Ensuring food safety modernization act produce safety rule compliance through water testing and sanitation validation programs

Manreet Singh Bhullar

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Part of the Food Science Commons

Recommended Citation
Bhullar, Manreet Singh, "Ensuring food safety modernization act produce safety rule compliance through water testing and sanitation validation programs" (2019). Graduate Theses and Dissertations. 17645. https://lib.dr.iastate.edu/etd/17645

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Ensuring food safety modernization act produce safety rule compliance through water testing and sanitation validation programs

by

Manreet Singh Bhullar

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Program of Study Committee:
Angela Shaw, Major Professor
Shannon Coleman
Keith Vorst
Ajay Nair
Anirudh Rajagopal Naig

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2019

Copyright © Manreet Singh Bhullar, 2019. All rights reserved.
DEDICATION

Family is the best treasure that I have in my life and I would dedicate the work in this dissertation to the continuous support and motivation of my family towards this accomplishment.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vi</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NOMENCLATURE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vii</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ACKNOWLEDGMENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>viii</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 1. GENERAL INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2. LITERATURE REVIEW</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>5</td>
</tr>
<tr>
<td>Major Outbreaks in Fresh Produce</td>
<td>5</td>
</tr>
<tr>
<td>Pathogens Associated with Fresh Produce Outbreaks</td>
<td>6</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>9</td>
</tr>
<tr>
<td><em>Salmonella</em> Species</td>
<td>11</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>12</td>
</tr>
<tr>
<td>Food Safety Regulations</td>
<td>13</td>
</tr>
<tr>
<td>FSMA Produce Safety Rule</td>
<td>14</td>
</tr>
<tr>
<td>Different Routes of Contamination of Fresh Produce</td>
<td>14</td>
</tr>
<tr>
<td>Agricultural Water</td>
<td>18</td>
</tr>
<tr>
<td>Contaminated Soils and Biological Soil Amendments of Animal Origin</td>
<td>19</td>
</tr>
<tr>
<td>Animal Use on the Farm (Wildlife and Domesticated Animals)</td>
<td>28</td>
</tr>
<tr>
<td>Worker Hygiene</td>
<td>31</td>
</tr>
<tr>
<td>Survival of Pathogens in the Produce Supply Chain</td>
<td>32</td>
</tr>
<tr>
<td>Postharvest Washing of Fresh Produce</td>
<td>33</td>
</tr>
<tr>
<td>Disinfection Methods</td>
<td>35</td>
</tr>
<tr>
<td>Chlorination</td>
<td>36</td>
</tr>
<tr>
<td>Long Term Survivor Cells</td>
<td>37</td>
</tr>
<tr>
<td>References</td>
<td>39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3. EXTENDING THE SAMPLE HOLDING TIME FOR TESTING EPA METHOD 1603 FOR PRODUCE GROWERS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>55</td>
</tr>
<tr>
<td>Introduction</td>
<td>56</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>57</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>59</td>
</tr>
<tr>
<td>References</td>
<td>63</td>
</tr>
</tbody>
</table>
CHAPTER 4. CHARACTERIZING THE MORPHOLOGICAL DIFFERENCES BETWEEN STATIONARY AND LONG-TERM SURVIVAL PHASE CELLS OF 
ESCHERICHIA COLI OUTBREAK STRAINS .................................................................67
  Abstract..................................................................................................................67
  Introduction ............................................................................................................68
  Materials and Methods ........................................................................................70
    Bacterial Cultures and Culture Conditions .........................................................70
    Preparation of Inoculum ....................................................................................70
    Light Microscopy of Gram Stains ......................................................................70
    Scanning Electron Microscopy ........................................................................71
  Results and Discussion ........................................................................................71
  Conclusion .............................................................................................................74
  References .............................................................................................................75

CHAPTER 5. LONG TERM SURVIVAL CELLS OF ESCHERICHIA COLI HAVE
HIGHER RESISTANCE AGAINST CHLORINE TREATMENT BOTH IN-VITRO AND
IN MODEL WASH SYSTEM TREATING ROMAINE LETTUCE ......................................78
  Abstract..................................................................................................................78
  Introduction ............................................................................................................79
  Materials and Methods ........................................................................................81
    Bacterial Cultures and Culture Conditions .........................................................81
    Preparation of Inoculum ....................................................................................81
    Minimum Bactericidal Concentration (MBC) of Chlorine ..................................82
    Preparation of Chlorine Solutions and Treatment .............................................83
    Determination of Sublethal Injury .....................................................................85
    Microbiological Analysis ..................................................................................85
    Scanning Electron Microscopy .........................................................................85
    Statistical Analysis ...........................................................................................86
  Results and Discussion ........................................................................................86
    Minimum Bactericidal Concentration ..............................................................86
    Chlorine Treatment of LTS and Stationary Cells – In-vitro .............................86
    Chlorine Treatment of LTS and Stationary cells – Lettuce Model .....................88
    Sublethal Injury .................................................................................................89
    Scanning Electron Microscopy .........................................................................91
  References .............................................................................................................94

CHAPTER 6. GENERAL CONCLUSION ........................................................................100
LIST OF FIGURES

Figure 2.1. The potential sources of contamination of fresh produce ........................................ 19
Figure 2.2. The concentration of HOCl at different pH levels .................................................. 38
Figure 2.3. Different growth phases of bacterial cells ................................................................. 40
Figure 3.1. Iowa map illustrating number of water samples collected in 2017 – 2018 ............... 58
Figure 3.2. Generic Escherichia coli cell counts of 32 contaminated samples (69 samples tested at no detectable Escherichia coli) at 6 hours and 24 hours sample holding time obtained from sixty Iowa produce farms in 2017–2018. ................. 62
Figure 4.1. LTS and stationary cell images of E. coli cocktail using light microscopy .............. 72
Figure 4.2. SEM images for LTS cells of E. coli cocktail .......................................................... 73
Figure 4.3. SEM images for stationary cells of E. coli cocktail ................................................. 74
Figure 5.1. Growth curve of long-term survival cells incubated at 35 °C for 50 days ............... 82
Figure 5.2. (a) Lettuce leaf coupon (6.15 cm²), (b) Inoculation of E. coli (50 uL) on coupons ....................................................................................................................................... 84
Figure 5.3. Log reduction of colony forming units per milliliter (CFU/mL) LTS and stationary cells of E. coli (Starting population of 7.04 and 7.84 CFU/mL) at different chlorine concentrations in-vitro. ....................................................................................................................... 87
Figure 5.4. Inactivation curves for stationary and LTS cells against chlorine treatment .......... 88
Figure 5.5. SEM images of stationary and LTS cells on control and inoculated leaves .......... 93
LIST OF TABLES

Page

Table 2.1. Foodborne outbreaks related to fresh produce (2005-2018) – adapted from NORS Dashboard (Centers for Disease Control and Prevention, 2019b) .................. 8

Table 2.2. List of major foodborne pathogens associated with fresh produce (Centers for Disease Control and Prevention, 2019b; Jung et al., 2014; Lynch et al., 2009) ...... 10

Table 2.3. The survival of human pathogens in different water sources ..................................... 24

Table 2.4. Summary of all Water Testing Methods ........................................................................ 27

Table 2.5. Updates compliance dates for water rule ..................................................................... 28

Table 3.1. Percentage of positive samples of generic Escherichia coli obtained from sixty Iowa fresh produce farm waters ................................................................. 60

Table 5.1. Free chlorine doses for different treatments ................................................................. 84

Table 5.2. Sublethal injury (%) and cell death (%) for stationary cells of E. coli (In-vitro) ......... 90

Table 5.3. Sublethal injury (%) and cell death (%) for LTS cells of E. coli (In-vitro) .................... 90

Table 5.4. Sublethal injury (%) in stationary and LTS cells (Lettuce model) ............................... 91
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSMA</td>
<td>Food Safety Modernization Act</td>
</tr>
<tr>
<td>PSR</td>
<td>Produce Safety Rule</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>USDA-ERS</td>
<td>United States Department of Agriculture Economic Research Service</td>
</tr>
<tr>
<td>MWQP</td>
<td>Microbial Water Quality Profile</td>
</tr>
<tr>
<td>LTS</td>
<td>Long Term Survival</td>
</tr>
<tr>
<td>BPW</td>
<td>Buffered Peptone Water</td>
</tr>
<tr>
<td>TSAYE</td>
<td>Tryptic Soya Agar with Yeast Extract</td>
</tr>
<tr>
<td>SMAC</td>
<td>Sorbitol MacConkey Agar</td>
</tr>
<tr>
<td>VBNC</td>
<td>Viable But Not Culturable</td>
</tr>
<tr>
<td>STEC</td>
<td>Shiga-toxin producing <em>E. coli</em></td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum Bactericidal Concentration</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like to thank my committee chair, Dr. Angela Shaw, and my committee members, Dr. Shannon Coleman, Dr. Keith Vorst, Dr. Ajay Nair, and Dr. Rajagopal Lakshman, for their guidance and support throughout the course of this research. I am grateful to all of my committee members for their expert advice and suggestions that have instilled in me valuable professional and life skill to be a successful individual. Dr. Angela Shaw, I am fortunate to be a PhD student in your lab and work under your mentorship, which has given a new shape and goal to my life. The exposure to so many professional development opportunities that you pushed me to get through has been rewarding. Your teachings about professional and personal skills have added a lot to my life’s approach. Dr. Shannon Coleman, I am grateful to have you in the committee, as you have been a great support to boost up my confidence in my teaching and presentation abilities. Your support in preparation for job interviews has helped me much to perform well. Dr. Keith Vorst, Dr. Ajay Nair, and Dr. Lakshman, you have been great supports, and your suggestions and advice to both my research projects and future endeavors has helped make timely decisions that have worked in favor of my interests. I am profoundly grateful to all my committee members and acknowledge that this journey would not have been as exciting as it has been without the support and guidance of you all.

To my family, I do not have to express my gratitude to show immense trust in my capabilities and allowing me to leave home for years to accomplish what I dreamt for my life. I am grateful to my granddad, Mr. Ajit Singh Bhullar, and my grandmom, Mrs. Amar Kaur Bhullar, for their godly blessings and words of wisdom that have paved paths of success in my life. Mom and Dad, Mrs. Kanwaljit Kaur Bhullar and Mr. Gurdev Singh Bhullar, you are two of the greatest parents in the world. Your education and teachings have been the building stone, and
I believe whatever I achieve in my life, you will be my heroes forever. And not the least, my fiancée, Ravneet Kaur Sandhu, your support and motivation always has kept me moving throughout the doctoral journey.

In addition, I would also like to thank my colleagues, Ellen Johnsen, Dan Fillius, Smaranda Andrews, and my friends Ana Monge, Lillian Nabwiire, Jacques Overdiep II, Bridget Perry, Niraja Shiva, Joshua Nazareth, and Chinwendu Ozoh, the department faculty and staff for their support, making my time at Iowa State University an enjoyable experience. Lastly, I want to offer my appreciation to all the undergraduate students and George Washington Carver Interns (Yazrah Randall, Briana Young, Keith Fennel, Jessyca, Kelvin, Daysis, and Mikuel), for their excellent assistance with the collection and analysis of water samples.
A large portion of fresh produce is consumed raw, and likewise, there have been many cases of foodborne illnesses associated with it. The contamination can occur at any point during the farm to fork continuum and may come from poor agricultural practices at the farm, deficit knowledge of food safety concerns among growers, and/or various physical, chemical, and biological hazards in the food supply chain. Agricultural water is a known vector for the transfer of foodborne pathogens onto fresh produce and has been implicated in recent foodborne outbreaks. Monitoring and management of microbial quality of agricultural water is a requirement under the Food Safety Modernization Act Produce Safety Rule (PSR). The water testing methods (n=9) mentioned in the PSR require no greater than a 6-hour time frame between the collection of the water sample and the delivery to the lab. This 6-hour timeframe is unrealistic for many farm locations in the Midwest. To address this issue, 101 agricultural water samples were collected from 60 different farms using method EPA 1603. A total of 32 samples were found contaminated with generic *E. coli*—mostly surface water (n=28, 87.5%). The results provide evidence that the sample holding time interval can be extended to a 24-hour time (p>0.05), which makes quantitative generic *E. coli* testing more accessible to growers. Cross-contamination through agricultural water has led to foodborne outbreaks with produce as well. These bacteria generally grow in four growth stages of lag, log, stationary, and death; however, research reported the fifth phase following the death phase called long term survival (LTS) cells, which have a higher resistance to antimicrobial treatments. Their sensitivity to chemical sanitizers is unknown. The study focused on quantifying the resistance of stationary and LTS cells against chemical sanitizer treatment (chlorine, sodium hypochlorite) and determined the effects of bacterial growth phase against chlorine treatment. The results reported higher
resistance to LTS cells in-vitro but statistically insignificant results in the lettuce wash model (p>0.05). Monitoring the diverse routes of agricultural water contamination is critical to ensure the safety of fresh produce and to ensure that more intensive measures are required in the food supply chain to protect public health.
CHAPTER 1. GENERAL INTRODUCTION

Fruits and vegetables are an integral part of the human diet, and the Dietary Guidelines of Americans (DGA) 2010 recommend daily intake of two cups of fruits and two and half cups of vegetables (Flock & Kris-Etherton, 2011). Interestingly, the production of fruits and vegetables has increased simultaneously with increased rates of consumption in the US. Grocery stores sold more than 400 fresh produce items in 2001 as compared to only 150 during the 1970s (French, Story, & Jeffery, 2001); the increase in produce items can be extrapolated to a significantly high number today. Knowing the adequate consumption of fruits and vegetables helps prevent some severe health concerns including diabetes, hypertension, cardiovascular disease, and obesity (Bazzano, 2006). Consumers have become more health-conscious and have started including larger portions of fruits and vegetables into their diet. Foodborne disease outbreaks typically originating from meat and poultry products has been the conventional notion; however, fruits and vegetables have also been implicated in many foodborne outbreaks in the recent past.

