaminated sprouts produced over time and in disparate locations (Dechet et al., 2014). The sprouting process which takes place in a warm and moist environment can further amplify the number of bacteria on the sprout, potentially leading to more infections (Fu, Reineke, Chirtel, & Vanpelt, 2008). In the PS Rule of FSMA, requirements specifically regarding sprouts safety are established to help prevent the

contamination of sprouts. The requirements include scopes on contamination prevention, irrigation water testing, environmental sampling, and corrective actions (FDA, 2019d). It needs to be mentioned that the microbiological standards of irrigation water used for sprouts are stricter than those for other produce products due to higher risks of sprouts contamination by irrigation water. On the other hand, it was noted that outbreaks attributed to tropical fruits started to emerge since 2010. In recent years, the U.S. consumption of tropical fruits has been increasing due to consumers' demand for more flavors and variations in produce and the change in American demographics as Hispanic and Asian populations has been on the rise (Johnson, 2016). Most tropical fruits in the U.S. are imported. In fact, the U.S. is one of the largest importers of tropical fruits representing 30% of the global tropical fruits purchase in 2015, with more than 50% of the total tropical fruits imported from Mexico (Ojeda, 2017). Therefore, it is vital to further strengthen compliance of the imported tropical fruits with safety standards. FDA established Foreign Supplier Verification Programs (FSVP) under FSMA requiring importers to perform certain risk-based activities to ensure compliance with applicable U.S. safety standards (FDA, 2019c). The first compliance dates for FSVP started on May 30, 2017; its impact on the safety of imported foods remains unclear and needs to be further evaluated. Besides, considering the significance of Mexico to the U.S. produce supply chain, the new U.S.-Mexico-Canada (USMCA) trade deal, if passed, is likely to have some impact on the safety of produce products in U.S. market. The lawmakers should carefully evaluate its potential impact on food safety.

Table 8: Number and percentage of reported foodborne outbreaks and associated illnesses, hospitalizations, deaths, attributed to produce, by food category – National Outbreak Reporting System, U.S., 1998-2017 [d]

Food Category	Outbreaks		Illnesses		Hospitalizations		Deaths	
	No.	%	No.	%	No.	%	No.	%
Vegetables								
Fungi	65	3	902	1	96	2	7	6
Sprouts	57	3	1845	3	199	4	5	4
Root and underground vegetables	437	21	13857	21	335	8	8	6
Seeded vegetables	613	30	23342	35	1899	43	29	23
Herbs	35	2	1517	2	80	2	0	0
Vegetable row crops	537	26	14234	21	865	19	18	14
Subtotal [a]	**1744**	**85**	**55697**	**56**	**3474**	**78**	**67**	**54**
Fruits								
Melons	86	4	4425	7	492	11	45	36
Pome fruits	56	3	1440	2	81	2	7	6
Stone fruits	8	0	209	0	26	1	1	1
Small fruits	73	4	2175	3	157	4	2	2
Tropical fruits	39	2	1180	2	204	5	3	2
Sub-tropical fruits	39	2	1466	2	19	0	0	0
Subtotal [a]	**301**	**15**	**10895**	**11**	**979**	**22**	**58**	**46**
Specified produce [b]	2045	62	66592	67	4453	88	125	86
Unspecified produce [b]	1228	38	32856	33	599	12	20	14
Unspecified vegetables [c]	1051	32	27189	27	486	10	18	12
Unspecified fruits	177	5	5667	6	113	2	2	1
Total	**3273**	**100**	**99448**	**100**	**5052**	**100**	**145**	**100**

[a], the subtotal of each produce subcategory reflects their frequencies of being implicated in outbreaks, they might not add up to the number of total produce outbreaks due to outbreaks that attributed to multiple produce items; the denominator for the percentages is the sum of subtotals of each produce subcategory; because of rounding, percentage numbers might not add up to 100

[b], the denominator for the percentages is the total produce outbreak numbers; because of rounding, percentage numbers might not add up to 100

[c], salads were classified into unspecific vegetables due to difficulties to classify them into any of the subcategories; the denominator for the percentages is the total produce outbreak numbers; because of rounding, percentage numbers might not add up to 100
[d], the structure of the table was adapted upon the work of Bennett et al. (2018)

Table 9: Number of reported foodborne illnesses outbreaks and median number of outbreak-associated illnesses attributed to produce by food category and year groups – National Outbreak Reporting System, U.S., 1998-2017 [a]

Food Category	1998-2001	2002-2005	2006-2009	2010-2013	2014-2017	Total
Fungi						
No. of outbreaks	9	12	15	11	18	65
Median no. illnesses (range)	3 (2-190)	7 (2-81)	6 (2-22)	3 (2-26)	4 (2-58)	4 (2-190)
Sprouts						
No. of outbreaks	12	10	11	12	12	57
Median no. illnesses (range)	36 (2-157)	16 (2-35)	20 (2-256)	8 (3-140)	20 (2-115)	20 (2-256)
Root and underground vegetables						
No. of outbreaks	144	119	64	52	58	437
Median no. illnesses (range)	8 (2-916)	16 (2-950)	17 (2-250)	15 (2-119)	19 (2-87)	14 (2-950)
Seeded vegetables						
No. of outbreaks	140	145	112	99	117	613
Median no. illnesses (range)	10 (2-886)	13 (2-880)	13 (2-1500)	17 (2-314)	10 (2-907)	12 (2-1500)
Herbs						
No. of outbreaks	5	6	3	7	14	35
Median no. illnesses (range)	35 (23-486)	9 (2-47)	46 (20-101)	27 (12-64)	17 (2-96)	21 (2-486)
Vegetable row crops						
No. of outbreaks	121	119	124	94	79	537
Median no. illnesses (range)	19 (2-300)	12 (2-935)	17 (2-238)	15 (2-99)	9 (2-252)	14 (2-935)
Melons						
No. of outbreaks	19	18	20	18	11	86
Median no. illnesses (range)	36 (2-736)	28 (8-126)	17 (3-594)	23 (6-261)	20 (4-136)	23 (2-736)
Pome fruits						
No. of outbreaks	12	10	7	15	12	56
Median no. illnesses (range)	22 (4-128)	16 (3-144)	8 (2-26)	9 (3-139)	5 (2-136)	10 (2-144)
Stone fruits						

No. of outbreaks	2	1	0	3	2	8
Median no. illnesses (range)	18 (2-34)	3 (NA)	NA (NA)	17 (7-131)	8 (2-13)	10 (2-131)
Small fruits						
No. of outbreaks	15	13	10	18	17	73
Median no. illnesses (range)	40 (3-100)	13 (2-98)	14 (3-30)	13 (2-139)	21 (3-137)	15 (2-139)
Tropical fruits						
No. of outbreaks	5	9	4	5	16	39
Median no. illnesses (range)	9 (2-100)	16 (3-68)	16 (3-31)	33 (13-129)	14 (3-213)	16 (2-213)
Sub-tropical fruits						
No. of outbreaks	13	4	10	5	7	39
Median no. illnesses (range)	9 (2-398)	22 (7-157)	20 (2-189)	8 (2-80)	4 (2-59)	10 (2-398)

No., number.

[a], the structure of the table was adapted upon the work of Bennett et al. (2018)

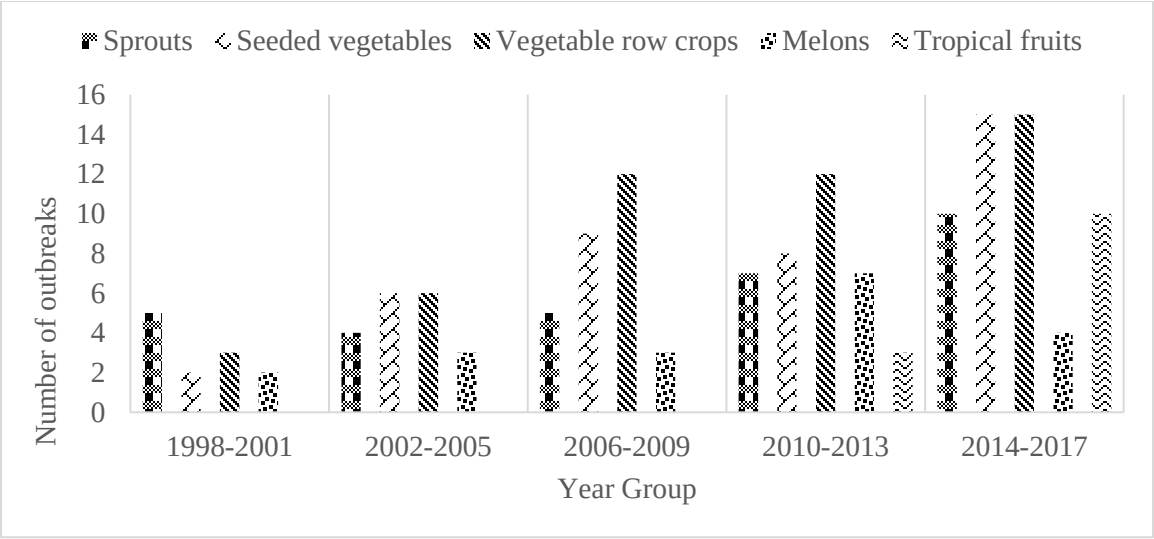

Figure 7: Number of multi-state produce outbreaks attributed to the five major produce subcategories – National Outbreak Reporting System, U.S., 1998-2017

3.3.2.2 Epidemic Analysis of Produce Outbreaks by Etiology

Overall, a large portion of produce outbreaks was attributed to norovirus. However, during the study period, a shift of the etiology of produce outbreaks towards a broader spectrum across the etiological agents was observed. In particular, the percentages of produce outbreaks associated with *Salmonella* and *E. coli* increased from 16% to 23% and from 4% to 9% respectively from 1998-2001 to 2014-2017 (**Figure 8**).

Produce outbreaks attributed to *E. coli* were most often linked to STEC O157, accounting for more than 77% of all produce outbreaks attributed to STEC (**Table 10**). STEC O157 is one of several Shiga-toxin producing serotypes known to cause human illnesses including asymptomatic shedding, non-bloody diarrhea, hemorrhagic colitis, hemorrhagic uremic syndrome, and death (Mead & Griffin, 1998). Although STEC O157 is not the most frequently implicated pathogen in foodborne illness outbreaks, it is known to cause outbreaks with high rates of hospitalizations imposing significant per-case economic burden (Hoffmann et al., 2015). For example, the multiple-state outbreak of fresh spinach in 2006, which resulted in 199 illnesses, 102 hospitalizations and 3 deaths, was attributed to STEC O157:H7 (CDC, 2006). It is evident that STEC O157 can transmit from irrigation water to the surface of produce products and persist for an extended amount of time (Ethan B. Solomon et al., 2002). In fact, many STEC O157

outbreaks associated with produce can be traced back to poor irrigation water quality including the two recent multi-state romaine lettuce outbreaks in 2018. On the other hand, even though STEC O157 serotypes play a dominating role in causing produce outbreaks associated with STEC, the potential risks associated with STEC non-O157 serotypes are not negligible. It was noted that the number of produce outbreaks attributed to STEC non-O157 serotypes increased during the study period (**Figure 9**). It agrees with the trends observed for the overall foodborne illness outbreaks reported in previous studies. Valilis et al. (2018) found that the incidence of STEC non-O157 infections in the U.S. has increased by more than 400% during 2007-2014. (Valilis, Ramsey, Sidiq, & DuPont, 2018). Furthermore, Cooley et al. (2013) evaluated produce, soil, livestock, wildlife, and water samples collected from a major leafy greens production region in California. It was found that 18.7% of the tested samples were positive for STEC, with STEC non-O157 at approximately 5-fold higher incidence than STEC O157, suggesting persistence of both STEC O157 and non-O157 in a leafy greens production environment (M. B. Cooley et al., 2013). It might explain our observation that vegetable row crops outbreaks were frequently attributed to STEC (**Table 10**). It is known that some of the vegetable row crops such as lettuce have leaves and rough surfaces that can trap water and favor the transmission of pathogens during irrigation (Stine et al., 2005). Therefore,

monitoring the microbial quality of irrigation water is crucial for the safety assurance of produce products, especially vegetable row crops.

Unlike STEC, a wide range of *Salmonella* serotypes were implicated in produce outbreaks. A large portion of these outbreaks was attributed to *Salmonella Enteritidis*, *Salmonella Newport*, *Salmonella Typhimurium* and *Salmonella Javiana* (**Table 11**). *Salmonella* is a ubiquitous enteric pathogen that comprises a large number of serotypes. It is known to have widespread occurrence and elevated survival capacities in the non-host environment causing a large number of foodborne illnesses (Levantesi et al., 2012). Traditionally, most foodborne *Salmonella* illnesses were thought to originate from animal products. However, produce outbreaks associated with *Salmonella* have been under the spotlight in recent years due to the increasing incidence. For example, the fresh papaya outbreak in 2019 causing 81 illnesses and 27 hospitalizations was attributed to *Salmonella Uganda*, and the pre-cut melons outbreak in 2019 causing 137 illnesses, and 38 hospitalizations was attributed to *Salmonella Carrau* (**Table 1**). It was observed that *Salmonella* was frequently implicated in seeded vegetable outbreaks (**Table 11**), particularly tomatoes (50 outbreaks). Hanning et al. (2009) summarized investigations of some *Salmonella* outbreaks due to contaminated tomatoes and found that contaminated irrigation water and contaminated wash water were often pinpointed as the source of

contaminations (Hanning, Nutt, & Ricke, 2009). It agrees with the fact that many produce outbreaks were widely dispersed, suggesting that contaminations occurred early in production. Besides, it was noted that melon was the most frequently implicated fruit in *Salmonella* outbreaks (**Table 11**). It aligns with the previous study by Tauxe et al. (1997), who found that watermelons and cantaloupes were among the common sources of *Salmonella* outbreaks (R. Tauxe et al., 1997). A survey of farm and processing facilities found that immersion in contaminated wash water in post-harvest facilities resulted in a majority of *Salmonella* contamination of melons (Gagliardi, Millner, Lester, & Ingram, 2003). Furthermore, the attachment of bacterial pathogens such as *Salmonella* to melons might be favored by surface irregularities such as roughness, crevices, and pits, thus reducing the ability of washing and chemical treatments to remove or inactivate attached pathogens (Ukuku & Fett, 2006).

Table 10: Number of STEC outbreaks attributed to produce by food category and STEC serotypes - National Outbreak Reporting System, U.S., 1998-2017

Food Category	No.	STEC serotypes						
		O157	O111	O26	O145	O6	O121	Other
Vegetables								
Fungi	0	0	0	0	0	0	0	0
Sprouts	10	7	0	1	1	0	1	0
Root and underground vegetables	9	5	0	0	0	2	0	2
Seeded vegetables	15	14	0	0	0	0	0	1
Herbs	5	3	0	0	0	1	0	1
Vegetable row crops	70	54	2	3	4	1	2	4
Unspecified vegetables	21	18	1	0	0	0	0	2
Subtotal	**130**	**101**	**3**	**4**	**5**	**4**	**3**	**10**
Fruits								
Melons	3	3	0	0	0	0	0	0
Pome fruits	12	8	2	0	0	0	1	1
Stone fruits	0	0	0	0	0	0	0	0
Small fruits	4	3	0	1	0	0	0	0
Tropical fruits	0	0	0	0	0	0	0	0
Sub-tropical fruits	1	1	0	0	0	0	0	0
Unspecified fruits	4	3	1	0	0	0	0	0
Subtotal	24	**18**	**3**	**1**	**0**	**0**	**1**	**1**
Total [a]	**154**	**119**	**6**	**5**	**5**	**4**	**4**	**11**

No., number.

[a], the total reflects the frequencies of all STEC serotypes being implicated in outbreaks, they might not add up to the number of total produce outbreaks associated with STEC due to outbreaks that attributed to multiple STEC serotypes.

Table 11: Number of Salmonella outbreaks attributed to produce by food category and Salmonella serotypes - National Outbreak Reporting System, U.S., 1998-2017

Food Category	No.	*Salmonella* serotypes							
		Enteritidis	*Typhimurium*	*Newport*	*Javiana*	*Braenderup*	*Heidelberg*	*Saintpaul*	Other
Vegetables									
Fungi	5	1	2	0	0	0	1	0	1
Sprouts	40	8	3	2	0	3	0	3	21
Root and underground vegetables	48	13	3	4	5	2	3	0	18
Seeded vegetables	120	17	10	23	13	8	5	9	35
Herbs	12	3	2	3	0	1	0	0	3
Vegetable row crops	34	6	4	4	4	2	1	1	12
Unspecified vegetables	93	25	11	5	5	4	3	0	40
Subtotal	**352**	**73**	**35**	**41**	**27**	**20**	**13**	**13**	**130**
Fruits									
Melons	38	2	6	9	2	0	3	1	15
Pome fruits	3	0	1	0	0	0	1	0	1
Stone fruits	1	0	0	0	0	0	0	1	0
Small fruits	10	0	1	2	0	0	0	2	5
Tropical fruits	21	3	1	2	0	2	1	1	11
Sub-tropical fruits	7	1	1	1	0	1	0	1	2
Unspecified fruits	18	4	0	5	2	0	1	1	5
Subtotal	**98**	**10**	**10**	**19**	**4**	**3**	**6**	**7**	**39**
Total [a]	**450**	**83**	**45**	**60**	**31**	**23**	**19**	**20**	**169**

No., number.

[a], the total reflects the frequencies of all *Salmonella* serotypes being implicated in outbreaks; they might not add up to the number of total produce outbreaks associated with STEC due to outbreaks that attributed to multiple *Salmonella* serotypes.

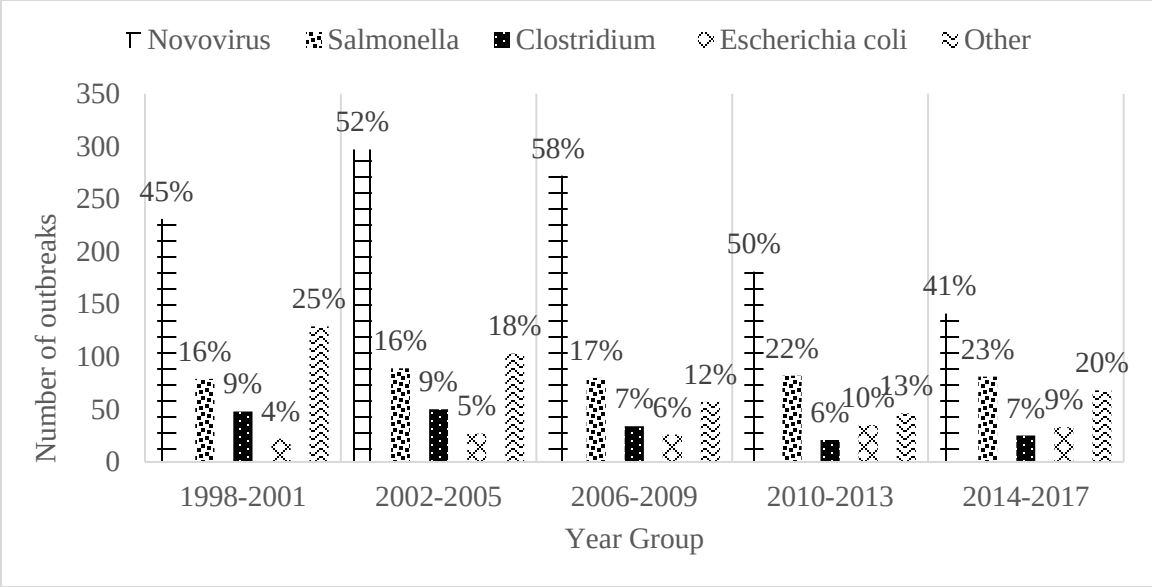

Figure 8: Numbers and percentages of produce outbreaks associated with the four primary etiological agents during the study period - National Outbreak Reporting System, U.S., 1998-2017