A large portion of fresh produce is consumed raw, and likewise there have been many cases of foodborne illnesses associated with it (Olaimat & Holley, 2012). The contamination can occur at any point during the farm to fork continuum and may come from poor agricultural practices at the farm, deficit knowledge of food safety concerns among growers, and/or various physical, chemical, and biological hazards in the food supply chain. The consumption of fresh fruits and vegetables have significant benefits that outweigh the risk of getting a foodborne illness that may be associated with consumer produce (Nyachuba, 2010); however, the potential to harm public health is unequivocally critical. The major pathogens associated with fresh produce outbreaks include bacteria (Escherichia coli (E. coli) O157:H7, Salmonella Spp.,
Listeria monocytogenes), viruses (Norovirus, Hepatitis A), and parasites (Giardia lamblia, Cryptosporidium parvum, Cyclospora cayetanensis, Toxoplasma gondii).

The Food Safety Modernization Act (FSMA) was signed into law in 2011, considering the rising burden of food outbreaks and their impacts on people's health. The FSMA rule book comprises seven different food safety regulations and includes the Produce Safety Rule (PSR) that defines standards for growing, harvesting, packing, and storing fresh produce. The PSR requires growers to come in compliance with the requirements of the law, until exempt. The PSR has been developed for improving and protecting the safety of fresh produce and recommends safety practices to minimize biological risks in the produce supply chain. The various modules focus on different factors that play a critical role affecting produce safety and include worker health and hygiene, biological soil amendments of animal origin, agricultural water quality, wildlife, and domesticated animals, and post-harvest handling.

Agricultural water has been the significant source of recent fresh produce outbreaks due to several challenges such as use of surface or recirculated waters to conserve drinking water supplies, inadequate water testing for quality control, and potential to cause significant contamination events with natural dispersal of pathogens. Risk assessment of water source and testing is vital to know the quality of water sources and determine its use. FDA recommends nine water testing methods under PSR to develop microbial water quality profile (MWQP) for fresh produce growers; however, all methods have a common user-unfriendly requirement to submit the water samples to certified labs within 6 hours. This timeframe resists growers to participate in water testing and poses a critical challenge to produce safety. In addition, FDA also recommends use of sanitizers or antimicrobials to enhance the safety of post-harvest water and
effectiveness of post-harvest washing; however, the efficacies of these chemical disinfectants are under question due to complex chemical reactions and treatment requirements.

The incidence of produce outbreaks informs a lot about food safety practices and emerging problems. With the recent lettuce outbreak of 2018, few regions of California and Arizona were implicated to be origin of the contamination. Under Leafy Greens Marketing Agreement, regulated in California, all produce growers are required to use sanitizers or antimicrobials during produce wash. However, the occurrence of produce outbreaks are common in California and reveals that disinfection is not adequate to ensure safety.

In addition, the evolution of microorganisms to adapt to unfavorable environments also pose significant threat to food safety. Bacterial cells are reported to grow into long term survival phases after the initial lag, log, stationary and death phase, during which they alter the morphological characteristics and become resistant to antimicrobial treatments. The scientific recommendations mentioned in PSR are based on microbiological studies conducted using stationary phase cells; however, the persistence of LTS cells in the natural production environments is common. The limited literature on LTS cells reported that these cells have higher resistance against high pressure and UV treatments, and cells require higher treatment doses for inactivation. The resistance is developed due to several phenotypic and genotypic gene expressions; however standardizing food treatment processes based on studies conducted using stationary cells may result in overestimation of the efficacy of treatment.

The following dissertation chapters focus on literature review providing background information on critical topics of produce safety, and research studies focusing on modification of agricultural water testing EPA Method 1603, illustrating proof of concept of long-term survival
cells and identifying efficacy of chlorination against long term survival cells and stationary phase cells.

References


CHAPTER 2. LITERATURE REVIEW

Background

Fresh produce safety has emerged as the utmost priority for all stakeholders around the world. The US fruit and vegetable market size has seen a phenomenal rise in its value in the past two decades. The value of fruits and vegetables produced domestically in the US has increased from $9.1 billion and $7.4 billion in 1995 to $21.3 and $13.1 billion in 2016, respectively. A similar increase was observed in amounts of imported produce, with rising in fruits and vegetables from 15% and 13% in 1995 to 40% and 29% in 2017 (USDA-ERS, 2019a, 2019b). In contrast, a higher number of foodborne outbreaks have been associated with fresh produce in the recent past which are attributed to increased consumption, international trade of produce, and more advanced surveillance of foodborne outbreaks (Havelaar et al., 2010; Lynch, Tauxe, & Hedberg, 2009; Tauxe et al., 1997). Remarkable scientific advancements in research and regulations have been enacted to safeguard the fresh produce supply and to address the food safety challenges in the US. However, produce safety needs better interventions to ensure higher levels of safety and tackle current problems.

Fresh produce is commonly consumed raw without undergoing any means of processing that involves a kill step, which makes it a potential vector of a human pathogen (Li et al., 2017). The safety of fresh produce is vulnerable to microbial contamination throughout the supply chain beginning from cultivation in the field until it reaches the plate of the consumer. In literature, it is reported that once produce gets contaminated with microbial hazards, it becomes challenging to decontaminate the produce further down the supply chain. The significant factors that contribute to contamination of fresh produce include irrigation water, biological soil amendments of animal origin (manure), and contaminated soil (Alegbeleye, Singleton, & Sant’Ana, 2018) and people
who come in contact with the produce. To establish standards for enhanced produce safety, it is imperative to understand the diverse routes of contamination and mechanisms of survival of the pathogen in the fresh produce supply chain.

**Major Outbreaks in Fresh Produce**

The health benefits of consuming fruits and vegetables are well illustrated in literature and are widely publicized through various health agencies in the world. The increased demand for fresh produce with increased intake in the daily diet of health-conscious consumers has resulted in the globalized production of fruits and vegetables. In contrast, fresh produce has been increasingly associated with foodborne outbreaks with rise from < 1% in the 1970s to 6% in 1990s underline the challenges related to fresh produce (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). From 1996 till 2010, approximately 23% of the total foodborne outbreaks were related to fresh produce (Jung, Jang, & Matthews, 2014). For some of the outbreak investigations, the microbial contamination was linked to primary production areas, highlighting demands for preventive measures that reduce chances of future fresh produce outbreaks.

To accommodate the consumer demand, utilization of advanced agronomic cultivation methods to grow crops under artificial environments, increased use of soil amendments, use of alternative water sources and increased global trade provides potential opportunity for human pathogens to compromise the microbial safety of fresh produce (Beuchat, 2002; Lopez-Galvez, Allende, Pedrero-Salcedo, Alarcon, & Gil, 2014). However, scientifically advanced progress has been made to enhance the safety of fresh produce with the implementation of stringent food laws, more research and studies are required to identify the niche vulnerabilities in the fresh produce supply chain to maximize minimization of food safety hazards to public health. This reflects a direct convergence among the increasing consumption of fresh produce, changes in production and distribution, and growing awareness of the problem on the part of public health officials.
Interestingly, leafy greens are most implicated produce type involved in fresh produce outbreaks (Luo et al., 2011; Olaimat & Holley, 2012), and the major contamination is due to *E. coli* O157:H7 and *Salmonella* spp. (Tomás-Callejas et al., 2012). The major outbreaks associated with fresh produce are reported in Table 2.1.

The contamination of fresh produce at any point in the chain of food production is possible, and the highest probability of contamination is reported during the production, initial processing, and final preparation phases (Lynch et al., 2009). Even though it is well known that biological hazards associated with fresh produce include zoonotic pathogens from human which can be classified into spore-forming, non-spore forming, parasites, and viruses; most of the produce outbreaks are implicated to bacterial source of origin (James, 2006; Lynch et al., 2009). Although the route of contamination in most of the produce-related outbreaks remains unclear, the application of whole-genome sequencing and field sampling methodologies have started providing critical information about the potential origins of contamination in the recent past. For example, outbreak-related to *E. coli* O157:H7 contaminated lettuce in 2018, the positive sample was collected from a nearby surface water reservoir and have been reported as the likely source of contamination as none other samples from various locations on the farm and the nearby region turned positive. Similar findings in other produce-related outbreaks assist researchers develop pathogen-food combinations and algorithmic models based on past data to potentially identify future outbreaks and sources of contamination. Examples include *E. coli* with lettuce/leafy greens, salmonellosis with melons, and Listeriosis with frozen produce.
Table 2.1. Foodborne outbreaks related to fresh produce (2005-2018) – adapted from NORS Dashboard (Centers for Disease Control and Prevention, 2019b)

<table>
<thead>
<tr>
<th>Produce</th>
<th>Year</th>
<th>Pathogen</th>
<th>Source</th>
<th>Location</th>
<th>Illnesses</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>2006</td>
<td>Shiga toxin-producing <em>Escherichia coli</em> (STEC)</td>
<td>Feral pig</td>
<td>Multistate</td>
<td>238</td>
<td>5</td>
</tr>
<tr>
<td>Celery</td>
<td>2010</td>
<td><em>Listeria monocytogenes</em></td>
<td>Unknown</td>
<td>Texas</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Strawberry</td>
<td>2011</td>
<td>Shiga toxin-producing <em>Escherichia coli</em> (STEC)</td>
<td>Black-tailed Deer Feces</td>
<td>Oregon</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Cantaloupe</td>
<td>2011</td>
<td><em>Listeria monocytogenes</em></td>
<td>Soil/Water and Biological Soil Amendments</td>
<td>Multistate</td>
<td>147</td>
<td>33</td>
</tr>
<tr>
<td>Cantaloupe</td>
<td>2012</td>
<td><em>Salmonella enterica</em> (serotypes Typhimurium, Newport)</td>
<td>Unknown (Imported from Gautemala)</td>
<td>Multistate</td>
<td>261</td>
<td>3</td>
</tr>
<tr>
<td>Frozen Vegetables</td>
<td>2013</td>
<td><em>Listeria monocytogenes</em></td>
<td>CRF Frozen Foods</td>
<td>Multistate</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Caramel Apple</td>
<td>2014</td>
<td><em>Listeria monocytogenes</em></td>
<td>Granny Smith and Gala apples from Bidart Bros.</td>
<td>Multistate</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>Cucumber</td>
<td>2015</td>
<td><em>Salmonella enterica</em> (serotype Poona)</td>
<td>Unknown (Imported from Mexico)</td>
<td>Multistate</td>
<td>907</td>
<td>6</td>
</tr>
<tr>
<td>Romaine Lettuce</td>
<td>2018</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>Irrigation Reservoir</td>
<td>Multistate</td>
<td>210</td>
<td>5</td>
</tr>
</tbody>
</table>
Pathogens Associated with Fresh Produce Outbreaks

The presence of foodborne pathogens on raw produce is of concern because the potential for human illness is much higher than when the pathogens are present on foods that will be cooked. A wide range of pathogens has been documented in fresh produce outbreaks in the US (Berger et al., 2010) and listed in Table 2.2. Fresh produce such as tomatoes were implicated in large multistate outbreaks of *Salmonella* infection in 1990, 1993, and 1999 in the United States (Cummings et al., 2001; Gupta et al., 2007). *Salmonella* spp., *E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, *Y. enterocolitica* and *Shigella* spp. were implicated in sprout-associated illnesses (Mahon et al., 1997; Peñas, Gómez, Frías, & Vidal-Valverde, 2008), including an outbreak of *E. coli* O157:H7 in Japan linked to consumption of radish sprouts that affected approximately 10,000 people (Michino et al., 1999). Cucumber, watercress, onions, parsley, spinach, coconut, cilantro, and celery have also been implicated in foodborne disease outbreaks (Steele & Odumeru, 2004).

Fruits can act as vehicles for disease transmission, for example, unpasteurized orange juice and apple cider were implicated in multiple outbreaks of *Salmonella*, *E. coli* O157:H7, and Cryptosporidium infection (Cody et al., 1999). Fresh fruits were implicated in outbreaks of *Salmonella*, Saphra Norwalk virus, Calicivirus, and the parasite *C. cayetanensis* (Gaulin, Frigon, Poirier, & Fournier, 1999).