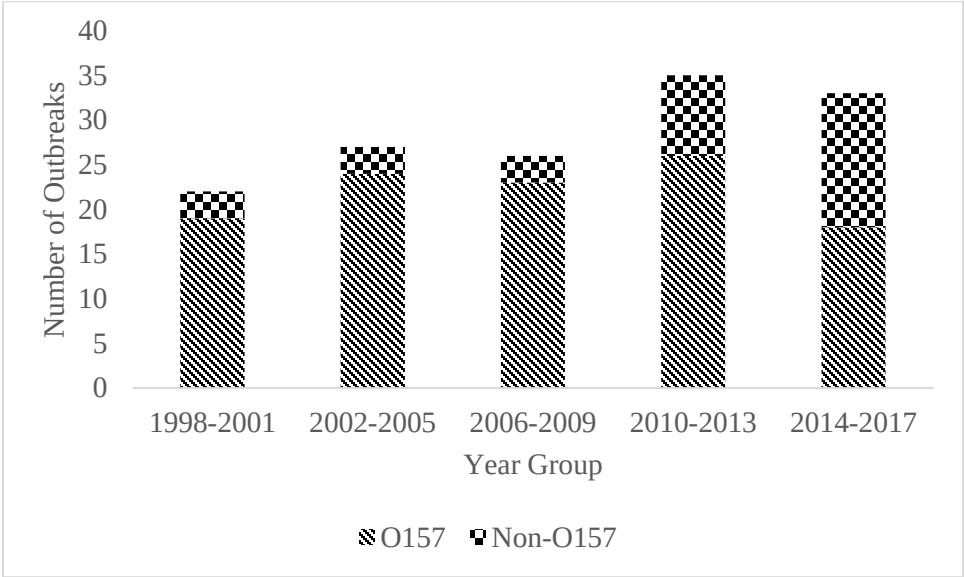

Figure 9: Numbers of produce outbreaks associated with STEC O157 and Non-O157 during the study period - National Outbreak Reporting System, U.S., 1998-2017

3.4 Summary

This report summarizes the epidemic analysis of foodborne illness outbreaks attributed to produce in the United States from 1998-2017. Norovirus remains the leading cause of produce outbreaks, most often implicated in single-state outbreaks. *Salmonella* and *E. coli* contribute to a large portion of multi-state produce illness outbreaks. The number of overall produce outbreaks has been decreasing due to regulatory efforts to improve production and service hygiene. However, there is a continuous increase of multi-state produce outbreaks indicating the vulnerability of the early stage of produce production to contaminations. In general, vegetables cause more produce outbreaks than fruits, particularly the seeded vegetables and the vegetable row crops. The increasing incidence of produce outbreaks associated with sprouts and tropical fruits suggests the need to further improve their safety regulations. The number of produce outbreaks caused by *Salmonella* and *Shiga-toxin producing E. coli* has been on the rise. It reconfirms the importance of preventions of these two pathogens in food production, particularly produce production. Preharvest elements, such as irrigation water, are believed to play an important role in produce contaminations. Therefore, robust preventive measures to ensure the microbial quality of irrigation water is essential to produce safety.

Chapter 4: A Novel Selective Medium for Sensitive Enrichment and Screening of Shiga Toxin-producing E. coli and Salmonella in Irrigation Water

4.1 Introduction

Fresh produce is one of the leading causes of foodborne illnesses in the United States. A wide variety of fresh produce has been implicated in *Shiga-toxin Producing E. coli* (STEC) and *Salmonella* outbreaks, including romaine lettuce, sprouts, cucumbers, peppers, tomatoes, and spinach (CDC, 2019a). In 2018, romaine lettuce was found to be the source of two major STEC outbreaks, which caused 272 infections, 121 hospitalizations and 5 deaths (CDC, 2018b, 2019b). The investigations by the FDA found that irrigation water was the source of contamination for both outbreaks (FDA, 2018, 2019e). The microbiological safety of fresh produce products remains challenging due to the open nature of fresh produce production, which makes it susceptible to contaminations from multiple sources, including soil, water, biological amendments, and wild animal activities (Murray, Wu, Shi, Jun Xue, & Warriner, 2017). Some pathogens can survive for an extended amount of time in the soil after irrigation, thus potentially leading to product contamination at harvest (Rajwar, Srivastava, & Sahgal, 2016). Therefore, it is crucial to adopt an effective monitoring method for the microbial quality of irrigation water. However, the detection and isolation of STEC and *Salmonella* present a technical challenge necessitating time-consuming and costly laboratory procedures that often exceed the technical and financial capabilities of many small growers and reference laboratories. In this study, we developed a colorimetric screening test for STEC and

Salmonella based on a highly selective enrichment medium. The test was adapted to microporous filtration membrane to permit screening of irrigation water.

4.2 Materials and Methods

4.2.1 Materials

Tryptic Soy Broth, MI Agar, Brain Heart Infusion were obtained from Becton Dickinson (Franklin Lakes, NJ). Tryptic Soy Agar, D-Raffinose, D-Arabinose, Bromocresol Purple, Peptone from casein, D-Xylose were obtained from Sigma-Aldrich (St. Louis, MO). D-Sorbitol was obtained from Fisher Scientific (Hampton, NH). Trehalose was obtained from GoldBio (St. Louis, MO). Bile salts was obtained from Honeywell Fluka (Charlotte, NC). CHROMagar™ STEC and CHROMagar™ were obtained from CHROMagar (Paris, France). The PDX-STEC medium was prepared according to instructions from the U.S. Patent: 9518283 (Olstein, 2016), with the addition of 0.025% (m/v) bromocresol purple. The modified PDX-STEC medium or mPDX-STEC medium was prepared by removing sulfanilamide and myricetin from the formulation. The modified tryptic soy broth (mTSB) was prepared by adding 0.15% (w/v) bile salts and 0.0008% (w/v) sodium novobiocin.

4.2.2 Bacterial strains

STEC and Salmonella strains were obtained from the Penn State University E. coli Reference Center in University Park, Pennsylvania, the Center for Disease Control and Prevention in Atlanta, Georgia, the U.S. Meat Animal Research Center (USDA Agricultural Research Services) in Clay Center, Nebraska, the American Type Culture Collection (ATCC) in Manassas, Virginia, and the University of Minnesota Veterinary Diagnostic Laboratory in Saint Paul, Minnesota. Bacterial cultures were maintained as glycerol stock at -20℃ and revived in TSB at 37℃ overnight before use.

4.2.3 Inclusivity and Exclusivity Study of PDX-STEC

Inclusivity study included 50 STEC strains and 103 Salmonella serotypes. Cultures were first serially diluted to determine the log dilution yielding plate counts of less than 10 CFU per plate (minimum concentration). Each inclusivity culture was diluted in PDX-STEC medium to 100 times the minimum concentration and cultured in 96-well microtiter plates (Thermo Fisher Scientific, MA) containing 200 µL PDX-STEC medium at 37℃ for 18-24 hours. Exclusivity study included 30 isolates of closely related non-STEC and non-*Salmonella* strains. Exclusivity cultures were cultured in the PDX-STEC medium at 37℃ for 18-24 hours without any dilutions. Each enrichment culture was streaked onto CHROMagar™ STEC or CHROMagar™ *Salmonella*, and TSA plates. Plates were incubated at 37℃ for 18-24 hours. STEC and *Salmonella* strains should grow

in the PDX-STEC medium and give typical results (mauve-colored colonies) on CHROMagar™ STEC and CHROMagar™ Salmonella plates, respectively. The exclusivity strains should grow poorly, or not at all, in the PDX-STEC broth. Results were recorded for each isolate on each plate.

4.2.4 Investigation of Fermenting Ability of STEC and *Salmonella*

The carbohydrate-fermenting-ability of the STEC and *Salmonella* isolates was examined with the following five carbohydrates: D-arabinose, D-trehalose, D-sorbitol, D-raffinose, and xylose. The fermenting abilities of the strains were determined by culturing the isolates in 96 well microtiter plates containing 200 µL of bovine heart infusion (BHI) broth supplemented with bromocresol purple (0.025%, w/v) and a particular carbohydrate (1%, w/v). Positive results were identified by the color of the medium changing from purple to brown or yellow after 24 hours incubation at 37℃.

4.2.5 Optimization of Sensitivity of PDX-STEC

Freshly prepared STEC O111, STEC O157, STEC O26, *Salmonella Enteritidis, Salmonella Newport, and Salmonella Typhimurium* cultures were serially diluted in sterile 0.1% peptone water. One hundred milliliters of sterile DI water samples were inoculated with 100 µL of the inoculum at different dilutions to obtain final inoculation

levels between 0 CFU and 10^5 CFU per 100mL. After 30 min at room temperature, the samples were filtered through a sterile membrane with 0.45 µm pores to collect cells onto the membrane following the procedure depicted in **Figure 10**. The membrane was immersed and incubated in 3 mL of PDX-STEC medium at 37°C for 24 hours. The limit of detection (LOD) for each STEC and *Salmonella* strain was identified as the lowest inoculum level tested which resulted in a color change of the medium from purple to brown or yellow. In addition to the original PDX-STEC medium, the LOD was also determined for a modified PDX-STEC medium (mPDX-STEC) made by subtracting sulfanilamide and myricetin from the formulation.