Commonly associated with processed, ready-to-eat (RTE), stored meat, and dairy products, *Listeria monocytogenes* have been found to contaminate fresh produce in various parts of the world (Meldrum et al., 2009). In the last few years, *Listeria monocytogenes* has caused many outbreaks in the US that includes chopped celery outbreak in Texas in 2010 (Gaul et al., 2012), a melon outbreak in Colorado in 2011, and caramel apple outbreak in California in 2014 (CDC, 2019c).
The trend in the US to buy local food has been on the rise, and farmers are motivated with the business model phrase “do not put your eggs in one basket” approach to mitigate financial risks and enhance sustainability. However, the increase in local market growth also attracts opportunities to produce contamination. Farmers’ markets lack adequate food safety measures such as electricity, temperature control, covered sheds, etc. In addition, farmers who sell at these markets are generally small scale and are exempt from most food safety regulations. Farmers sell diverse food items, and the same results in potential cross-contamination of foods if both fresh produce and animal products are sold by the same farmer. The chances of poultry products sold at farmers market carrying more human pathogens than grocery store products are reported to be higher in the literature (Scheinberg, Doores, & Cutter, 2013).

Table 2.2. List of major foodborne pathogens associated with fresh produce (Centers for Disease Control and Prevention, 2019b; Jung et al., 2014; Lynch et al., 2009)

<table>
<thead>
<tr>
<th>Type of pathogen</th>
<th>Examples</th>
</tr>
</thead>
</table>
| Bacteria         | *Escherichia coli*  
|                  | *Salmonella* spp.  
|                  | *Campylobacter* spp.  
|                  | *Vibrio* spp.  |
| Parasites        | *Cyclospora*  
|                  | *Cryptosporidium parvum*  
|                  | *Giardia* spp.  
|                  | *Toxoplasma gondii*  |
| Viruses          | *Norovirus* (NoV)  
|                  | *Hepatitis A virus*  
|                  | *Norwalk and Norwalk-like Rotavirus*  |
| Fungi            | *Alternaria* sp.  
|                  | *Fusarium* sp.  
|                  | *Aspergillus niger*  |
The common human pathogens prevalent in the fresh produce outbreaks are discussed in this dissertation include *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes*.

**Escherichia coli**

Produce outbreaks associated with *E. coli* have been greatly recognized in the recent past (Berger et al., 2010). The fresh produce implicated in *E. coli* outbreaks includes romaine lettuce, strawberry, spinach, and other leafy greens. Not all *E. coli* are harmful, and few strains are predominantly present in the human intestinal tract. However, diarrhea-causing *E. coli* are pathogenic and are divided into six categories that cause illness as severe as acute diseases including hemolytic uremic syndrome (Palermo, Exeni, & Fernández, 2009). Both Shiga-toxin producing *E. coli* (STEC) and non-shiga toxin-producing *E. coli* (Non-STEC) are the leading human pathogens in the United States. STEC are gram-negative, non-spore forming, mesophilic facultative anaerobes, with best growth at pH pf 6-7.5 and heat tolerance up to 71 °C (Smith, Fratamico, & Gunther, 2014).

The fecal matter of ruminant animals (cattle, deer, goat) is the big reservoirs of Shiga-toxin producing *E. coli* (STEC) and commonly contaminate beef and beef products. Non-ruminant animals are also reported to carry STEC cells; however, the probability of serving as a reservoir for outbreak rather than a vector for transmission is very low. However, contamination with STEC in leafy greens and other fresh produce that is often raw has been on the rise (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). The washing of fecal matter from animals into the water sources results in the contamination of drinking water, irrigation water, and the produce that comes in contact with contaminated water. By knowing the potential sources of contamination of produce, STEC contamination of fresh produce can be minimized with controlled use of animals on the farm, monitoring microbial water quality and water sources through testing, and adequate food safety training of employees on the farm (Smith et al., 2014).
Growth media characteristics for detection of E. coli

A growth medium is a nutrient base used to support the growth of microorganisms or cells, and common growth media in use are nutrient broths and agar plates. Selective media is used for the growth of targeted microorganisms, and the growth of unwanted microorganisms is restricted with use of known antibiotics or enzymatic sensitivity. An example of media used for selective detection of *E. coli* is Mac Conkey-Sorbitol ChromoSelect Agar. It is recommended for selective isolation of *Escherichia coli* 0157:H7 from food and animal feeding stuff. MacConkey Sorbitol Agar is based on the formulation described by Rappaport and Henigh. The medium contains sorbitol instead of lactose, and it is recommended for the detection of enteropathogenic strains of *E. coli* 0157:H7, which ferments lactose but does not ferment sorbitol and hence produce colorless colonies. Sorbitol fermenting strains of *E. coli* produce pink-red colonies due to production of acid from sorbitol, absorption of neutral red, and a subsequent color change of the dye when pH of the medium falls below 6.8. *E. coli* 0157:H7 has been recognized as a cause of hemorrhagic colitis. Strains of *E. coli* possessing β-D-glucuronidase appear as blue colored colonies on the medium. Enteropathogenic strains of *E. coli* 0157 do not possess beta-D-glucuronidase activity and thus produce colorless colonies. *E. coli* fermenting sorbitol and possessing beta-D-glucuronidase activity produce purple colored colonies. Most of the gram-positive organisms are inhibited by crystal violet and bile salts. The addition of Tellurite-Cefixime Supplement makes the medium selective. Potassium Tellurite selects the serogroups 0157 from other *E. coli* serogroups and inhibits Aeromonas species and Providencia species. Cefixime inhibits Proteus species.

*Salmonella* Species

*Salmonella* spp. include gram-negative and non-spore forming bacterial cells, which are mesophilic facultative anaerobes, with the best growth at pH of 4.0 -9.0 and inactivated at
pasteurization temperatures (Jay, Loessner, & Golden, 2008). The Centers for Disease Control and Prevention (CDC) estimates a total of 1.2 million illnesses, 23000 hospitalizations and 450 deaths annually in the United States with most illnesses resulting from foodborne outbreaks (CDC, 2019d). *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori*. The major serovars of *Salmonella* spp. associated with produce outbreaks include Typhimurium, Poona, Newport, Miami, Enteriditis, Montevideo, and Stanley (CDC, 2019a).

In addition, *Salmonella* can survive in the environment for longer periods of time. Birds, reptiles, mammals, and insects carry *Salmonella* as natural inhabitants in the intestinal tract (Jay et al., 2008). Fecal matter from animals and birds contaminate soil and water through runoff and can contaminate produce upon contact. *Salmonella* is reported to persist in the hog production environment for five weeks in soil and up to seven weeks in the shelters (Jensen, Dalsgaard, Stockmarr, Nielsen, & Baggesen, 2006); thus creating potential chances of cross-contamination on mixed farms. This has been implicated in contaminating several produce items, not limited to alfalfa sprouts, cantaloupe, fruit salad, lettuce, mamey, mung bean sprouts, tomatoes, and a watermelon (Harris et al., 2003).

*Salmonella* is a ubiquitous human pathogen, and despite the implementation of strict food safety practices, its incidence has not reduced significantly in the food supply chain. Food safety interventions such as monitoring animal use on the farm and testing the microbial quality of irrigation and post-harvest water are critical to minimizing exposure of fresh produce to *Salmonella* contamination.

**Listeria monocytogenes**

Soil, manure, and water have been reported to carry *Listeria monocytogenes*, a member of the genus *Listeria* (Jeyaletchumi et al., 2012). *Listeria monocytogenes* is a gram-positive unlike *E. coli* and *Salmonella* spp., non-spore forming, facultative anaerobe, and can grow at
extreme temperatures of -0.4 °C to 45 °C (Jay et al., 2008). *Listeria* has been reported to cause a severe human disease called listeriosis, with 1600 sicknesses and 260 deaths annually in the United States (CDC, 2019b). 19% of total deaths caused by consumption of contaminated food in the United States are attributed to *Listeria* (Scallan et al., 2011).

*Listeria monocytogenes* has been implicated in several fresh produce outbreaks in the past including chopped celery outbreak in Texas in 2010, resulting in 10 deaths; melon outbreak in Colorado in 2011, infecting 30 people; and caramel apple outbreak in California, resulting in 35 infections and 7 deaths (Zhu, Gooneratne, & Hussain, 2017). Listeria is isolated from the fecal matter of humans and animals and is abundant in the environment (Nightingale et al., 2004; Vela et al., 2001). The contamination of fresh produce could occur with cross-contamination from contaminated water, soil, fecal matter, deposition from the environment, contaminated equipment, and humans (Zhu et al., 2017). *Listeria* carries the ability to survive in soil for long and prefers to thrive in high moisture soils (McLaughlin, Casey, Cotter, Gahan, & Hill, 2011). The high prevalence in the environment, and ability to form biofilm makes *Listeria* a potential pathogen of interest in food production and manufacturing (Valderrama & Cutter, 2013).

*Listeria’s* widespread prevalence in farming environments and implications in fresh produce outbreaks make it an important human pathogen of interest. The application of good food safety practices in the pre-harvest environment and using effective treatment methods to reduce biofilm formation will help decrease its survival and contamination in the fresh produce supply chain (Zhu et al., 2017).

**Food Safety Regulations**

**FSMA Produce Safety Rule**

The FSMA Produce Safety Rule was signed into law in January 2011 by US President Barack Obama and was finalized in November 2015. The FDA introduced the new food safety
regulation focusing on prevention strategies rather than a reaction to foodborne outbreaks. The law covers all the food commodities (including fresh fruits and vegetables) that are generally consumed raw and can cause illness in humans if contaminated. The regulation establishes science-based minimum standards for safe production and harvesting of fresh fruits and vegetables. These standards are based on a foundation of Good Agricultural Practices (GAPs). The rule is divided into several parts, including standards for worker health, hygiene, and training; agricultural water, both for production and post-harvest uses; biological soil amendments (e.g., compost, manure); domesticated and wild animals; equipment, tools, buildings, and sanitation; and production of sprouts (FDA, 2019a). Not all fresh produce farms are covered under the FSMA PSR. The rule does not apply to farms that do not grow raw agricultural commodity (RAC), grows food grains, grows food for personal or on-farm consumption, or the annual food sales from the farm are less than $28,106 in the year 2018 (FDA, 2019b). The rule also provides a qualified exemption to farms that have annual sales less than $562,119 in year 2018 and sell their products to qualified end-users (See appendix).

Recently, FDA launched a Food Safety Dashboard under the FDA-TRACK Program to follow the progress and impact of FSMA regulation; and identify needs of continuous improvement to food safety regulations (FDA, 2019e).

The key requirements under the FSMA Produce Safety Rule include:

**Agriculture water:** The rule establishes two sets of criteria to monitor the microbial quality of water based on use; pre-harvest water - the water used for the production of fresh produce (including irrigation, pesticide application, dust abatement, frost protection, harvesting) and post-harvest water (including washing, cooling, holding activities). The pre-harvest must have ≤ 126 generic *E. coli* cells/100 mL of water sample, and postharvest must be free of any generic *E.
*coli*/100 mL of water sample. There are nine certified quantitative and seven qualitative water testing methods mentioned in the Produce Safety Rule (FDA, 2019a). Details about the methods and water testing requirements are discussed in later sections of the literature review. Water Summit (February 27-28, 2018): FDA received stringent feedback from stakeholders on water quality criteria mentioned in the PSR, raising concerns about complexity and ease of implementation. FDA has extended dates of compliance for water rule to address these concerns and identify ways to simplify the water standards. The summit was an opportunity to discuss ways of enhancing the safety of water use on fresh produce farms and reducing the risks of future foodborne illnesses. Currently, the FDA is reviewing the comments and identifying potential needs to modify the water standards, and thus ‘water rule’ is stated to be incomplete (Wall et al., 2019).

**Biological Soil Amendments:** FDA recommends the use of composted manure and disapproves of using raw manure in fresh produce fields due to increased chances of cross-contamination with human pathogens. The PSR mandates microbial and process parameters for thermophilic composting that minimizes the food safety risks to produce. However, there are certain questions to which FDA does not have an immediate answer such as time interval between application of manure and harvest of produce; although FDA does not object farmers complying with USDA’s National Organic Program guidelines that recommend a time interval of 90 and 120 days for compost and manure application (FDA, 2018b, 2019a). Soil Summits (March 28-29, 2017, Geneva, New York; December 12-13, 2017, Houston, Texas; January 21-22, 2018, Atlanta, Georgia): The FDA organized the soil summits to address the knowledge needs and concerns of growers and educators, and shared research initiatives under progress related to manure risk assessment and its application to FSMA PSR. FDA marked the section of the PSR as ‘Reserved’
that regulates the application of untreated Biological Soil Amendments Of Animal Origin (BSAAO) due to increased concerns from the stakeholders regarding its implementation. Currently, the FDA is conducting extensive research to find out the minimum interval of application of untreated BSAAOs and identifying compliance with USDA NOP guidelines. Since risk assessment studies are time-consuming, the delay in finalization of the BSAAO rule may take additional few years (PSA, 2019).

**Domesticated and Wild Animals:** The PSR addressed the issues of food safety at mixed farms, including grazing animals and working animals on fresh produce farms. The rule established minimum scientific standards for controlled use of animals on the farm to minimize food safety risks and enlist recommendations such as field regions with foreseeable hazards on-field, or wildlife intrusion should be quarantined and not harvested for consumption. The FSMA PSR does not prohibit use of domesticated and working animals in the produce fields; however, their presence should be monitored to minimize the food safety risks. The best way to minimize risks is to not allow working animals in fields near to harvest and when the edible portion of the crop is growing. Although the rule does not require establishing grazing time intervals, however FDA recommends growers to do so minimizing the risks. PSR also focuses on maintaining biodiversity and does not encourage to destroy wildlife habitat or exclude animals at farms (FDA, 2019a).