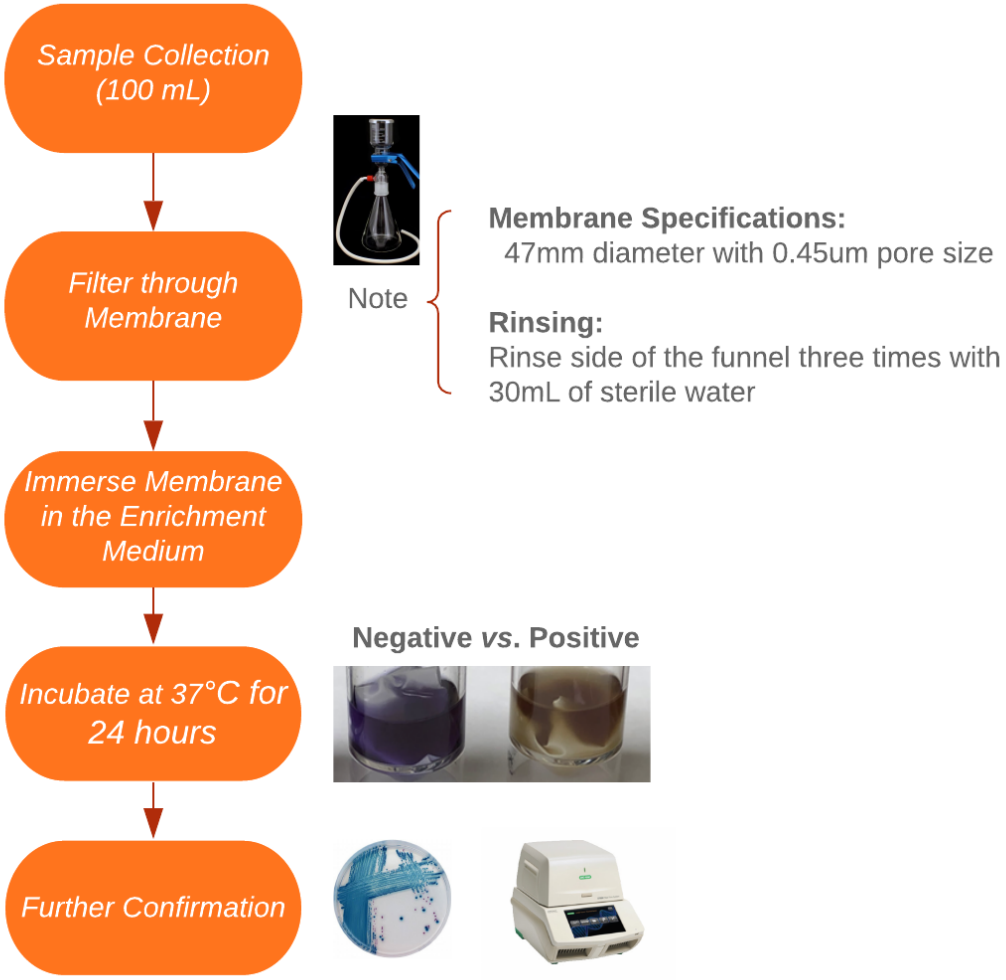

Figure 10: Diagram of the adapted procedure for water analysis

4.2.6 Comparison of Sensitivity and Selectivity of mPDX-STEC with mTSB

Groundwater samples were collected from well water in Minnesota and Wisconsin. Water samples (n=16) were co-inoculated with STEC *O157 and S. Enteritidis*, or STEC O111 and *S. Typhimurium*, or STEC O26 and *S. Newport* at low levels (~5 CFU per 100 mL). After 30 min at room temperature, each sample was filtered through a sterile membrane with 0.45 µm pores to collect cells onto the membrane following the procedure depicted in **Figure 10**. The membrane was immersed and incubated in 3 mL of the mPDX-STEC medium (n=8) or mTSB (n=8) at 37℃ for 24 hours. The color change of the samples in the mPDX-STEC medium was recorded at the end of the incubation. A loopful of culture enrichment was streaked onto CHROMagar™ STEC and CHROMagar™ *Salmonella* bi-plate to identify positive samples. In addition, experiments were repeated with ground water samples collected from a different source with enrichment temperature at both 37℃ and 42℃.

4.2.7 Evaluation of Microbial Quality of Water Samples from Different Sources

Seventeen surface water samples were collected from different sites along the St. Croix and Mississippi rivers from both Minnesota and Wisconsin, as well as lakes in Minnesota, and two ground water samples were collected from two wells in Minnesota

and Wisconsin (**Table 12**). One hundred milliliters of the surface water sample were filtered through a sterile membrane with 0.45 µm pores to collect cells onto the membrane following the procedure depicted in **Figure 10**. The membrane was immersed and incubated in 3 mL of mPDX-STEC medium at 37℃ or 42℃ for 24 hours. A loopful of aliquot from each culture enrichment was streaked onto CHROMagar™ STEC and CHROMagar™ Salmonella bi-plate to identify positive samples. Suspect colonies were picked, and DNA purifications were conducted according to the instruction of a commercial DNA purification kit. Real-time PCR analysis targeting *stx*1, *stx*2, and *eae* genes of STEC and the *inv*A gene of *Salmonella* were performed using a Chai open PCR dual-channel instrument (**Table 13**). In addition, the number of generic *E. coli* of each water sample was determined using EPA Method 1604 (Oshiro, 2002).

Table 12: Sampling locations of surface water

Sample ID Number	Location
1	Hasting, MN - Lake Rebecca - US Lock and Dam 2
2	Vermillian River - Public Access, County Roads 54 & 68 Ravina Township
3	Bay City, MN - Small Inlet to Lake Pepin/Boat launch
4	Bay City, MN - Public Beach (Mississippi River- Lake Pepin)
5	Maiden Rock, WI - Public Beach (Mississippi River- Lake Pepin)
6	Stockholm, WI - Campground (Mississippi River- Lake Pepin)
7	Pepin, WI - YMCA Camp Beach (Mississippi River- Lake Pepin)
8	Linstrom, MN - South Lindstrom Lake Beach
9	Taylor Falls, MN - St. Croix Interstate Park (St. Croix River, boat launch)
10	Taylor Falls, MN - North Lions Park (St. Croix River boat launch)
11	Center City, MN - Wild River State Park, (St. Croix River boat launch)
12	White Bear Lake, MN - Otter Lake boat launch
13	White Bear Lake, MN - Bald Eagle Lake - Spring running into Lake
14	White Bear Lake, MN - White Bear Lake. Memorial Park Beach
15	Vadnais Heights, MN - Sucker Lake
16	Vadnais Heights, MN - Vadnais Lake
17	Roseville, MN - Lake McArrons, boat launch

Table 13: Sequence of DNA probe and primers used in this study (Eggers et al., 2018)

	Probe [a]	Primers [b]
STEC virulence markers		
stx$_1$	5' 56-FAM-CTG GAT GAT/zen/ CTC AGT GGG CGT TCT TAT GTA A-3IABkFQ 3'	(F) 5' TTT GTY ACT GTS ACA GCW GAA GCY TTA CG 3' (R) 5' CCC CAG TTC ARW GTR AGR TCM ACD TC 3'
stx$_2$	5' 56-FAM-TCG TCA GGC /zen/ ACT GTC TGA AAC TGC TCC-3IABkFQ 3'	(F) 5' TTT GTY ACT GTS ACA GCW GAA GCY TTA CG 3' (R) 5' CCC CAG TTC ARW GTR AGR TCM ACD TC 3'
stx$_3$	5' 56-FAM-ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC-IABkFQ 3'	(F) 5' CAT TGA TCA GGA TTT TTC TGG TGA TA 3' (R) 5' CTC ATG CGG AAA TAG CCG TTM 3'
Salmonella virulence markers		
invA	5' 56-FAM-TAC CGG CCT /zen/ TCA AAT CGG CA-3IABkFQ-3'	(F) 5' TCA TCG CAC CGT CAA AGG AACC-3' (R) 5' GTG AAA TTA TCG CCA CGT TCG GGC AA 3'

[a], mixed nucleotide key: Y (C, T), W (A, T), R (A, G), M (A, C), D (A, G, T)
[b], (F), forward primer; (R), reverse primer

4.2.8 Statistical Analysis

To determine the LOD values of PDX-STEC and mPDX-STEC, triplicate experiments on different dates were performed. All data obtained were interpreted by an analysis of variance (ANOVA) using Tukey and FisherLSD tests at a significance level of 5%. Data were analyzed using OriginPro, version 9.0 (OriginPro software, OriginLab Corporation, Northampton, MA). The comparisons between probabilities of detection of pathogens using mPDX-STEC and mTSB were conducted using Fisher's Exact Test. A p value of larger than 0.05 was considered to indicate no significant difference in the numbers of positive test portions given by the conditions being compared. Data were analyzed using RStudio version 1.2.1335. The correlations between the generic E. coli populations and the present of STEC or Salmonella in water samples were analyzed by calculating Pearson correlation coefficient using RStudio version 1.2.1335. A Pearson correlation coefficient at 1 means a perfect positive correlation, and a Pearson correlation coefficient at -1 means a perfect negative correlation. A p value larger than 0.05 was considered to indicate no statistical significance of the reported correlation.

4.3 Results and Discussion

4.3.1 Investigation of Inclusivity and Exclusivity of PDX-STEC

Both STEC and *Salmonella* comprise a large number of serotypes. Among all the STEC serotypes, STEC O157 is particularly important as it accounted for most of the produce outbreaks attributed to STEC (**Table 10**). However, it was noted that the number

of produce outbreaks attributed to STEC non-O157 serotypes has been increasing (**Figure 9**). Therefore, it is important to test the inclusivity of the PDX-STEC medium for both STEC O157 and STEC non-O157 serotypes. Besides, inclusivity is particularly important for *Salmonella* as a wide range of *Salmonella* serotypes were implicated in foodborne illness outbreaks associated with produce (**Table 11**). The inclusivity study results are depicted in **Table 14** and **Table 15**. A significant fraction of the STEC strains used were of unknown origin because they were obtained from commercial culture collections. The proportion of STEC inclusivity strain obtained from USDA Agricultural Research Services was primarily derived from beef, veal or feedlot fecal matter. All the tested STEC strains grew well in PDX-STEC medium, except one STEC O91 isolate. However, it was noted that the other two STEC O91 isolates grew well in the PDX-STEC medium. It is not uncommon that isolates of the same bacteria serotype from difference sources exhibit heterogeneous characteristics. Mellmann et al. (2009) investigated the diversity and relatedness of one hundred STEC O91 isolates. The authors were able to further divide the STEC O91 isolates into 10 different sequence types exhibiting different characteristics such as virulence (Mellmann et al., 2009). Furthermore, STEC O91 does not impose as significant public health risks as some other STEC serotypes. In fact, there was only one foodborne illness outbreak reported to CDC was attributed to STEC O91

(CDC, 2017). All the regulated STEC serotypes, i.e., O157, O111, O121, O145, O45, O26, and O103, as well as several other STEC serotypes, grew well in PDX-STEC medium (**Table 14**). All the tested *Salmonella* serotypes grew well in the PDX-STEC medium.

Irrigation water from different water sources contain a variety of background microflora. Although most of them are not pathogenic to humans, thus imposing little health risks, they may interfere with the selective enrichment for pathogens comprising its outcome. Therefore, it is crucial to evaluate the exclusivity of PDX-STEC against common background bacteria that might interfere with the enrichment of STEC and *Salmonella*. The exclusivity study results are depicted in **Table 16**. All the commensal *E. coli* strains grew poorly or not at all in the PDX-STEC medium. Of the 30 exclusivity bacteria tested, only *Citrobacter braakii* and *Enterobacter cloacae* grew in the PDX-STEC medium. The remaining 28 strains, including *E. aerogenes* and *E. aergoviae* grew poorly or not at all. Overall, the PDX-STEC medium exhibited great inclusivity and exclusivity for selective enrichment of STEC and *Salmonella* from irrigation water.