**Worker Training and Health Hygiene:** The PSR defines practices that prevent contamination of produce and contact surfaces by the sick worker or people with poor hygiene on the farm. The contamination from workers can be minimized using good hygiene practices such as washing hands thoroughly as needed after toilet use and before touching the fresh produce. The rule also focuses on implementing good practices for visitors to minimize the risk of contamination on the
farm. Worker training is must for all farm people who come in direct contact with the produce and include principles of food hygiene and food safety, importance of health and personal hygiene, and other pieces of training related to worker’s job. Only one person from a farm is required to attend a Produce Safety Alliance training and provide trainings to other workers at the farm (FDA, 2019a).

**Equipment, Tools, and Buildings:** The PSR made recommendations for sanitation practices of tools, equipment, and the building reducing the chances of cross-contamination of fresh produce from dirty surfaces. It includes using basic sanitation practices such as housekeeping, eliminating pests and debris, and minimizing standing water. Other recommendations include reducing risks in the packaging areas by keeping it clean, separating produce handling areas from other farm activities, providing proper hygiene facilities for workers, and keeping covered and non-covered produce separate.

Cleaning and sanitation efforts can be targeted by defining zones in the packing areas. The rule clearly defines the differences between cleaning and sanitizing; stating, a dirty surface cannot be sanitized, and it must be cleaned before sanitizing. Recommendations regarding the sanitary design of the building and equipment are made to enhance cleaning and sanitation practices. Certain modifications are made to the equipment over time (such as retrofitting) and ensuring these new changes do not introduce additional food safety risks to the produce is important. The requirements of this module extend to greenhouses, germination chambers, toilets and hand-washing facilities (FDA, 2019a).

**Different Routes of Contamination of Fresh Produce**

The farm to fork continuum does not follow standardized operations and knowing that every farm is unique in its own operations, poses a great challenge to understand the routes of contamination. Farming systems vary in different production zones within the United States and
have different environmental risk factors, including topography, climate, and land-use interactions (Alegbeleye et al., 2018). The major routes that contaminate fresh produce include water, soil, and raw manure (Lynch et al., 2009; Uyttendaele, Jaykus, Amoah, Chiodini, Cunliffe, Jacxsens, Holvoet, Korsten, Lau, McClure, et al., 2015). The following figure (Figure 2.1) well illustrates the sources of contamination and the interaction among them, which could lead to potential contamination of fresh produce.

![Diagram of potential sources of contamination of fresh produce]

**Figure 2.1. The potential sources of contamination of fresh produce**

**Agricultural Water**

Irrigation water is a key element of crop production, and different water sources are used to meet the irrigation demands of crops in limited rainfall regions (Kirby, Bartram, & Carr,
However, agricultural water is implicated in several produce-related outbreaks in the recent past (including *E. coli* O157:H7 contaminated romaine lettuce outbreak of 2018) (FDA, 2019c; Uyttendaele, Jayks, Amoah, Chiodini, Cunliffe, Jacxsens, Holvoet, Korsten, Lau, & McClure, 2015). Although the Clean Water Act regulates the microbial quality of water used for humans and environmental health, there are no microbial standards regulated for irrigation water in the United States (Rock et al., 2019). Literature reports irrigation water to be a potential source of pathogenic bacteria, including *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* (Y. Pachepsky, Shelton, McLain, Patel, & Mandrell, 2011; Steele & Odumeru, 2004).

Sources of irrigation water that can cause contamination include groundwater, surface water, and human wastewater. Municipal water has been least associated with foodborne outbreaks since it is treated and tested regularly for the presence of fecal coliforms and human pathogens. Groundwater is found in aquifers beneath the earth’s surface, and contamination occurs during surface runoff through the well with no backflow prevention device or seepage through the soil, for shallow wells. Surface water includes that of various freshwater sources, such as ponds, lakes, rivers, and creeks, and open access to the environment compromises the microbial quality of water. Wastewater refers to human sewage and is commonly used for irrigation in countries where water is limited, including Canada and the United States. The use of wastewater irrigation can increase the available water supply and provide important nutrients for crops, but improperly treated wastewater can contain high levels of foodborne pathogens and affect safety of fresh produce (Steele and Odumeru, 2004). For example, Hepatitis A contaminated lettuce outbreak and spring onions were associated with sewage water irrigation (Heaton & Jones, 2008).
Irrigation factors that affect the potential and magnitude of contamination of fresh produce include water source (surface, ground, rural), method of irrigation (furrow, overhead, drip), type of crop (root crop, leafy green, stem, flower, seed) and land use practices (mixed farming) (Olaimat & Holley, 2012; Y. Pachepsky et al., 2011; Steele & Odumeru, 2004). Among water sources, surface waters have been reported to have the highest bacterial contamination, followed by groundwater and potable water (Bhullar, Shaw, Hannan, & Andrews, 2020; Uyttendaele, Jaykus, Amoah, Chiodini, Cunliffe, Jacxsens, Holvoet, Korsten, Lau, McClure, et al., 2015). Sprinkler irrigation method poses the largest risk of microbial contamination as compared to furrow and drip irrigation methods, as the harvestable portion of the crop comes in direct contact with contaminated water (Y. Pachepsky et al., 2011). Irrigating covered produce with contaminated water near to harvest time adds significant risk to the safety of produce as it increases the chances of survival of pathogens on the produce (Alegbeleye et al., 2018). Also, the method of transporting water to the field adds potential microbial hazards to the quality of water, such as, furrows interact with microbial reservoirs of soil and sediments; water in pipes interact with biofilms that are difficult to clean over time (Y. A. Pachepsky, Blaustein, Whelan, & Shelton, 2014; Y. Pachepsky et al., 2011). A similar issue exists with hydroponics growing system where the incidence of biofilms is common; however, crop foliage does not come in direct contact with water and thus poses lowered chances of microbial contamination from irrigation water (Jung et al., 2014).

Another factor impacting the safety of crop grown is the internalization of pathogens into the plant foliage (such spinach leaves), which is reported to be highest under sprinkler irrigation system (Warriner, Huber, Namvar, Fan, & Dunfield, 2009). The adhesion of human pathogens to plant surfaces and internalization into the plant tissues have rendered several processing methods
ineffective and has emerged as a compelling public health concern (Doyle & Erickson, 2008; Warriner et al., 2009). The survival of pathogens in the irrigation water poses another significant challenge to ensure the safer use of water for covered produce operations (Table 2.3). Survival is affected by several factors, including pathogen type, pathogen load, pH of water, temperature, and UV exposure (Jokinen, Hillman, & Tymensen, 2019; Steele & Odumeru, 2004). The nutrient availability in the irrigation is also reported to enhance the survival of *E. coli* in waters (Wanjigi, Fox, & Harwood, 2016); however, a different study reported significant cell viability for *E. coli* in nutrient deficit water environments (Takano, Pawlowska, Gudel, Yomo, & Tsuru, 2017). Water used for irrigation encounters the harvestable portion of the crop, multiple times during the growing phase (depends on frequency and method of irrigation), thus depositing multiple batches of pathogen loads on to the plants. Thus, the potential of survival of pathogenic microbes in irrigation waters poses considerable threat to safety of fresh produce (Alegbeleye et al., 2018).

Food Safety issues that are currently associated with fresh produce are linked to two major causes: naturally occurring contamination on produce fields due to inadequate implementation of preventive practices (Stuart, 2010) and paucity of technical knowledge among growers that can prevent food contamination (Parker et al., 2012). Large scale farms are perceived to have an effective food safety program that includes third-party audits and advanced processing facilities, whereas all these resources are unavailable to small scale growers. With this established assumption, experts from the food science industry believe higher risk of contamination at small scale growers’ farms (Viswanathan, 2013). There are several factors listed in the FSMA Produce Safety Rule that are of utmost importance to protect the safety of fresh produce and public health, with prominent focus on agriculture water quality. The rule classifies fresh produce farms based on the annual food sales from the farm and is classified into
three categories: Very small businesses (>\$28,106 – 281K), Small businesses (>\$281K - 
\$562,119), and all other businesses (>\$562,119). All these different types of farms need to be in 
compliance under the Produce Safety Rule; however, their effective dates are different.

In FSMA, Produce Safety Rule, agricultural water is defined as water that comes 
in direct contact with a harvestable portion of covered produce §112.44(b) (FDA, 2017a). 
Agricultural water is divided into two sections: production water ad postharvest water. All water 
that is used for growing activities (including irrigation, pest management, dust abatement, frost 
protection, harvesting) in the field is termed as production water. Postharvest water includes all 
water that is used during washing, packing, cooling, holding, or packing activities. And, covered 
produce includes all the fruits and vegetables that are often consumed raw. Potable water for 
human and environmental health is regulated by US government under Clean Water Act; 
however, there are no microbial indicator standards that regulate quality of agricultural water 
(Rock et al., 2019). The standards listed in FSMA Produce Safety Rule are based on generic 
E. coli numbers and do not truly reflect the microbial quality of water used for produce 
production. The contamination risks from irrigation water vary with water source, irrigation 
method, crop type, and agroclimatic conditions (UV radiation, relative humidity) (Uyttendaele, 

The Produce Safety Rule focuses on the implementation of minimum science-
based methods to minimize foodborne outbreaks in fresh produce, with a specific focus on 
agricultural water. To the present date, the rule requires fresh produce growers to develop a 
Microbial Water Quality Profile (MWQP) for each water source used on the farm through water
Table 2.3. The survival of human pathogens in different water sources

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Water Source</th>
<th>Inoculum (CFU/mL)</th>
<th>Findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Surface Water</td>
<td>8 x 10^4</td>
<td>Water aeration and sterilization enhanced persistence, higher survival in lake and puddle waters as compared to drinking and river water</td>
<td>(Avery, Williams, Killham, &amp; Jones, 2008)</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Wastewater</td>
<td>10^3 – 10^7</td>
<td>Failed to establish and proliferate in wastewater</td>
<td>(Ravva, Sarreal, Duffy, &amp; Stanker, 2006)</td>
</tr>
<tr>
<td><em>Salmonella</em> Typhimurium</td>
<td>Groundwater</td>
<td>Not Specified</td>
<td>Highly stable in groundwater</td>
<td>(Dowd &amp; Pillai, 1997)</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Groundwater</td>
<td>10^7</td>
<td>Water composition affects pathogen survival</td>
<td>(Cook &amp; Bolster, 2007)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Groundwater</td>
<td>10^4</td>
<td>Detected throughout the year, before and after manure application, strong correlation with an application time of manure</td>
<td>(VanderZaag, Campbell, Jamieson, Sinclair, &amp; Hynes, 2010)</td>
</tr>
</tbody>
</table>
testing and reporting number of generic *E. coli* cells per 100 mL of water sample. The water quality criteria are based on the initial PSR rule and is currently under review to modify the requirements that are more implementable and user-friendly to enhance the water safety in fresh produce production. The water rule under PSR will be updated in the coming few years. The current criteria for water sampling are listed in Table 2.4.

Table 2.4. Water testing frequency for different water sources on a covered farm

<table>
<thead>
<tr>
<th>Source</th>
<th>Testing Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Public Water Supply</td>
<td>Copy of test results or current certificates of compliance</td>
</tr>
<tr>
<td></td>
<td>4 or more times during the growing season or over the period of a year</td>
</tr>
<tr>
<td>Ground Water</td>
<td>1 or more samples rolled into profile every year after the initial year</td>
</tr>
<tr>
<td>Surface Water</td>
<td>20 or more times over a period of 2 to 4 years</td>
</tr>
<tr>
<td></td>
<td>5 or more samples rolled into profile every year after an initial survey</td>
</tr>
</tbody>
</table>

The agricultural water quality criteria for production water to meet the minimum standards for use in direct application on fresh produce is as follows:

- 126 or less colony-forming units (CFU) generic *E. coli* per 100 mL of water sample based on water geometric mean (GM), AND
- 410 or less CFU generic *E. coli* per 100 mL of water sample based on a statistical threshold value (STV)

Note: The criteria for postharvest water require zero generic *E. coli* in 100 mL of the water sample.

The geometric mean and standard threshold value criteria are used to evaluate the microbial quality of agricultural water, determine compliance, and find an appropriate use of water sources
on the farm. The geometric mean is the average value of generic \textit{E. coli} numbers from different test reports for a water source. And STV values represent the variability in water quality, and the standards require 90\% of test results should be lower than 410 cells/100 mL of water samples. The MWQP must be established over a minimum time period of 2 years to maximum of 4 years. There are several online tools to assist growers with calculations of GM and STV values including a spreadsheet developed by University of California Davis (https://ucfoodsafety.ucdavis.edu/sites/g/files/dgvnsk7366/files/inline-files/268306.xlsx).

The rule enlists different quantitative and qualitative (presence/absence) water testing methods for both production and postharvest water. Currently, there are nine water testing methods listed in the FSMA Produce Safety Rule for quantitative analysis of \textit{E. coli} and seven testing methods for testing the presence/absence of generic \textit{E. coli} in water samples (FDA, 2018a). The summary of all quantitative test methods is listed in Table 2.5.

The key concern in all the FDA approved water testing methods is the 6-hour sample holding time, i.e., the time between sample collection and the start of analysis. Not only in Midwest but in several regions of the United States, this timeframe is not realistic for fresh produce growers and needs modification to accommodate more growers under the Produce Safety Rule. In October 2018, FDA issued additional provisions to agricultural water module of the Produce Safety Rule, adding more equivalent water testing methods and extending the compliance dates for water rule (FDA, 2019d). The updated compliance dates are listed in Table 2.6. The extension in compliance dates is added to get feedback from industry stakeholders and provide FDA with time to reconsider the standards that regulate water quality. Thus, water rule is still under construction to ensure that existing standards and methods enhance public safety, minimize risks, and accommodate growers and industry’s needs.
Table 2.5. Summary of all Water Testing Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Type of test</th>
<th>Time for Incubation</th>
<th>Distinguishing step</th>
<th>Enumeration criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1103.1</td>
<td>Membrane Filtration</td>
<td>35°C for 2h 44.5°C for 22h</td>
<td>Urea substrate</td>
<td>Yellow, Yellow-green and yellow-brown colonies for <em>E. coli</em> 6-hour sample holding time</td>
</tr>
<tr>
<td>1604</td>
<td>Membrane Filtration (MI Agar 5mL)</td>
<td>35°C for 24h</td>
<td>Enzyme breakdown</td>
<td>Blue color for <em>E. coli</em> 6-hour sample holding time</td>
</tr>
<tr>
<td>9213 D</td>
<td>Membrane Filtration (bromocresol purple and Bromophenol red)</td>
<td>35°C for 2h 44.5°C for 22h</td>
<td>Urea substrate</td>
<td>Yellow and yellow-brown for <em>E. coli</em> 6-hour sample holding time</td>
</tr>
<tr>
<td>9222B</td>
<td>Membrane Filtration (Fuchsin Indicator)</td>
<td>35°C for 24h</td>
<td>Enzyme breakdown</td>
<td>Bright blue halo under UV for <em>E. coli</em> 6-hour sample holding time</td>
</tr>
<tr>
<td>D5392-93 (Similar to 1103.1)</td>
<td>Membrane Filtration</td>
<td>35°C for 2h 44.5°C for 22h</td>
<td>Urea substrate</td>
<td>Yellow, Yellow-green and yellow-brown colonies for <em>E. coli</em> 6-hour sample holding time</td>
</tr>
<tr>
<td>Hach 10029</td>
<td>Membrane Filtration (2mL reagent)</td>
<td>35°C for 24h</td>
<td>Enzyme breakdown</td>
<td>Blue color for <em>E. coli</em> under a wide field microscope 6-hour sample holding time</td>
</tr>
<tr>
<td>Colilert-24</td>
<td>Reagent based</td>
<td>35°C for 24h</td>
<td>Color change</td>
<td>Color comparison Dark Yellow = presence Blue under UV = <em>E. coli</em> presence 6-hour sample holding time</td>
</tr>
<tr>
<td>Colilert-18</td>
<td>Reagent based</td>
<td>35°C for 18h</td>
<td>Color change</td>
<td>Color comparison Dark Yellow = presence Blue under UV = <em>E. coli</em> presence 6-hour sample holding time</td>
</tr>
</tbody>
</table>
Table 2.6. Updates compliance dates for water rule

<table>
<thead>
<tr>
<th>Farm Size</th>
<th>Date of Compliance</th>
</tr>
</thead>
<tbody>
<tr>
<td>All other businesses (&gt;562,119)</td>
<td>January 26, 2022</td>
</tr>
<tr>
<td>Small businesses (&gt;281K - 562,119)</td>
<td>January 26, 2023</td>
</tr>
<tr>
<td>Very small businesses (&gt;28,106 - 281K)</td>
<td>January 26, 2024</td>
</tr>
</tbody>
</table>

Contaminated Soils and Biological Soil Amendments of Animal Origin

Animals manure has been used on farms as a source of fertilizer for decades, but it also carries a high number of microbial populations that may include human pathogens. Foodborne diseases owing to animal waste may occur when raw or improperly treated manure is used as a soil amendment or organic fertilizer to grow fruits and vegetables. Human pathogens associated with animal manure are present in the gastrointestinal tract of healthy farm animals (Doyle et al., 2001), and the presence of these pathogens in animal waste that is not properly treated can lead to foodborne and waterborne diseases.

Epidemiological investigations have confirmed that leading causes of bacterial illnesses resulting from food consumption have predominant zoonotic transmission routes. Some most common and important pathogens causing food or waterborne illnesses are bacteria - *E. coli* O157:H7 and other shiga-toxin-producing *E. coli*, *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Shigella* spp., *Clostridium perfringens*, *Vibrio* spp.; Viruses - enteroviruses, rotaviruses, adenoviruses, caliciviruses, hepatitis E viruses; protozoans - *Cryptosporidium parvum*, *Giardia lamblia*, *Toxoplasma gondii*, Cyclospora; and helminths: *Ascaris suum*, *Trichuris trichiura* (Manyi-Loh et al., 2016; Olson, 2001). Literature reports that *Salmonella enterica* serovar Newport can survive for 184, 332, and 405 days in manure, manure-amended, and manure-amended sterilized soil (You et al., 2006). Similar results are reported in the latest study, concluding that probability of survival of *E. coli* is higher in poultry litter,
followed by horse manure and dairy manure solids (Sharma et al., 2019). Another study reported that dairy-based composts are less likely to support survival of *E. coli* as compared to poultry-based composts, and these findings can assist fresh produce growers in making farming decisions during application of manure (Neher, Cutler, Weicht, Sharma, & Millner, 2019). Cowpat has been reported to act as reservoir for *E. coli* O157:H7 and *Salmonella* Typhimurium and these pathogens can survive in cowpats for a year and can even over winter. Their persistence can serve as a source of re-infection in the following year posing significant threat to food safety on mixed farms (Nyberg, Andersson, & Elving, 2019). Recent research explores various environmental factors that can affect pathogen population in the soil, such as the presence of higher pH in the soil promotes longer survival time for *E. coli* O157:H7 (Xing, Wang, Brookes, Salles, & Xu, 2019).

Animal manure is a widely accepted potential source of human pathogens that can contaminate food and water if it is applied to the production field untreated. Different routes of contamination include the presence of wildlife or domesticated animals on the field, runoff from the pasture or pasture grazing, contaminated surface water, and manure-based soil amendments. The transfer of human pathogens to fresh produce could occur either with direct contact with the produce (such as growing root crops in manure applied fields) or with water splash onto the produce surface or sprinkler irrigation and with a good possibility lies of transfer through vectors (insects) (Warriner et al., 2009).

The FSMA Produce Safety Rule recommends a grower to use composted manure in fresh produce fields and has set standards for the thermophilic composting process (FDA, 2019a). Composting is defined as a process to produce stabilized compost in which organic material is decomposed by the actions of the microorganisms under thermophilic conditions for a set period
(3 days), at a set temperature of 131 °F or 55 °C. Temperature is the primary method of pathogen reduction in the composting process, and length of composting depends on management factors such as aeration, turning, cover, moisture, and type of manure. Only a composting process that has been scientifically validated ensures pathogen reduction. Scientifically valid composting methods include (a) *Static composting* that maintains aerobic (oxygen) conditions at a minimum of 131° F (55° C) for 3 consecutive days and is followed by adequate curing; and (b) *Turned composting* that maintains aerobic conditions at a minimum of 131° F (55° C) for 15 days (can be non-consecutive), with a minimum of 5 turnings and followed by curing. Composting is a continuous process that transforms readily available nutrient sources into mineralized, environmentally stable forms of nutrients. Various factors control the efficacy of thermophilic composting and include oxygen, aeration, nutrients (C:N ratio), moisture, characteristics of compost pile (porosity, structure, texture, particle size), pH, temperature, turning and time (Alsanius et al., 2016; Rynk et al., 1992). The microbial standards for thermophilic composting include:

*Salmonella* species: Not detected using a method that can detect three most probable numbers (MPN) per 4 gram (or milliliter, if the liquid is being sampled) of total solids

*Fecal coliforms:* Less than 1,000 MPN per gram of total solids (dry weight basis) or milliliter if the liquid is being sampled

Interestingly, organic growers worldwide are encouraged to apply animal manure to enrich soils and enhance biodiversity; and organic proponents assert enhanced food safety of organic foods than conventional produce; though there is no research to support this claim (Maffei, Batalha, Landgraf, Schaffner, & Franco, 2016; Warriner et al., 2009). The FSMA PSR
recommends guidance for minimizing contamination of fresh produce and enhance food safety. Numerous resources in the form of guidance documents, factsheets and flowcharts are available at the FDA FSMA website to assist growers with food safety information.

**Animal Use on the Farm (Wildlife and Domesticated Animals)**

The agriculture has become more intensive proximity of fresh produce fields to the animal production farms has resulted in new ecological connections and has a significant effect on microbial quality of fresh produce (Lynch et al., 2009). Cattle and other animals (both domestic and wild) are widely reported to carry high numbers of *E. coli* O157:H7 and the interaction of these animals in and around the produce fields poses a challenge to microbial safety. For example, the *E. coli* O157:H7 contaminated spinach outbreak of 2006, which resulted in 3 deaths and more than 200 illnesses in the United States, has been implicated in animal use on the farm (CDC, 2006). The investigations by the federal and state agencies did not reveal any risk factors for the contamination. However, the California Food Emergency Response team reported the potential risks that include presence of feral pigs in and around the spinach fields, and the proximity of irrigation wells to the surface waterways that have open access to fecal contamination from cattle and wildlife (Langholz & Michele, 2013; Team, 2007).

The contamination from wildlife may occur with direct fecal deposition onto the plants or indirectly through contamination of growing environment variables such as water and soil (Langholz & Michele, 2013). However, the mechanism of contamination routes from exposure to wildlife and animals on the farm is still not clear in the research studies. *E. coli* O157 has been isolated from avian species (Canada goose, Duck, Sparrow, Swan, Wild turkey), large mammals (Deer, Bighorn sheep, Elk, Moose), small mammals (Bat, Fox, Hare, Rabbit, Raccoon, Rodent) and invertebrates (Fly, Housefly, Slug) (Langholz & Michele, 2013). A review of 657 studies reported that inadequate experimental designs, outdated testing methods, and data reporting
makes it challenging to define policy and decision making to minimize potential food safety risks on the farms (Ilic et al., 2012; Langholz & Michele, 2013). Since the elimination of animals and wildlife on the farm is unrealistic and ecologically unjustified, preventive approaches such as use of buffer zones and avoiding practicing mixed farming may significantly lower contamination of fresh produce. Buffer zones are a more common practice in organic farming; however, its benefits can be extended to enhancing fresh produce safety at farms that have higher risk of wildlife intrusion (New Jersey Department of Agriculture, 2008). A study reported the filtration effect of *E. coli* through vegetative buffers on cattle-grazing land and reported lowered run-off of *E. coli* into the surface water reservoirs (Tate, Atwill, Bartolome, & Nader, 2006). In contrast, a study evaluated the effect of food safety buffer zones on habitat and biodiversity around the farm and reported loss of vegetation and wildlife, accompanied with increased cost for the grower (Gennet et al., 2013). Continuous investment in research is needed to study diverse vectors of contamination from wildlife and define preventive measures and minimize impact on public health.

**Worker Hygiene**

In the fresh produce industry, there are ample opportunities for the transfer of human pathogens from worker's hands onto the produce. Several pre-harvest and post-harvest activities require touching the produce at multiple points through harvesting till packing, and if adequate measures are not followed, the chances of microbial contamination are higher (T V Suslow et al., 2003). Workers can spread foodborne illnesses through hand contact with fecal matter or contaminated surface to the food. A study evaluated the data from 81 foodborne illnesses caused by food workers and reported that 89% of outbreaks were caused due to transfer of pathogens from worker hands (Guzewich & Ross, 1999). This marks the need for improved worker handwashing practices, following which United States Food and Drug Administration (USFDA)
included guidelines for handwashing in the Food Code for retail establishments (FDA, 2017b). Also, the microbial testing of aprons of food handlers reported unsatisfactory levels of *Staphylococcus aureus, E. coli, L. monocytogenes, Bacillus*, coliforms, yeast and mold (Sibanyoni & Tabit, 2019). The possibility of transfer of human pathogens is high when workers are not provided with adequate facilities on the farm, such as toilets and handwashing stations. As per the requirement of Occupational Safety and Health Administration (OSHA), the farm needs to provide one toilet and one hand washing station for every 20 workers on a farm and in a vicinity of 0.25 mile, to prevent contamination of produce if they workers face adverse health conditions on the farm (OSHA, 2019).

Worker health and hygiene are some of the critical factors that affect the safety of fresh produce. The foodborne outbreaks occur when contaminated food is consumed and results in sickness, i.e., through fecal-oral route of contamination, and includes food handlers or workers as a critical factor in the filed touching the food at multiple points throughout the farm to fork continuum (Beuchat & Ryu, 1997). For example, workers were implicated as source of contamination of green onions outbreak of 2003 with Hepatitis A (CDC, 2003; Calvin, Avendaño, & Rindermann, 2004; Wheeler et al., 2005).

Knowing that the fresh produce industry is very labor-intensive and there are many produce items (such as strawberries, lettuce, grapes) that are commonly hand-harvested and have little to partially mechanized harvest (USDA-ERS, 2010). Direct hand contact with the fresh produce is used to sort, tie or bind, pack and re-pack produce, and significantly compromises the microbial safety if adequate hygiene measures are not used (T V Suslow et al., 2003).