Table 14: STEC inclusivity for PDX-STEC

No.	Serotype	Origin	PDX-STEC Growth [a]
1	O111:H8	Unkown	+
2	O45:H2	Unkown	+
3	O157:H7	Unkown	+
4	O104:H4	Unkown	+
5	O157:H7	Unkown	+
6	O91:H21	Unkown	-
7	O157:H7	Unkown	+
8	O145:H28	Unkown	+
9	O104:H4	Unkown	+
10	O111:H8	Unkown	+
11	O157:H7	Unkown	+
12	O26:H11	Unkown	+
13	O157:H7	Unkown	+
14	O103:H11	Unkown	+
15	O103:H2	Unkown	+
16	O111:H28	Unkown	+
17	O5:ND	Feces	+
18	O74:ND	Feces	+
19	O109:ND	Feces	+
20	O177:ND	Feces	+
21	O121:ND	Feces	+
22	O121:ND	Carcass	+
23	O121:ND	Carcass	+
24	O118: ND	Veal	+
25	O84: ND	Veal	+
26	O69: ND	Veal 4	+
27	O111:H8	Unkown	+
28	O145:NM	Unkown	+
29	O26:H11	Unkown	+
30	O26:H11	Human	+
31	O26:H11	Beef	+
32	O45:H2	Human	+
33	O45:H2	Beef	+
34	O45:ND	Beef	+
35	O103:H2	Beef	+
36	O103:H2	Beef	+
37	O91:H21	Beef	+
38	O91:H21	Beef	+

39	O145:NM	Human	+
40	O145:H28	Beef	+
41	O26:H11	Beef	+
42	O111:ND	Beef	+
43	O111:ND	Beef	+
44	O157:H7	Beef	+
45	O157:H7	Beef	+
46	O157:H7	Beef	+
47	O157:H7	Beef	+
48	O145:H28	Beef	+
49	O145:NM	Human	+
50	O111:H8	Human	+

[a], +, positive ; -, negative.

Table 15: Salmonella inclusivity for PDX-STEC

No.	Serotype	Origin	PDX-STEC Growth [a]
1	*S. Abaetetuba*	Freshwater	+
2	*S. Aberdeen*	Infantile diarrhea	+
3	*S. Adelaide*	Meat meal	+
4	*S. Bilthoven*	Unkown	+
5	*S. Agona*	Soybean meal	+
6	*S. Abony*	Human feces	+
7	*S. Alachua*	Swine	+
8	*S. Albany*	Unkown	+
9	*S. Amsterdam*	Unkown	+
10	*S. Anatum*	Chicken feed	+
11	*S. Arizonae*	Unkown	+
12	*S. Artis*	Unkown	+
13	*S. Avechaveleta*	Unkown	+
14	*S. Bareilly*	Unkown	+
15	*S. Berkeley*	Unkown	+
16	*S. Bergen*	Unkown	+
17	*S. Blockley*	Environment	+
18	*S. Bovismorbificans*	Vietnam	+
19	*S. Bongori*	Unkown	+
20	*S. Brandenburg*	Swine	+
21	*S. Bredney*	Unkown	+
23	*S. Budapest*	Unkown	+
24	*S. California*	Animal feed	+
25	*S. Canoga*	Unkown	+
26	*S. Chandans*	Unkown	+
27	*S. Carrau*	Frozen shrimp	+
28	*S. Cerro*	Poultry feed	+
29	*S. Champaign*	Unkown	+
30	*S. Chester*	Frozen tilapia fish	+
31	*S. Chittagong*	Unkown	+
32	*S. Choleraesuis*	Fish	+
33	*S. Dusseldorf*	Unkown	+
34	*S. Cubana*	Swine feed	+
35	*S. Diarizonae*	Unkown	+
36	*S. Dahlem*	Unkown	+
37	*S. Derby*	Polluted water	+
38	*S. Dublin*	Cattle	+
39	*S. Ealing*	Dried baby milk	+

40	*S. Emek*	Frozen catfish	+
41	*S. Enteritidis*	Ice cream	+
42	*S. Gallinarum*	Poultry	+
43	*S. Give*	Lobster tail	+
44	*S. Gloucester*	Sesame seeds	+
45	*S. Goodwood*	Feces	+
46	*S. Hadar*	Turkey	+
47	*S. Heidelberg*	Poultry	+
48	*S. Havana*	Unkown	+
49	*S. Hvittingfoss*	Frozen frog legs	+
50	*S. Ikeja*	Frozen Shrimp	+
51	*S. Indiana*	Unkown	+
52	*S. Infantis*	Frozen lobster tail	+
53	*S. Javiana*	Frozen shrimp	+
54	*S. Johannesburg*	Meat meal	+
55	*S. Kentucky*	Cottonseed meal	+
56	*S. Kingston*	Unkown	+
57	*S. Kumasi*	Frozen crab meat	+
58	*S. Lexington*	Unkown	+
59	*S. Lille*	Chicken feed	+
60	*S. Limete*	Unkown	+
61	*S. London*	Polluted water	+
62	*S. Manchester*	Unkown	+
63	*S. Manhattan*	Avian	+
64	*S. Mbandaka*	Soybean meal	+
65	*S. Meleagridis*	Frozen shrimp	+
66	*S. Minnesota*	Swine	+
67	*S. Mississippi*	Unkown	+
68	*S. Montevideo*	Raw eggs	+
69	*S. Muenchen*	Frozen shrimp	+
70	*S. Moscow*	Unkown	+
71	*S. Nashua*	Poultry feed	+
72	*S. Newbrunswick*	Frozen shrimp	+
73	*S. Newington*	Wild poultry	+
74	*S. Newport*	Frozen lobster tail	+
75	*S. Ohio*	Animal feed	+
76	*S. Oranienburg*	Egg	+
77	*S. Panama*	Infantile diarrhea	+
78	*S. Paratyphis*	Sewage	+
79	*S. Paratyphis*	Frozen frog legs	+
80	*S. Pomona*	Unkown	+

81	*S. Pensacola*	Unkown	+
82	*S. Poona*	White pepper	+
83	*S. Potsdam*	Unkown	+
84	*S. Rubislaw*	Frozen shrimp	+
85	*S. Saint Paul*	Milk powder	+
86	*S. Salford*	Unkown	+
87	*S. Senttenbas*	Unkown	+
88	*S. Senftenberg*	Sewage	+
89	*S. Tel Aviv*	Unkown	+
90	*S. Stanley*	Reptile	+
91	*S. Sterrenbos*	Frozen shrimp	+
92	*S. Uganda*	Unkown	+
93	*S. Tallahassee*	Unkown	+
94	*S. Tennessee*	Soybean meal	+
95	*S. Thomasville*	Poultry meal	+
96	*S. Thompson*	Ice cream	+
97	*S. Typhimurium*	Salted dune egg	+
98	*S. Urbana*	Reptile	+
99	*S. Vallore*	Unkown	+
100	*S. Virchow*	Basil	+
101	*S. Waycross*	Urine	+
102	*S. Weltevreden*	Dried ling shrimp	+
103	*S. Worthington*	Chicken feed	+

[a], +, positive ; -, negative.

Table 16: Exclusivity strains for PDX-STEC

No.	Strain	Origin	PDX-STEC Growth [a]
1	*E. coli* K12-W3110	Feces	-
2	*E. coli* 261	Feces	-
3	*E. coli* A11-3a	Feces	-
4	*E. coli* ATCC 10799	Unkown	-
5	*E. coli* ATCC 25922	Unkown	-
6	*E. coli* 3TF1	Feces	-
7	*E. coli* 3BF2	Feces	-
8	*E. coli* ECOR-17	Feces	-
9	*E. coli* W-21	Feces	-
10	*E. coli* GB4 H7	Feces	-
11	*E. coli* MDR 0215	Feces	-
12	*E. coli* ECOR-71	Feces	-
13	*Citrobacter brakii*	Unkown	+
14	*Edwardsiella tarda*	Unkown	-
15	*Enterobacter aerogenes*	Unkown	-
16	*Hafnia alvei*	Unkown	-
17	*Klebsiella oxytoca*	Unkown	-
18	*Klebsiella pneumoniae*	Unkown	-
19	*Morganella morganii*	Unkown	-
20	*Proteus mirabilis*	Unkown	-
21	*Providencia stuartii*	Unkown	-
22	*Serratia marcescens*	Unkown	-
23	*Shigella flexnerii*	Unkown	-
24	*Shigella boydii*	Unkown	-
25	*Yersinia enterocolitica*	Unkown	-
26	*Yersinia ruckeri*	Unkown	-
27	*Providencia alcalifaciens*	Unkown	-
28	*Ralstonia insidiosa*	Unkown	-
29	*Enterobacter cloacae*	Unkown	+
30	*Enterobacter gergoviae*	Unkown	-

[a], +, positive ; -, negative.

4.3.2 Characterization of Carbohydrate-Fermenting Abilities

Results of analysis of the carbohydrate-fermenting abilities of 44 STEC strains and 25 *Salmonella* strains for the five carbohydrates are shown in **Table 17**. All STEC and *Salmonella* serotypes were not able to ferment arabinose within 24 hours except three STEC O26, one STEC O145, and two *S. Typhimurium* strains. Similarly, only STEC O26, two STEC O145 strains, and two *S. Typhimurium* strains were able to ferment raffinose. In general, sorbitol exhibited good fermentability by most of the tested STEC and *Salmonella* strains with an exception that two STEC O111 and three STEC O157 strains were not able to ferment sorbitol within 24 hours. It was unexpected that sorbitol was fermentable by one of the tested STEC O157 strains. It is believed that typical *E. coli* O157: H7 does not ferment sorbitol at 24 hours (Farmer & Davis, 1985). In fact, sorbitol has been commonly used in media formulations to differentiate *E. coli* O157: H7 from other fecal *E. coli*, such as in the MacConkey Agar with Sorbitol (SMAC). However, sorbitol-fermenting *E. coli* O157 strains are not uncommon. Many studies have reported isolations of sorbitol-fermenting *E. coli* O157 from clinical specimens (Gunzer et al., 1992; Karch et al., 1990). Furthermore, sorbitol positive STEC O157:H7 mutant has also been previously reported (Fratamico, Buchanan, & Cooke, 1993). The STEC O157 strain exhibited sorbitol fermenting ability in current study was from an unknown source. It is possible that it belongs to one of the sorbitol positive STEC O157 strains.

All tested STEC and *Salmonella* strains were able to ferment both xylose and trehalose within 24 hours. It agrees with the previous study by Hiramatsu et al. (2002). The authors reported that all the tested STEC O26, O157, and O111 strains were able to

ferment xylose and trehalose (Hiramatsu et al., 2002). It was noted that fermentation of trehalose by some of the tested STEC and *Salmonella* strains were more efficient than fermentation of xylose, which resulted in a larger pH drop reflecting on a color change to a greater extent. Besides, it has been previously reported that xylose had poor fermentability among certain minor serotypes of STEC, such as O119:H4, O121:H19, O165 [HUT] (Seto, Taguchi, Kobayashi, & Kozaki, 2007). Moreover, Shamanna and Sanerson (1979) reported a 50% reduction of the uptake of xylose by *Salmonella Typhimurium* LT2 when the osmotic shock was applied (Shamanna & Sanderson, 1979). Therefore, trehalose appears to be a better option as the sole carbohydrate source offering advantages of applicability to a broader range of STEC serotypes and consistent efficacy for *Salmonella* under stress. In fact, trehalose has been shown to be an osmoregulatory solute in many bacteria, including *E. coli* and *Salmonella*, crucial for the prevention of osmotic dehydration of the cells under stressed growth environments (Dupray et al., 1995; Giaever et al., 1988).