**Survival of Pathogens in the Produce Supply Chain**

The contamination in fresh produce can occur at any stage of production and processing until it is consumed. To cause an outbreak, food must be contaminated with human pathogens,
and the pathogen survives until the food is consumed, to a number that can cause illness. The major stages for highest contamination chances are in the field during production, initial processing (produce wash and handling), and during final preparation in the kitchen. Recent work by plant pathologists and food microbiologists indicates that the connection between the fresh produce and human pathogens may be beyond the passive transfer mechanism (Tyler and Triplet, 2008). Nevertheless, these organisms are well adapted to gut microbiome, and they can also well flourish on and in plants.

The bacterial pathogens can reach the interior of the plants by a variety of routes, including natural apertures (stomatal openings), surface injuries (pest attack or plant defect), and uptake through roots (Steele & Odumeru, 2004). As a fact, once the pathogen is internalized in the plant tissue, it almost impossible to remove it by washing or any other disinfection technique. The internalization of a pathogen within the fresh produce is a big challenge as bacteria can move in the plant by the capillary action of water (Burnett et al., 2000). Literature reports that plant defense system (cell walls and wax layers) naturally restricts entry of pathogens to inside of the plant tissue (Heaton & Jones, 2008).

The establishment of pathogen in produce can occur through different mechanisms, including attachment and internalization. Pathogen attachment is higher in produce with uneven surfaces, cavities, cracks as they entrap the pathogen and protect from disinfection (Alegbeleye et al., 2018). Several factors, including UV radiation, unfavorable temperatures, and relative humidity poses a challenge for microbial populations to survive on the plants. However, once pathogens colonize the produce surface, there are good chances of biofilm formation, which poses a vital threat to produce safety due to increased protection of pathogen cells from disinfection methods (Lemon, Higgins, & Kolter, 2007; Yao & Habimana, 2019).
The transfer of pathogen from the produce surface to the inside of plant tissue has been widely reported in literature and termed as internalization (Burnett, Chen, & Beuchat, 2000; Eissenberger, Drissner, Walsh, Weiss, & Schmidt, 2020; Erickson et al., 2019; Ge, Lee, & Lee, 2012; Golberg, Kroupitski, Belausov, Pinto, & Sela, 2011; Gomes et al., 2009; Guo, Chen, Brackett, & Beuchat, 2001; Itoh et al., 1998; Janisiewicz et al., 1999; Kroupitski, Gollop, Belausov, Pinto, & Sela, 2019; Moriarty, Semmens, Bissonnette, & Jaczynski, 2019). Internalization could occur through two different modes, i.e. active and passive internalization. Active internalization occurs when the pathogen is transferred from natural apertures in the phyllosphere (plant part above the ground), and passive internalization occurs through roots and growth from contaminated seeds (Matthews, 2014; Meireles, Giaouris, & Simões, 2016). Most of these studies are conducted in controlled environments; however, there are few studies that were conducted in field scenarios and reported low probability of internalization in the field settings (Erickson et al., 2010; Zhang et al., 2009). Internalized human pathogens pose a significant threat to fresh produce safety, and further research is required to evaluate true incidence of internalization and mechanisms that favor the transfer of pathogenic cells inside the plant surface in order to develop preventive interventions.

**Postharvest Washing of Fresh Produce**

The contaminated fresh produce is the leading cause of foodborne outbreaks with contamination from major human pathogens, including shiga toxin-producing *E. coli* (STEC), *Salmonella* spp., and *Listeria monocytogenes* (Callejón et al., 2015). The contamination of fresh produce during post-harvest activities adds significant risk as compared to pre-harvest contamination, as produce is closer to consumption. The water used in most post-harvest activities comes in direct contact with the produce, compromising the microbial quality of food if contaminated. It was believed that field contamination could be removed using disinfection
approaches during post-harvest washing (Feliziani, Lichter, Smilanick, & Ippolito, 2016); however it was recently known that postharvest washing results in limited disinfection of pathogen load and enhances cross-contamination (Gombas et al., 2017). Thus, regulatory agencies including FDA has changed their focus to preventing contamination beginning from the first stage of produce cultivation and minimize risks that come along the harvested produce from the field.

Postharvest washing not only washed the soil or dirt off the produce, but also reduces the microbial load of the produce (Barrera, Blenkinsop, & Warriner, 2012). Different post-harvest methods aim at achieving 5-log reductions of the microbial load in the lab setup conditions (Gombas et al., 2017; Olaimat & Holley, 2012), however, in commercial settings, the average inactivation of only 1-2 logs is achieved irrespective of the sanitizer type and treatment time (Barrera et al., 2012). The efficacy of postharvest treatments also depends on the adhesion of pathogen to the surface of produce (Murray, Wu, Shi, Jun Xue, & Warriner, 2017) and the organic load that neutralizes the antimicrobial effect of the sanitizer used (Shen, Luo, Nou, Wang, & Millner, 2013). The greatest challenge with standardizing the postharvest treatment processes include the lack of a risk-assessment model, although a log reduction of 2 – 5 log CFU is considered acceptable (Gombas et al., 2017). The inactivation kinetics models for target organisms in produce wash treatments are yet to be determined for effective sanitizer treatments.

**Disinfection Methods**

Fresh produce is commonly consumed raw and does not involve any kill step to ensure safety. Although, the Produce Safety Rule focuses on best practices to minimize risks during growing, harvesting, packing and storing of produce, antimicrobial and sanitizer treatments during postharvest processing are employed to enhance the safety of produce. Several methods can be used for preventing cross-contamination and include chemical treatments (sanitizers and
antimicrobials), irradiation treatment, and natural/biological methods (Goodburn & Wallace, 2013). The commonly used treatment methods to reduce microbial load and prevent cross-contamination in the produce industry is use of chemicals for their higher efficacy. The chemical treatments include chlorine, chlorine dioxide, bromine, iodine, trisodium phosphate, quaternary ammonia compounds, acids, hydrogen peroxide, and ozone. Research studies reported varying degrees of disinfection with different sanitizers depending on type of produce, sanitizer type, contact time, organic load in produce, water source, and pH (Erickson et al., 2010). However, chlorine is the most commonly used disinfection method in produce industry.

**Chlorination**

Chlorination, in the form of free chlorine (HOCl), added as sodium hypochlorite, is the commonly used sanitizer. The efficacy of free chlorine is maximum at a pH of 6.5 and concentration of 50 – 200 ppm (See Figure 2.2). Three different forms of chlorine can be used to make hypochlorite solutions including chlorine gas, sodium hypochlorite, and calcium hypochlorite (Trevor Suslow, 1997). Several studies have reported chlorination as an effective disinfection treatment for fresh produce contamination (Feliziani et al., 2016; Garrido, Marín, Tudela, Allende, & Gil, 2019; María I. Gil, López-Gálvez, Andújar, Moreno, & Allende, 2019; Maria I Gil, Selma, López-Gálvez, & Allende, 2009; Shen et al., 2013; Tudela et al., 2019).

Chlorine quantity in an aqueous solution is expressed in two terms called total chlorine and free chlorine. Total chlorine is the sum of chlorine (reacted with organic matter or other contents) and free chlorine (chlorine available for disinfection). The major benefits of chlorination include ease of use, comparative stability in the solution as compared to other sanitizers, rapid disinfection rate, low cost, and readily available. The major disadvantages of chlorination are, free chlorine is highly corrosive to equipment at lower pH range, forms harmful gases (Chloramines), reduced efficacy at low temperatures, pH maintenance for effective
disinfection, limited inactivation in the presence of organic matter, and wastewater treatment. Apart from these drawbacks, there has been an emerged concern with use of chlorine in produce industry, and that is the chemical risk of forming and accumulating disinfection by-products (DBPs) such as chlorate (Maria Isabel Gil, Marín, Andujar, & Allende, 2016; Shen, Norris, Williams, Hagan, & Li, 2016) and industry is looking forward to using alternative sanitizing treatments including hydrogen peroxide, organic acids, and ozone (López-Gálvez, Allende, Selma, & Gil, 2009).

![Figure 2.2. The concentration of HOCl at different pH levels](image)

Several lab studies reported high efficacy of chlorine against human pathogens in produce wash water with an average range of chlorine concentration of 1 – 20mg/L. At industrial scale, few studies reported minimum chlorine concentration of 10 mg/L for effective disinfection of leafy greens (Luo et al., 2018), and contradicting to this, a study reported survival of microorganisms at dose of greater than 20 mg/L of free chlorine, which may be due to inadequate regulation pf pH (López-Gálvez, Tudela, Allende, & Gil, 2019). This added discrepancy addresses the knowledge gap of lacking understanding of inactivation kinetics and
modeling with sanitizer disinfection processes. Research has reported that different product types require concentrations of free chlorine to achieve industrial disinfection standards (Tudela et al., 2019).

Numerous studies have reported the mechanism of kill action of free chlorine. According to (Estrela et al., 2002), sodium hypochlorite degrades the fatty acids of the cell into salts and glycerol, lowering the structural stability of the cytoplasmic membrane necessary for carrying out various functions of the cellular metabolism. Another study reported the sequence of events that lead to death of cells upon treatment with sodium hypochlorite; (1) cell membrane lipids are damaged, (2) respiration is terminated with destruction of membrane-bound enzymes, (3) intracellular micro (ions) and macromolecules (DNA and proteins) leak, (4) cell homeostasis deteriorated, and (5) finally leading to cell death (Ersoy, Dinc, Cinar, Gedik, & Dimoglo, 2019).

**Long Term Survivor Cells**

Microorganisms reproduce (grow in number) when exposed to a favorable environment by a process called binary fission. During the process, a cell asexually divides into two cells, creating a replica of the original cell. These cells have a surface area to volume ratio called s/v ratio. The formation of new daughter cells from parent cells increases the s/v ratio as more nutrient is needed to synthesize energy and cellular components (Ray & Bhunia, 2013). For ideal growth of microbial cells, environmental factors including food, temperature, acidity (pH), water activity, oxidation-reduction potential, and nutrients need to be optimum. The plot (Figure 2.3) illustrates the growth curve of microbial cells at different times; *lag phase* – during this phase the cell population does not change, and cells assimilate nutrients and increase in size. The population remains unchanged because of change in size, both cell mass and optical density show some increase; *log or exponential phase* - the cell number starts increasing, first slowly and then very rapidly. The cells in the population differ initially in metabolic rate and varied growth,
and follows first-order reaction kinetics; stationary phase – during this phase, nutrient shortage and accumulation of waste products occur coupled with signaled cell death to keep the living population stable; and death phase – in this phase the rate of cell death is higher than the rate of cell multiplication. Depending on the strain and conditions of the environment, after a long period of time (may even be a few years) some cells may still remain viable (Ray & Bhunia, 2013). Recent research on the characterization of these cells has termed this phase of bacterial growth as ‘long term survival’ cells.

Figure 2.3. Different growth phases of bacterial cells

With the increasing resistance of bacterial cells to survive antimicrobial treatments, including sanitizers, the survival of bacteria after chlorination is a rising concern for food safety. In most of the bacterial inactivation studies, quantifying the antimicrobial potential of various chemicals and natural agents, the use of stationary phase cells is not uncommon. Bacteria grows in 4 identified phases, including lag, log, stationary and death phase (Figure 1). However, research reports that cells beyond the death phase enter long term survival phase. In this phase, cells tend to attain resistance to unfavorable conditions, and are hardened to kill with the use of
commercial antimicrobial agents. In the 5th phase (Long-term Survival Phase), the cells are in the dormancy and consume the minimum food. The dead cells serve as the food for the healthy cells (Finkel, 2006; Wen, Karthikeyan, Hawkins, Anantheswaran, & Knabel, 2013). The change in morphological characters of the cells from rod-shaped to coccoid indicates the development of resistance in the cell wall against antimicrobials.

The growth of any bacterial culture in a nutrient media is supported only to a certain time beyond which the cells no longer have nutrients to repair and sustain metabolic functions. The cell death results in loss of cellular integrity, and surviving cells catabolize the dead cells (also called programmed cell death or apoptosis) to synthesize energy from their amino acids, carbohydrates from the cell wall, lipid from the cell membrane and DNA material (Finkel & Kolter, 2001). During late-term survival phase, cell division and cell death are balanced as nutrient status in LTS phase can only support few cells (Finkel, 2006). As new cells are reproduced, the older must die to maintain the balance.