Table 17: Fermentation of selected carbohydrates by STEC and Salmonella strains [c]

Pathogen	Serotype	Carbohydrates				
		Arabinose	Raffinose	Xylose	Sorbitol	Trehalose
STEC	O157	4 [a] (0) [b]	4 (0)	4 (4)	4 (1)	4 (4)
	O111	9 (0)	9 (0)	9 (9)	9 (7)	9 (9)
	O26	8 (3)	8 (8)	8 (8)	8 (8)	8 (8)
	O103	6 (0)	6 (0)	6 (6)	6 (6)	6 (6)
	O6	1 (0)	1 (0)	1 (1)	1 (1)	1 (1)
	O121	4 (0)	4 (0)	4 (4)	4 (3)	4(4)
	O45	6 (0)	6 (0)	6 (6)	6 (6)	6 (6)
	O145	6 (1)	6 (2)	6 (6)	6 (2)	6 (6)
Salmonella	*Heidelberg*	1 (0)	1 (0)	1 (1)	1 (1)	1 (1)
	Javiana	1 (0)	1 (0)	1(1)	1 (1)	1 (1)
	Muenchen	1 (0)	1 (0)	1 (1)	1 (1)	1 (1)
	Typhimurium	6 (2)	6 (2)	6 (6)	6 (6)	6 (6)
	St. Paul	2 (0)	2 (0)	2 (2)	2 (2)	2 (2)
	Newport	7 (0)	7 (0)	7 (7)	7 (7)	7 (7)
	Braenderup	4 (0)	4 (0)	4 (4)	4 (4)	4 (4)
	Enteritidis	3 (0)	3 (0)	3 (3)	3 (3)	3 (3)

[a], number of strains examined
[b], number of strains exhibiting positive results
[c], the results were confirmed from at least 3 replicates

4.3.3 Evaluation and Optimization of Sensitivity of PDX-STEC

The PDX-STEC exhibited good sensitivity for STEC O26, *S. Typhimurium*, *S. Newport*, and *S. Enteritidis*. The LOD values for these serotypes were below 10 CFU per 100 mL of water samples (**Table 18**), which would be considered of acceptable pathogen concentrations in water in terms of the risk level of infections according to the estimates reported by Stine at al. (2005). The authors determined that a concentration of *Salmonella* in irrigation water at 2.5 CFU/mL would result in a 1:10,000 annual risk of infection from irrigated produce which is the goal set by the U.S. EPA for risk of waterborne pathogen infections and has been widely used for risk assessments of the use of reclaimed wastewater for food crop irrigations (Stine et al., 2005). However, it was noted that the extent of color change of the PDX-STEC medium of positive samples was not always very significant (**Figure 11 A**) at end of the 24 hours incubation, suggesting that the growth of pathogens in the PDX-STEC medium was suppressed to a certain degree. The compromised growth was further confirmed by the continuous shift of color of the PDX-STEC during the extended incubation (**Figure 11 B**). One possible explanation is that the injuries occurred to the targeting cells during the filtering process (**Figure 10**) might decrease their recoveries in the enrichment medium. Kenner et al. (1961) observed that the recovery of fecal *Streptococci* from surface water samples in enrichment medium was reduced by 50% when using membrane filters (Kenner, Clark, & Kabler, 1961). Furthermore, the sample preparation steps might impose additional stress to the targeting cells. Hoadley & Cheng (1974) found that the injuries of cells occurred during sample preparations prevented recovery of viable cells on selective media and suggested to

reduce the selectivity of enrichment media against injured cells. (Hoadley & Cheng, 1974). Besides, selective media usually contain combinations of antimicrobial agents and tend to have less nutrients than non-selective media, which might compromise the recoveries of cells to a certain extent during the enrichment. The compromised recoveries of both STEC and *Salmonella* have been previously reported for several selective enrichment media (Juven et al., 1984; P. Taormina, Rocelle, Clavero, & Beuchat, 1998). Most importantly, it was noted that the PDX-STEC medium was not sensitive for screening STEC O157 and STEC O111 as their LOD values were both above four log units (**Table 18**). It is particularly problematic as STEC O157 is the most frequently implicated STEC serotype in foodborne illness outbreaks. Good sensitivity for STEC O157 is essential for a selective enrichment to be applicable for STEC screening.

Therefore, we were able to develop a modified formulation of the PDX-STEC by removing myricetin and sulfanilamide. Myricetin is a common plant-derived flavonoid exhibiting antibacterial activities against several microorganisms such as *Enterobacter* and *Klebsiella*. In general, it is believed to inhibits many commensal *E. coli* but have no effect against STEC or *Salmonella* at concentrations of about 1 milligram per liter (Olstein, 2016). However, the PDX-STEC medium contains efflux pump inhibitors such as 1-(1-naphthylmethyl)-piperazine and 4-chloroquinoine, which might increase the activities of antimicrobial agents such as myricetin and lower their minimum inhibitory concentrations (MIC) against STEC and *Salmonella*. Furthermore, it was previously reported that myricetin was able to inhibit *E. coli* DnaB helicase, which is an essential enzyme for DNA replication and elongation, thus potentially hindering the proliferation

of *E. coli* including the Shiga-toxin producing serotypes (Griep, Blood, Larson, Koepsell, & Hinrichs, 2007). It agrees with another study from Lee at al. (2010) which reported bacteriostatic effects from flavonoids on the growth of STEC O157 (Lee, Moon, Kim, Mendonca, & Paik, 2010). Sulfanilamide is another antimicrobial agent in the PDX-STEC formulation. It is an organic sulfur compound exhibiting antimicrobial activities against most Gram-positive and many Gram-negative bacteria. A previous study characterized the antibiotic resistance of STEC O157 and found little resistance of STEC O157 to sulfanilamide (Bel, 2018). On the other hand, the removal of myricetin and sulfanilamide should impose limited effects on reducing selectivity of the PDX-STEC medium. First of all, the mPDX-STEC contains combinations of selective agents such as aminocoumarins, cycloheximide, supravital stain, ascorbic acid, bromobenzoic acid, and polyketides (Olstein, 2016), which will compensate the selectivity from myricetin and sulfanilamide. Besides, the mPDX-STEC still contains sulfathiazole, which is another sulfa drug exhibiting similar toxicity of sulfanilamide. Moreover, slightly lowering the antimicrobial properties of selective medium is unlikely to substantially reduce selectivity against background bacteria as they tend to be more sensitive to antimicrobial activities. For example, sediment *E. coli*, which is a common source of background *E. coli* community in freshwater environments, was found to be more susceptible to antibiotics than some of other *E. coli* such as STEC O157 (Bel, 2018).

It was noted that, by removing myricetin and sulfanilamide from the formulation, the sensitivity of the mPDX-STEC medium for STEC O157 and O111 was significantly improved (**Table 18**). Furthermore, the extent of color change of the mPDX-STEC

medium of positive samples at end of the 24 hours incubation was more substantial when compared to the PDX-STEC medium (**Figure 11 C**), suggesting an improved growth condition from the mPDX-STEC medium. It improves the ease of identification of positive samples during screening.

Table 18: Limit of Detection of PDX-STEC and mPDX-STEC for STEC and *Salmonella* Screening in Water Samples

Pathogen	Serotype	Limit of Detection (CFU/100mL)	
		PDX-STEC	mPDX-STEC
STEC	O157	74000 ± 12000 [a]	4.53 ± 0.86 [b]
	O111	52833 ± 16833 [a]	3.04 ± 0.45 [b]
	O26	6.57 ± 2.77 [a]	4.15 ± 0.35 [a]
Salmonella	*Typhimurium*	0.99 ± 0.29 [a]	6.22 ± 1.25 [a]
	Newport	6.05 ± 1.05 [a]	5.52 ± 0.72 [a]
	Enteritidis	6.30 ± 1.27 [a]	3.20 ± 0.31 [a]

For each STEC or *Salmonella* serotypes, values with different superscript letters indicate that the difference of LOD between two medium formulations is significant at the 0.05 level.

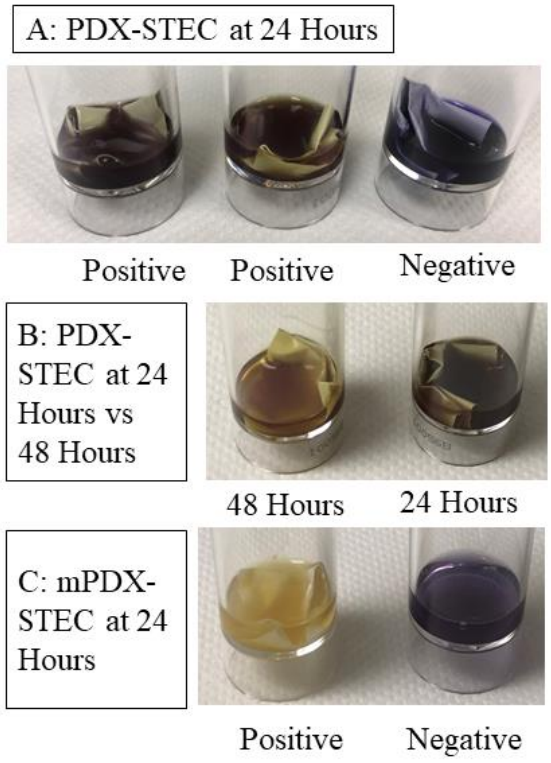

Figure 11: Examples of positive results from PDX-STEC and mPDX-STEC

4.3.4 Comparison of Sensitivity and Selectivity of mPDX-STEC with mTSB

The results of the comparison of paired STEC-Salmonella enrichment recoveries in mPDX-STEC and mTSB from ground water (No. 18 in Table 20) are depicted in **Table 19**. Both mPDX-STEC and mTSB exhibited similarly good sensitivity for detection of low levels (less than 5 CFU per 100mL) of paired STEC-Salmonella. The inoculated sample sets incubated in both mPDX-STEC and mTSB did not miss any of the *Salmonella* completely and only missed small portions of STEC. One notable exception appeared to be STEC O26. Significantly fewer positive STEC O26 plates were identified from enrichments in mTSB than mPDX-STEC (38% vs 100%). The results agreed with the previous study from Eggers et al. (2018). The authors found that the STEC and Salmonella selective (SSS) broth, which had similar formulation as the mPDX-STEC, exerted greater recovery of STEC in ground beef enrichments than the use of mTSB (Eggers et al., 2018). It was also reported that the recovery of *Salmonella* from ground beef was comparable for SSS broth and mTSB broth (Eggers et al., 2018). It needs to be noted that the results presented and analyzed in **Table 19** were based on identifying and confirming at least one colony of STEC or *Salmonella* to consider the replicate positive. However, the CHROMagar^{TM} STEC plates streaked from mTSB enrichments showed only a small number of STEC O111 and STEC O26 colonies (**Figure 12**). By contrast, the populations recovered on CHROMagar^{TM} STEC from mPDX-STEC enrichments were dense for all the tested STEC serotypes (**Figure 12**). The observation suggested that the growth of STEC O111 and STEC O26 was greatly suppressed in mTSB. It could be problematic for detection of low levels of STEC in water samples containing complex

background microflora as they might outcompete STEC during the enrichment potentially leading to false negative results. Furthermore, it would be challenging to identify a few suspect pathogen colonies from large population of non-suspect background microflora on CHROMagar™ plates.