Literature reports another bacterial state of cells called viable but nonculturable (VBNC) state. VBNC are the living bacterial cells that are unable grow into colonies when culturing procedure are done; however, these cells are metabolically active (Afari & Hung, 2018; Afari, Liu, & Hung, 2019; Oliver, 2000, 2010). LTS cells are different than VBNC as LTS cells are grow on nutrient medium using culturing procedures. Limited research is reported in the literature identifying the similarities and differences between the LTS and VBNC state of cells. Also, further research is required to identify the virulence factors associated with LTS phase of bacterial cells. Evaluating the significance of the use of LTS cells in validating antimicrobial treatments and sanitation methods used in the food industry will ensure higher standards of food safety.
References


Alegbeleye, O. O., Singleton, I., & Sant’Ana, A. S. (2018). Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: A review. *Food Microbiology, 73*, 177–208. https://doi.org/10.1016/j.fm.2018.01.003


CHAPTER 3. EXTENDING THE SAMPLE HOLDING TIME FOR TESTING EPA METHOD 1603 FOR PRODUCE GROWERS

Manreet Singh Bhullar ¹, Angela Shaw ¹*, Joseph Hannan ² and Smaranda Andrews ¹

¹ Department of Food Science and Human Nutrition, Iowa State University, Ames Iowa 50011, USA; manreet.bhullar@gmail.com (M.S.B.); smaranda@iastate.edu (S.A.)
² Iowa State University Extension and Outreach, Adel Iowa 50003, USA; jmhannan@iastate.edu

* Correspondence: angelaml@iastate.edu; Tel.: 1-515-294-0868

Modified from a manuscript published in Water (MDPI)

Abstract

Agricultural water is a known vector for the transfer of foodborne pathogens onto fresh produce. Development of pre-harvest and post-harvest microbial profiles of agricultural water used by fresh produce growers, processors, and holdings is a requirement under the Food Safety Modernization Act Produce Safety Rule. One of the United States Environmental Protection Agency (US EPA) approved agricultural water testing methods is US EPA Method 1603, which requires no greater than a 8-hour time frame between the collection of the water sample and initiation of analysis. To ensure samples are analyzed, it is recommended to submit samples to labs within 6 hours of collection. This 6-hour timeframe is unrealistic for many produce growers due to there being few laboratories certified to conduct testing and the geographic location of the farms. Agricultural water samples (n = 101) from well water and surface water were collected from 60 different farms to determine if holding samples for 24 hours yielded significantly more generic Escherichia coli (E. coli) than 6 hours using EPA 1603 method. A total of 32 samples were found contaminated with generic E. coli. Of these positive samples, surface water accounted for 87.5% of the samples (n = 28). There was no significant disparity between populations of generic E. coli at 6- and 24-hour sample-test time interval (p > 0.05). These
results provide evidence that the sample-test time interval can be extended to 24-hour time, which makes quantitative generic *E. coli* testing for agricultural water as mandated by the FSMA Produce Safety Rule more accessible to growers.

Keywords: agricultural water; EPA 1603; water testing; fruit; vegetable; Produce Safety Rule

**Introduction**

Fresh produce, which is commonly eaten raw, utilizes water in pre- and post-harvest activities including irrigation, pest management, washing, cooling, sanitation, and personal handwashing. Agriculture production is the major consumer of surface and groundwater in the United States, with 70% of agricultural water used for irrigation (Shanan, 1998). The microbial quality of water used in fresh produce production is a critical food safety concern, especially considering that irrigation water has been reported as a source of contamination in fresh produce (Faour-Klingbeil, Murtada, Kuri, & Todd, 2016; Steele & Odumeru, 2004).

Agricultural water microbial quality has been linked to many foodborne outbreaks in produce (M. F. Craun, Craun, Calderon, & Beach, 2006; Reynolds, Mena, & Gerba, 2008). For instance, in January 2018, a romaine lettuce outbreak in Yuma, Arizona, was linked to irrigation water sourced from a stream that was contaminated with *Escherichia coli* O157:H7 (*E. coli* O157:H7) from a nearby cattle feedlot. This outbreak affected 172 persons in 32 states, with 45% of people hospitalized, and resulted in one death (Centers for Disease Control and Prevention, 2019).

Development of an agricultural water profile can help farmers mitigate their food safety risk. There are nine approved agricultural water testing methods available under the Food Safety Modernization Act (FSMA) Produce Safety Rule (U.S. Food and Drug Administration, 2015). United States Environment Protection Agency (US EPA) method 1603 "detection of *E. coli* in water by membrane filtration using modified membrane Thermotolerant *E. coli* (mTEC) media"
is one of the nine approved methods. This method has a maximum 8-hour time interval between collection of the sample and start of the analysis. These FSMA water requirements also apply to all the imported covered produce from other countries into the United States. With respect to water regulations globally, there are no requirements in place to regulate microbial water quality other than third party audit requests such as Safe Quality Food (SQF). Short water-holding time is a challenge in many states in the US since there are few certified laboratories equipped to do this test, and many produce farms are not located close enough to a lab to deliver the sample within the time constraint or without spending an entire day away from the farm (U.S. Environmental Protection Agency, 2002). In literature, there are studies that analyzed the extension of sample holding time, but limited information was found related to agricultural water and EPA method 1603, the only Food and Drug Administration (FDA) -approved water testing method (McDaniels et al., 1985; Pope et al., 2003; Selvakumar, Borst, Boner, & Mallon, 2004). To modify the requirements for water testing methods under the FSMA Produce Safety Rule, the researcher must submit comments and data through the FDA Dockets Management platform. These comments can be made with draft rule and final rule. Data must include the complete methods, results, and conclusions from the study. The objectives of this study were to determine the prevalence of generic *E. coli* in agricultural water used in Iowa fresh produce farms and determine if the current sample holding time for US EPA 1603 method can be extended from 6 hours to 24 hours.

**Materials and Methods**

Two-hundred mL water samples (*n* = 101) were collected from 60 fresh produce farms located throughout Iowa. A listserv of produce growers in Iowa was utilized to solicit participation in this study. Farms were chosen based on distribution throughout the state and water sources, and the ability to collect water samples within the 6-hour transportation time as
recommended under US EPA Method 1603 (See Figure 3.1). The water sampling was conducted during the growing season in Iowa (May–August) in 2017 and 2018. A subset of the total samples (n = 11) were sampled multiple times during the study, to accommodate growers’ requests and assist with developing records and microbial water quality profile. Samples were collected from two different types of water sources: Well water (deep wells (n = 63, >250 ft) and shallow wells (n = 6, <60 ft), all capped), 67%) and surface water (n = 32, (ponds, lakes, rain harvested water), 31%). Municipal water samples were not collected for this study as the water is treated and tested by the city daily. Samples were collected in sterile water cups (Samco Scientific, Mexico) and prepared for analysis at two-time frames: 6 hours and 24 hours.

Figure 3.1. Iowa map illustrating number of water samples collected in 2017 – 2018
The temperature of the water (°C) from the source was recorded at the time of sampling, and the water samples were stored in the range of 4–10 °C until the time of analysis per US EPA Method 1603 (U.S. Environmental Protection Agency, 2002). However, for 24-hour analysis, the samples were held at <10 °C for the first 6 hours and then stored at 4 °C for the remaining 18 hours. Samples were delivered to Iowa State University BSL2 Food Microbiology Laboratory and analyzed according to the US EPA Method 1603 standard (U.S. Environmental Protection Agency, 2002). As part of this study and not included in this manuscript, the water sample results were provided to the growers to include as part of their farm water profile.

All cell count numbers were documented in colony forming unit (CFU)/100 mL values and transformed to a logarithmic scale to meet the requirements for statistical analysis. The mean differences between bacterial counts at 6 hours and 24 hours were calculated using paired t-test in SAS statistical computing environment (SAS 9.4, Cary, NC). Statistical significance was established at p < 0.05.

**Results and Discussion**

The quality of agricultural water used on produce farms is a critical factor in reducing food safety risks. There is limited information available for understanding the impact of different sources of irrigation water used by produce farms, each posing varying degrees of food safety risks (Decol et al., 2017). In this study, the number of *E. coli* cell counts varied among water source samples. Among the 101 samples collected, 32 samples (31%) contained *E. coli* colonies ranging from 1 to 68,000 cells per 100 mL water sample. According to the FSMA Produce Safety Rule, the *E. coli* population in agricultural water must not exceed a geometric mean of 126 cells/100 mL and the standard threshold value of 410 cells/100 mL. Nine of these positive samples (8.75%, all surface waters) were contaminated beyond the FDA’s maximum allowed limit (U.S. Food and Drug Administration, 2015). Water sources contaminated above the FDA
set standards to require corrective actions (such as employing bacterial die-off time, re-inspecting agricultural water system, water treatment) for direct use on covered produce (U.S. Food and Drug Administration, 2015). The contamination in surface waters (87.5%) was found to be highly prevalent, followed by well waters (5.8%), as shown in Table 3.1.

Table 3.1. Percentage of positive samples of generic *Escherichia coli* obtained from sixty Iowa fresh produce farm waters

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of Samples</th>
<th>Contaminated Samples</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Water</td>
<td>32</td>
<td>28</td>
<td>87.5</td>
</tr>
<tr>
<td>Well Water</td>
<td>69</td>
<td>4</td>
<td>5.8</td>
</tr>
</tbody>
</table>

The results from this study agree with other studies that have shown that susceptibility of surface water (including ponds, lakes, and streams) to microbial contamination is much higher than groundwater (i.e., well water) sources (Uyttendaele et al., 2015). Surface water has been shown through research to be more prone to contamination than any other water source because it is open to the environment, animals have easy access to it, rainfall events contribute to run off, and sewage and manure sludge could potentially be directly seeping into watersheds (James, 2006; Steele & Odumeru, 2004). Also, surface water has been reported to carry a high number of enteric pathogens (Crowe, Mahon, Vieira, & Gould, 2015). The literature also reported that a higher incidence of waterborne illnesses is associated with drinking water at times of intense rainfall (Curriero, Patz, Rose, & Lele, 2001; Thomas et al., 2006). However, no trends were observed in the data from this study regarding high contamination of surface waters (88%) such as the same water source or the same region of the state. Contamination in groundwater is common in the United States. Between 1991 and 1992, most (76%) of the 34 drinking water disease outbreaks have been traced back to contaminated wells (Moore et al., 1993; Reynolds et al., 2008). Moreover, during 1981–1990, contaminated groundwater was responsible for 43% of
diseases and outbreaks reported a total of 42 drinking water-associated outbreaks that resulted in
1006 illnesses, 124 hospitalizations, and 13 deaths (Benedict et al., 2017).

The *E. coli* analysis was conducted at two different time frames, 6 hours, and 24 hours. No statistical difference (*p* > 0.05) was observed between *E. coli* colony counts during these time intervals (Figure 1). While it is a small sample size, these results support the extension of the sample holding time to 24 hours with samples stored at <10 °C for agricultural water samples from the surface, well, and municipal water sources. Our results differ from a study reported in 2003 that analyzed *E. coli* in water from 24 different sites across the USA and showed that the water analysis could be done beyond 8 hours to detect *E. coli* if stored at 10 °C. However, another study reported water samples should be analyzed within 8 hours to get accurate results (Pope et al., 2003). The results from a study conducted on stormwater and sewage show that water samples kept at 4 °C were not statistically different when analyzed for fecal coliforms at 7 hours and greater than 24 hours holding times (Selvakumar et al., 2004). The study also supported the conclusion that sample storage could be extended to 24 hours for analysis of microorganisms without altering the mean levels of *E. coli*, which is in accordance with the results of this paper. Another similar studies supported the results reported in this study, stating that extending sample holding time from 8 to 30 hours does not affect the bacterial population and also mentioned the possibility to extend sample storage time to 62 hours (Aulenbach, 2010; Bushon, Brady, & Lindsey, 2015). Extending sampling holding period promotes water testing by making it easier to access certified laboratories in the region through overnight shipment, coordination of delivery with other growers, or transporting by self. These standards will also assist with conformance with drinking water standards held throughout the world. For example,
studies conducted in the European Union have reported use of ISO methods (commonly used ISO 7899-2 method) as per the requirements of European Drinking Water Directive 98/83/EG. These methods use 24-hour sample holding time before the analysis of water samples, which is in agreement with the conclusion of this study (Schets et al., 2005; Tanaro et al., 2014). Moreover, in Australia their drinking water standards are similar to the United States but hold stricter requirements for frequency and level of *E. coli* levels using AS/NZS 5667.5:1998 which requires samples held less than 24 hours (Standards Australia/standards New Zealand, 1998).

![Figure 3.2](image-url)

Figure 3.2. Generic *Escherichia coli* cell counts of 32 contaminated samples (69 samples tested at no detectable *Escherichia coli*) at 6 hours and 24 hours sample holding time obtained from sixty Iowa produce farms in 2017–2018.

The average temperature of water samples collected at the source was 18.6 °C with minimum and maximum readings of 9 °C and 30 °C. No significant correlation ($r = 0.20$, $p > 0.05$) was observed between water temperature and the *E. coli* cell count among different
types of water sources. A study reported a significant correlation between *E. coli* and water temperature \((r = 0.26, p < 0.001)\) that sampled surface water from canal waters at multiple time points during a day (Lothrop et al., 2018). It can be hypothesized that this disparity between the studies is due to the sample size and regional differences between the southwest USA and Iowa, which might have helped reduce the microbial numbers. The southwest USA has increased exposure to the sun, warm air temperatures (monthly high of 21 °C), and low precipitation (monthly average of 0.97 cm) (Lothrop et al., 2018).

Water plays a crucial food safety role in the production of fresh fruits and vegetables. Numerous direct points of contact between water and food from farm to fork make it a potential source of contamination and a food safety concern. The strict regulation of agriculture water quality for produce farms under the FSMA Produce Safety Rule means that all covered farms are required to have quantitative water tests using one of the nine certified methods, including EPA 1603. Considering the limitations of the 6-hour sample holding time with all approved methods and few certified labs, many growers resist participating in water testing. Extending the holding time from 6 hours to 24 hours would help growers become compliant with the new regulation and would minimize food safety risks on their farm. This adjustment in the water testing method would also aid the FDA in its approach to preventing and reducing foodborne illnesses at the farm level, as defined in the FSMA Produce Safety Rule, and ultimately resulting in better protection of the US food system as a whole.