Table 19: Comparison of paired STEC-*Salmonella* enrichment recoveries in mPDX-STEC and mTSB (Water Sample No.18)

STEC: Salmonella Serotypes	Enrichment Media	% Positive mPDX-STEC indicator	% Positive STEC Plates	% Positive Salmonella Plates
Negative Control	mPDX-STEC	0	0 [a]	0 [a]
	mTSB	NA	0 [a]	0 [a]
STEC O157: *S. Enteritidis*	mPDX-STEC	88	88 [a]	100 [a]
	mTSB	NA	88 [a]	100 [a]
STEC O111: *S. Typhimurium*	mPDX-STEC	100	100 [a]	100 [a]
	mTSB	NA	88 [a]	100 [a]
STEC O26: *S. Newport*	mPDX-STEC	100	100 [a]	100 [a]
	mTSB	NA	38 [b]	100 [a]

For each STEC: *Salmonella* pair, values with different superscript letters indicate that the difference of probability of detection between two enrichment media is significant at the 0.05 level.

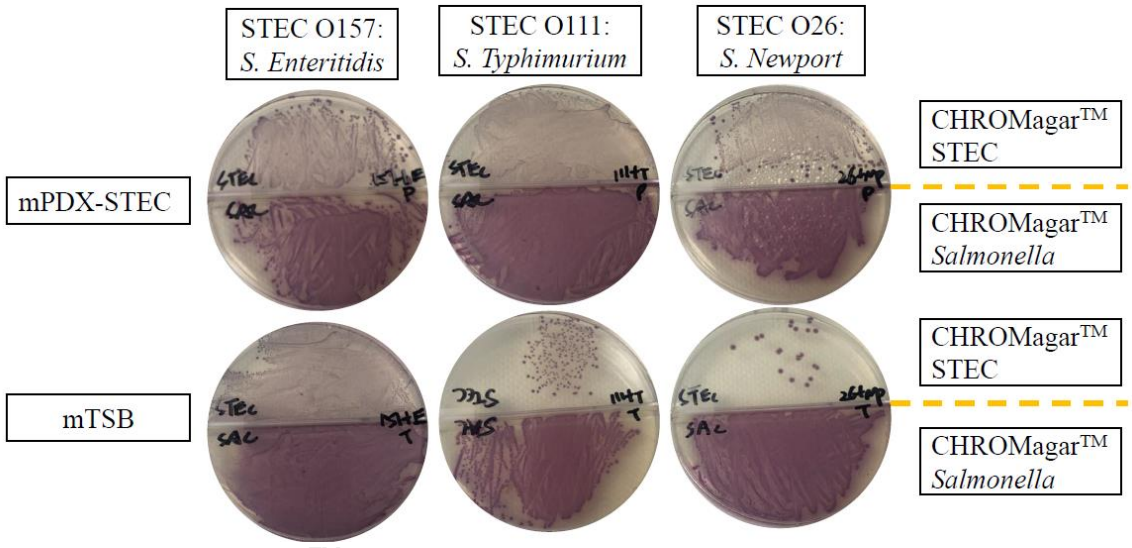

Figure 12: CHROMagar™ platings of mPDX-STEC and mTSB enrichments

In addition, none of the negative controls caused color change of mPDX-STEC at end of the enrichment (**Table 19**), indicating good selectivity of the developed medium for screening STEC and *Salmonella* from water samples. However, water from different natural sources such as ground and surface tend to have diverse background microflora. Therefore, for some water samples, it might be necessary to apply additionally restrictive conditions to further improve selectivity of the screening method, such as adjusting growing conditions like temperature. Most bacteria have optimal growth at the temperature of 37℃ or below. An elevated temperature might still allow growth of the targeting microorganisms while suppressing the growth of some background microflora therefore potentially improving the selectivity of the enrichment. One of the water samples (No. 19) was tested negative for both STEC and Salmonella, but still caused color change of mPDX-STEC at end of the enrichment at 37℃. By increasing the incubation temperature to 42℃, the proportion of false positive results of the control samples was significantly reduced (**Table 20**) suggesting improved selectivity. In the meanwhile, the recovery of both STEC and *Salmonella* enriched in mPDX-STEC was not negatively affected by the evaluated incubation temperature. In fact, the recovery of *Salmonella* strains enriched in mPDX-STEC was improved at the elevated incubation temperature. In addition, it was noted that the relative sensitivity of the mPDX-STEC and mTSB media remained consistent with results previously obtained from water samples of a different source (**Table 19** and **Table 20**), indicating good robustness of the developed method. On the other hand, it was interesting to note that the elevated incubation temperature led to numerically more positive confirmations of *S. Typhimurium* in mTSB, but the recovery of STEC O111 in mTSB was reduced at the same time. Similarly,

although the elevated incubation temperature improved the recovery of STEC O26 in mTSB, the proportion of positive *S. Newport* plates was reduced by 50% at the same time. The observations suggested that the sensitivity for detection of STEC and *Salmonella* enriched in mTSB was very sensitive to the competition between target pathogens. It might be problematic for simultaneous detection of pathogens with different growth rates. By contrast, a similar trend was not observed for mPDX-STEC, suggesting its better capacity to simultaneously support the growth of multiple targeting pathogens under restrictive growing conditions. It should be mentioned that although the use of elevated temperature increases the selectivity of enrichment, it may also slow down the recovery of injured target microorganisms (Taskila et al., 2012). Therefore, it is important to carefully choose the most suitable conditions based on characteristics of the samples and targeting pathogens.

Table 20: Comparison of paired STEC-*Salmonella* enrichment recoveries in mPDX-STEC and mTSB at 37℃ and 42℃ (Water Sample No.19)

STEC: Salmonella Serotypes	Temperature	Enrichment Media	% Positive mPDX-STEC indicator	% Positive STEC Plates	% Positive Salmonella Plates
Negative Control	37℃	mPDX-STEC	100 [1]	0 [a, 1]	0 [a, 1]
		mTSB	NA	0 [a, 1]	0 [a, 1]
	42℃	mPDX-STEC	25 [2]	0 [a, 1]	0 [a, 1]
		mTSB	NA	0 [a, 1]	0 [a, 1]
STEC O157: *S. Enteritidis*	37℃	mPDX-STEC	100 [1]	100 [a, 1]	75 [a, 1]
		mTSB	NA	100 [a, 1]	100 [a, 1]
	42℃	mPDX-STEC	100 [1]	75 [a, 1]	88 [a, 1]
		mTSB	NA	100 [a, 1]	75 [a, 1]
STEC O111: *S. Typhimurium*	37℃	mPDX-STEC	100 [1]	63 [a, 1]	38 [a, 1]
		mTSB	NA	63 [a, 1]	38 [a, 1]
	42℃	mPDX-STEC	75 [1]	63 [a, 1]	63 [a, 1]
		mTSB	NA	25 [a, 1]	75 [a, 1]
STEC O26: *S. Newport*	37℃	mPDX-STEC	100 [1]	88 [a, 1]	38 [a, 1]
		mTSB	NA	25 [b, 1]	75 [a, 1]
	42℃	mPDX-STEC	100 [1]	100 [a, 1]	63 [a, 1]
		mTSB	NA	88 [a, 2]	38 [a, 1]

For each STEC: *Salmonella* pair, values with different superscript letters indicate that the difference of probability of detection between two enrichment media is significant at the 0.05 level; values with different superscript numbers indicate that the difference of probability of detection of the medium at two different temperatures is significant at the 0.05 level.

4.3.5 Evaluation of Microbial Quality of Water Samples from Different Sources

The populations of generic *E. coli* in water samples and STEC and Salmonella screening results using mPDX-STEC are shown in **Table 21**. Water samples No.1 to No.17 were surface water collected from ponds, rivers and lakes in Minnesota and Wisconsin. Water samples No.18 and No.19 were ground water collected from two wells. The results showed that most of the surface water samples were tested positive for either STEC or *Salmonella* or both, with 9 of the 17 surface water samples tested positive for STEC, 14 of the 17 surface water samples tested positive for *Salmonella*, and 7 of the 17 surface water samples tested positive for both STEC and *Salmonella*. It was not unexpected that large number of the surface water samples were tested positive for STEC and/or *Salmonella*. The water samples were collected between September and October in which excessive precipitation had occurred. It is known that weather fluctuations tend to have a significant impact on the microbial quality of surface water. Surface water is particularly susceptible to microbial contaminations by flood runoffs. Paruch et al. (2015) found that an increase in microbial concentrations in surface water was immediately observed after the first rainfall events, and the contaminations with *E. coli* and intestinal parasitic protozoa were detected with concentrations up to three times higher during the wet/cool period than during the dry/warm period (Paruch et al., 2015). However, it should be noted that one of the samples tested positive for STEC (No.9) on CHROMagar^{TM} STEC medium and five of the samples tested positive for *Salmonella* (No.10, No.11, No.15, No.16, No.17) on CHROMagar^{TM} *Salmonella* medium were unable to be confirmed with qPCR analysis. It was previously reported that

CHROMagar™ STEC medium had a positive predicative value between 40% and 51.3%, and a negative predicative value between 98% and 98.8% (Gouali, Ruckly, Carle, Lejay-Collin, & Weill, 2013). Similarly, some non-*Salmonella* organisms were reported being capable of producing mauve-colored colonies on CHROMagar™ *Salmonella* medium (Maddocks, Olma, & Chen, 2002). Given the extremely diverse microflora existing in surface water, it was possible that false positive results were obtained from CHROMagar™ plates in current study. But on the other hand, the chromogenic agar plating not only allows rapid visualization of the target pathogens but also renders the sample ready for subsequent characterization. It would be beneficial to adopt a combined approach of culture medium and molecular detection for food pathogen screening.

The tested surface water samples showed a wide range of generic *E. coli* population, ranging from 2.5 CFU per 100 mL to 560 CFU per 100mL. The PS Rule of FSMA requires that generic *E. coli* concentrations not exceed a geometric mean (GM) of 126 CFU per 100 mL of water and a statistical threshold value (STV) less than 410 CFU per 100 mL of water (FDA, 2019d). Although the results obtained in current study were from single time samplings at each site, it showed that most of the collected surface water samples would potentially meet the FSMA microbial standard for irrigation. It suggests that solely relying on generic *E. coli* population for risk assessment of irrigation water would impose potential risks of product contaminations with pathogens. In the current study, the Pearson correlation coefficients between presence of STEC and generic *E. coli* populations was 0.328 ($p = 0.170$). The data showed only a medium correlation between the presence of STEC and the population of generic *E. coli*, and it was not statistically

significant. Similarly, a weak and statistically non-significant correlation was observed for the presence of *Salmonella* and generic *E. coli* populations (Pearson correlation coefficient = 0.195, p = 0.423). The results agree with some recent studies which have identified a weak correlation between indicator bacteria and pathogenic bacteria (Benjamin et al., 2013; Dechesne & Soyeux, 2007; Jenkins et al., 2011). Benjamin et al. (2013) investigated the occurrence of generic *E. coli*, *E. coli* O157 and *Salmonella spp.* in water and sediment from leafy green produce farms and streams in central California, and found that the generic *E. coli concentration* was not significantly associated with the presence of either *E. coli* O157 or *Salmonella* (Benjamin et al., 2013). Similarly, Dechesne and Soyeux (2007) found no recurring evidence showing the correlation between fecal indicators and pathogen presence (Dechesne & Soyeux, 2007). It reaffirms the necessity to directly screen pathogens for risk assessment of irrigation water. On the other hand, the two ground well water samples tested negative for both STEC and *Salmonella*, and the generic *E. coli* was non-detectable in the samples. In addition, one of the surface samples collected from a spring running into a lake (No.13) was negative for both STEC and *Salmonella*. This demonstrates that ground water is generally of good microbial quality and less susceptible to microbial contaminations than surface water.

The results showed that mPDX-STEC medium appeared to be promising for screening of STEC and *Salmonella* in water samples. In the current study, the screenings were performed at both 37℃ and 42℃. At both incubation temperatures, it was noted that the color change was identified for all the samples tested positive for STEC and/or *Salmonella* on CHROMagar^{TM} medium, indicting good screening sensitivity for water

samples from different sources. A color change was observed for one of the tested ground water samples (No.19) when the incubation was carried out at 37℃, giving a false positive result, suggesting that an elevated incubation temperature might be necessary to maximize the selectivity of the screening method. On the other hand, it was also noted that four surface water samples (No.10, No.11, No.12, and No.17) which were tested positive for *Salmonella* on CHROMagar™ *Salmonella* medium after enrichment in mPDX-STEC at 37℃, tested negative when the incubation temperature was increased to 42℃, suggesting that other microorganisms had outcompeted the *Salmonella* strain(s) in the mPDX-STEC at the elevated incubation temperature. It is known that the sample preparation steps such as the filtering process might impose additional stress and even injure the targeting cells thus decreasing their recoveries during selective enrichment (Hoadley & Cheng, 1974; Kenner et al., 1961). The evaluated incubation temperature might further slower down the growth of the stressed or injured targeting cells, particularly cells with slower growth rates and sensitive to stresses. However, it needs to be mentioned that three of the four presumptively *Salmonella* positive samples (No.10, No.11, No.17) were unable to be confirmed by qPCR analysis, suggesting potentially false positive results from CHROMagar™ *Salmonella* medium after enrichment in mPDX-STEC at 37℃. Therefore, incubation at 37℃ might be preferred when enrichment sensitivity is at the top priority of the testing, while an elevated incubation temperature could be applied when used for screening large numbers of samples for known pathogen types, to minimize potential interference from the background microflora.

Table 21: Screening of microbial quality of water samples from different sources using mPDX-STEC and MI agar

Sample ID	Generic *E. coli* (CFU/100mL) [a]	Temperature	Colorimetric Indictor [b]	STEC [g]	*Salmonella* [h]
1	13.00 ± 2.45	37℃	+	-	+ [c]
		42℃	+	-	+ [c]
2	12.67 ± 1.70	37℃	+	-	+ [c]
		42℃	+	-	+ [c]
3	20.00 ± 2.94	37℃	+	-	+ [c]
		42℃	+	-	+ [c]
4	5.67 ± 1.70	37℃	+	+ [c]	+ [c]
		42℃	+	+ [c]	+ [c]
5	5.33 ± 2.05	37℃	+	-	+ [c]
		42℃	+	-	+ [c]
6	22.00 ± 3.74	37℃	+	+ [c]	-
		42℃	+	+ [c]	-
7	19.00 ± 2.16	37℃	+	-	+ [c]
		42℃	+	-	+ [c]
8	113.50 ± 0.50	37℃	+	+ [c]	-
		42℃	+	+ [c]	-
9	113.50 ± 2.50	37℃	+	+ [s]	+ [c]
		42℃	+	+ [s]	+ [c]
10	106.50 ± 12.50	37℃	+	+ [c]	+ [s]
		42℃	+	+ [c]	-
11	100.50 ± 8.50	37℃	+	+ [c]	+ [s]
		42℃	+	+ [c]	-
12	560.00 ± 20.00	37℃	+	+ [c]	+ [c]
		42℃	+	+ [c]	-
13	9.50 ± 0.50	37℃	-	-	-
		42℃	-	-	-
14	8.00 ± 0.00	37℃	+	-	+ [c]
		42℃	+	-	+ [c]
15	17.50 ± 1.50	37℃	+	+ [c]	+ [s]
		42℃	+	+ [c]	+ [s]
16	222.00 ± 11.00	37℃	±	-	+ [s]
		42℃	±	-	+ [s]
17	2.50 ± 0.50	37℃	+	+ [c]	+ [s]
		42℃	+	+ [c]	-
18	Non-detectable	37℃	-	-	-
		42℃	-	-	-
19	Non-detectable	37℃	+	-	-
		42℃	-	-	-

[a], results were summarized from duplicate samples counted on MI agar plates.

[b], +, presumptively positive result based on color change; -, presumptively negative result based on color change; ±, weak color change.

[g, h], +, presumptive positive result based on at least one mauve-colored colony; -, presumptively negative result; positive result with superscript letter c indicates that the positive result was confirmed with qPCR analysis; positive result with superscript letter s indicates that the positive result was unable to be confirmed with qPCR analysis.

4.4 Summary

This study summarizes the development, optimization and evaluation of a novel selective medium for sensitive enrichment and screening of STEC and *Salmonella* in irrigation water. The PDX-STEC medium showed enriching effect which was inclusive of a wide range of STEC and *Salmonella* serotypes, while exhibiting exclusivity against common background bacteria. The incorporation of bromocresol purple and D-trehalose enabled presumptive screening of common serotypes of STEC and *Salmonella* using PDX-STEC medium. Modifications on medium formulation were made to improve screening sensitivity reducing limits of detection for six major STEC and *Salmonella* serotypes to less than 6.22 CFU per 100 mL of water. The comparative evaluation of the recovery rates of STEC and *Salmonella* in mPDX-STEC and mTSB media suggested that the enriching performance of mPDX-STEC was equivalent to mTSB for all the tested STEC and *Salmonella* serotypes except STEC O26 for which the mPDX-STEC exhibited superior enriching efficiency. The developed method was applied to evaluate the microbial quality of water samples from different sources. The results suggested that mPDX-STEC was able to enrich and screen for STEC and *Salmonella* in diverse water samples. An elevated incubation temperature increased screening selectivity of mPDX-STEC but hindered recoveries of *Salmonella* in some water samples. Therefore,

incubation temperatures should be adjusted according to characteristics of the water samples, targeting pathogens and testing priorities to achieve optimal screening sensitivity and selectivity.

Chapter 5: Conclusions

In conclusion, this research has been successful in achieving the outlined objectives. The study has potentially contributed to improvements of produce safety through two directions.

The first (Chapter 3) described the changing epidemiology of produce outbreaks in the United States from 1998-2017. The analysis included epidemic trends of overall foodborne illness outbreaks, overall produce outbreaks, and produce outbreaks by food categories and etiology. It established the baseline before large scale compliance with the Produce Safety Rule was required. The analysis showed that the early stage of produce production is particularly susceptible to microbial contaminations. Preharvest elements, such as irrigation water, are believed to play an important role in produce contaminations. It reaffirmed the importance of robust preventive measures to ensure the microbial quality of irrigation water.

The second (Chapter 4) described the development, optimization and evaluation of a novel selective medium for sensitive enrichment and screening of STEC and *Salmonella* in irrigation water. The developed method exhibited excellent screening sensitivity and selectivity for STEC and *Salmonella* allowing cost effective detection within 24 hours. Furthermore, the study showed that the developed selective medium was

equivalent or superior to the reference medium in terms of recovering STEC and *Salmonella* from water samples. Evaluations on water samples from different sources also validated the applicability of the developed method for screening of STEC and *Salmonella* in irrigation water.

As a result, this project has opened new perspectives to improve produce safety through risk managements which are adapted to the ever-changing food safety epidemiology, and foodborne pathogen detections which are more efficient and cost effective. Several projects are now initiated based on this work's results, including:

- Additional epidemic analysis of produce outbreaks. It may include analysis of the newest epidemic data on produce outbreaks which would offer additional insights into the potential impact of the Produce Safety Rule of FSMA. Furthermore, analysis might be extended to other major produce exporting countries to evaluate the safety of global produce supply chain.
- Adaptation of the developed medium for environmental surface sampling in the food processing facilities. Potential challenges include recovery of low concentrations of injured cells on the surfaces. Some preliminary results showed that pre-buffering swab sponges with sodium pyruvate in

combination with a 2-hour resuscitation step were able to improve the recovery of STEC and *Salmonella* in PDX-STEC. Research is currently being conducted on developing a new growth stimulant which could be used to promote recovery of STEC and *Salmonella* during enrichment.

- Development and evaluation of novel water treatment technologies, such as oscillating magnetic field is being looked at to potentially reduce foodborne pathogens in irrigation water at the source.

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