**References**


CHAPTER 4. CHARACTERIZING THE MORPHOLOGICAL DIFFERENCES BETWEEN STATIONARY AND LONG-TERM SURVIVAL PHASE CELLS OF *ESCHERICHIA COLI* OUTBREAK STRAINS

Manreet Singh Bhullar 1, Angela Shaw 1,* , Aubrey Mendonca 1

1 Department of Food Science and Human Nutrition, Iowa State University, Ames Iowa 50011, USA;
*Correspondence: angelaml@iastate.edu; Tel.: 1-515-294-0868

Modified from a manuscript to be submitted in Foodborne Pathogens and Disease

Abstract

Bacteria is widely known to grow in four growth phases: lag, log, stationery and death phase; however, cells do not die beyond the death phase and remain viable in nutrient deficient environments. Long term survival (LTS) cells are the cells that are deprived of nutrients and survive in dormant stages without multiplying for a long time. During this phase, cells tend to develop resistance towards antimicrobial treatments. LTS cells are more commonly studied as persister cells in the field of medicine and immunology and are phenotypic variants of stationary cells that can tolerate lethal concentrations of antibiotics and are different from antibiotic resistant cells as this tolerance is not genetically defined. The chapter focuses on characterizing the long-term survival cells of outbreak strains of *Escherichia coli* (*E. coli*) (a common human pathogen) and visualizing the phenotypic or morphological differences that occur during the growth beyond the stationary phase of bacterial growth curve. The stationary cells (24 h) and long-term cells (21 days) are imaged using light microscopy and scanning electron microscopy, and the differences in cell wall structure and potential mechanisms involved are explained with support of published literature. The imagery of phenotypic alterations in the cell structure provides critical information about the higher resistance of LTS cells against commercial processing treatments. Continuous research in understanding the mechanism of transition of
stationary phase cells into LTS phase would be required to find interventions that can potentially mitigate food safety risks arising due to high tolerance cells in food supply chain.

Keywords: Long term survival cells, persister cells, tolerance, starved cells

**Introduction**

The traditional growth curves of bacteria mentioned in the vast literature and fundamental textbooks reflect a lot of information on the first four phases of growth: lag, log, stationary, and death phase. Bacteria grows by binary fission, and thus follow an exponential phase during growth, when all cells are exposed to favorable growth environment (Angert, 2005). Long term cells are more commonly studied as persister cells in the field of clinical medicine and immunology (Dörr, Lewis, & Vulić, 2009; Keren, Shah, Spoering, Kaldalu, & Lewis, 2004; Wu, Vulić, Keren, & Lewis, 2012). Limited nutrient media in laboratory setup experiments could only allow the cells to grow to a limit beyond which apoptosis (programmed cell death) occurs and cells transition to dormant stage (Finkel, 2001, 2006). The bacteria may exist in long term survival phase in many natural environments, maintaining cell density and viability until favorable conditions prevail. Harsh conditions and limited nutrients push the cells to transition to prolonged stationary phase and exhibit low metabolic rate (Kolter, 1993). The LTS phase marks the expression of a wide variety of stress-response genes and alternative metabolic pathways for survival (Finkel, 2006a). Literature reports this model of bacterial growth as ‘feast or famine model’ since bacteria cannot consume virtually all readily metabolizable nutrients and converting it to biomass (Novitsky & Morita, 1978).

Hypothesizing real world scenarios in food production environments, which lack essential nutrients for bacterial growth including carbon, phosphate, and nitrogen to support the bacterial growth unlike laboratory growth media, the competition for nutrient is extreme. The ability of the bacteria to survive for extended times under nutrient deprived conditions marks the
challenge to the safety of food under diverse condition of food supply chain. A little is known about the behavior of these cells against commercial food processing treatments and prevalence in the food supply chain.

Shiga-toxin producing *E. coli* (STEC) is one of the important foodborne pathogens linked to food safety outbreaks (Centers for Disease Control and Prevention, 2019; Deering, Mauer, & Pruitt, 2012; Fremaux, Prigent-Combaret, & Vernozy-Rozand, 2008; Smith & Fratamico, n.d.; Xing, Wang, Brookes, Salles, & Xu, 2019). The major source of STEC is fecal shedding from ruminant animals (cattle, goat, deer), which is further dispersed and disseminated with water runoffs into the environment. STEC can survive for long period of time in the natural environment such as watershed, soil, manure, and plants (Franz & Van Bruggen, 2008; Fremaux et al., 2008; Neher, Cutler, Weicht, Sharma, & Millner, 2019; Oliveira, Viñas, Usall, Anguera, & Abadias, 2012). The STEC cells are reported to tolerate the antimicrobial effects of UV radiation, unfavorable temperatures, water and nutrient deficiency, and antimicrobials (Fremaux et al., 2008).

Other studies have reported the long term survival of *E. coli* O157:H7 in water (Wang & Doyle, 1998), and manure amended soils (Islam, Doyle, Phatak, Millner, & Jiang, 2005) and literature also reports LTS cells exhibit higher resistance to processing treatments including UV (Wang et al., 2018), thermal processing and high pressure processing (Wen, Anantheswaran, & Knabel, 2009). To the best of our knowledge, there are limited studies reporting the morphological changes in long term STEC cells and pathways through which these cells survive the processing treatments in the fresh produce supply chain. This study investigates the structural changes in cell walls of stationary and LTS cells of STEC and provides possible mechanisms for development of tolerance against antimicrobials.
Materials and Methods

Bacterial Cultures and Culture Conditions

Four outbreak strains of *E. coli* (O145, O26, O121, O157:H7) were obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University, Ames. The stock cultures were maintained at -80 °C in Brain Heart Infusion (BHI) broth (Difco, Becto Dickinson, Sparks, MD) using 10% (v/v) glycerol.

Preparation of Inoculum

For the overnight culture of stationary phase cells, the frozen cultures were thawed and inoculated in Tryptic Soya Broth (Difco, Becto Dickinson, Sparks, MD) supplemented with 0.6% (w/v) yeast extract for 24 hours at 35 °C. Two consecutive 24-h transfers were made to grow a healthy population of cells for all the *E. coli* strains and were used as stationary cells. To prepare LTS phase cells, 1mL of each bacterial strain from second subculture was transferred to 99mL of TSBYE in 100mL sterile bottles, incubated at 35 °C. The LTS cells were harvested after 21 days. 10 mL of each strain for both stationary and LTS cells were combined to obtain a 4-strain mixture. Cells were harvested by centrifugation using Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., Eat Lyme, CT) at 10,000 rpm for 10 mins, 22 °C. The harvested cells were washed twice in 30 mL Dubelco’s Phosphate Buffer Saline (DPBS) and resuspended in DPBS to make bacterial suspension with target population around 7 log colony forming units (CFU)/mL.

Light Microscopy of Gram Stains

Gram stains of cell cultures (4-strain mixture) for both stationary and LTS phase were examined with a 100x oil immersion objective lens using a microscope equipped with a camera (Leica ICC50 W, Leica Microsystems Inc., Buffalo Grove, Illinois, USA).
Scanning Electron Microscopy

Cell cultures were fixed with 1% paraformaldehyde and 3% glutaraldehyde in cacodylate buffer (0.1M) at pH 7.2 for at least 1h / 4˚C. After fixation, samples were rinsed 3x (15 m each) in cacodylate buffer (0.1M). Then samples were post-fixed in 1% osmium tetroxide in cacodylate buffer (0.1M) for 1 hour. After several washes with deionized water, samples were dehydrated through graded ethanol series (25%, 50, 70, 85, 95, 100) 2 changes each for 15 minutes. Samples were critical point dried using a Denton Vacuum, Inc. drying apparatus, Model DCP-1 (Denton Vacuum, Moorestown, NJ). Dried materials were mounted on aluminum stubs with carbon tape and colloidal silver paint and sputter-coated with platinum using a Cressington HR208 Sputter Coater (cressington.com). Images were captured using a Hitachi SU-4800 field emission scanning electron microscope at 10 kV (hitachi-hightech.com/us/).

Results and Discussion

The photomicrographs of stationary and LTS cells from light microscopy and SEM are evaluated to identify morphological differences in cells. The cell structures in stationary phase cells are rod-shaped, such as of a healthy bacterium cell, whereas in LTS cells structural conformation are evident reporting the morphological alterations such as change in cell shape and decrease in cell length (See Figure 4.1, 4.2, and 4.3). These alterations assist LTS cells to survive in limited nutrient availability conditions and the findings are in agreement with previously reported study (Wen et al., 2009). The LTS cells of E. coli are reported to survive the death phase and remain viable for months (Naas, Blot, Fitch, & Arber, 1994; Vasi & Lenski, 1999; Zambrano, Siegele, Almiron, Tormo, & Kolter, 1993). One potential plausible mechanism of survival in the LTS phase is the utilization of nutrient from the dead cells (Zinser & Kolter, 1999). The key mechanism in the development of antimicrobial resistance among the nutrient deprived cells could be the starvation induced growth arrest which is controlled by activity of
starvation-signaling stringent response (SR) (Nguyen, Joshi-Datar, et al., 2011). The ability of gram-negative bacterial strains such as *E. coli* O157:H7 to form dormant vegetative cells under nutrient deficit media and biofilms, leads to starvation induced antimicrobial tolerance (Nguyen, Joshi-Datar, et al., 2011; Wang et al., 2018). The mechanism of development of resistance in the cells may be due to both genetic mutation or phenotypic adaptation to unfavorable environment (Carter, Louie, Huynh, & Parker, 2014; Davidson & Surette, 2008). However, in late term survival phase, cells are the phenotypic variants of the stationary phase cells and potentially do not possess any genetic mutation. Under favorable conditions of nutrient availability, LTS cells regain the characteristics of healthy stationary cells.

There are multiple physiological, morphological and gene expression changes associated with transition of stationary cells to long term survival phase and the most significant is the GASP phenotype, that enables LTS cells to overcome the cells from new growth in younger cultures (Finkel, 2006b). Also, there is an assumption that the toxin-antitoxin (TA) gene pairs in the death phase play a critical role in the transition adaptation of LTS cells (Finkel, 2006b; Jensen & Gerdes, 1995).

![Clumping of cells](image1)

![Higher proportion of rod-shaped cells](image2)

*Figure 4.1. LTS and stationary cell images of *E. coli* cocktail using light microscopy*
The formation of clumping of cells in the LTS phase (figure 1) could be due to several factors. In natural environments, microorganism rarely live as planktonic cells and prefer growing as aggregates, also called microbial granules (Liu, Yang, Qin, & Tay, 2004). Unfavorable environmental condition bring adaptive modification in bacterial organisms such as release of outer membrane vesicles, which results in a mechanism observed in gram negative organisms reported increased cell surface hydrophobicity and ability to form biofilms (Baumgarten et al., 2012). Literature reports the cell density and viability in a bacterial culture is maintained by the process of apoptosis (controlled death) (Finkel, 2006a). This process is regulated by quorum sensing, during which cells signal command within the population for self-death knowing the cells number are higher than the available nutrients (Bassler, 2002; Finkel, 2006b). Further, the nutrients from the dead cells serve as food for the surviving population and these dead cells are also reported to signal “exit apoptosis” message to the surviving population (at 90% inactivation of cells) (Wen et al., 2009), and the phenomenon is called is cryptic growth (Finkel, 2006b; Kolter, 1993). Also, it is well established in scientific reports that bacteria becomes highly tolerant to unfavorable environment conditions (such as presence of antimicrobials) when nutrients are limited (Nguyen, Joshi-datar, et al., 2011).

Figure 4.2. SEM images for LTS cells of *E. coli* cocktail
As observed in the SEM images, there exists a significant difference in the cell length of the stationary and LTS cells. During and after the death phase of bacterial growth, bacterial cells exhibit programmed cell death (PCD), which brings with it cell shrinkage, RNA degradation, and leakage of cell contents (Hochman, 1997). Saprotrophic bacteria is reported to survive in the death phase in a dormant state exhibiting long term survival (Lappin-Scott & Costerton, 1990) and is reported in bacterial populations of *E. coli* (Finkel, 2006a). Additionally, Wen et al. (2009) reported the formation of cocci shaped cells of *Listeria monocytogenes* in long term survival; however, the mechanism of transition from stationary or death phase to long term survival is unclear. It is also hypothesized that bacterial viable cells at the end of the death phase synthesize higher levels of ATP synthase, stimulating higher proton export, increasing acidification in the cells, and terminating programmed cell death, leading to transition to the LTS phase (Wen et al., 2011).

**Conclusion**

The increase in resistance of prolonged stationary phase cells or LTS cells pose a significant threat to food safety and global health. Understanding the diverse mechanisms and pathways of persistence of LTS cells and their morphological resilience against antimicrobials would be the subject of future research. The critical findings from these studies are required to
effectively implement ramifications to various processing interventions in the food supply chain, protecting public health and ensuring higher levels of food safety.

References


CHAPTER 5. LONG TERM SURVIVAL CELLS OF ESCHERICHIA COLI HAVE HIGHER RESISTANCE AGAINST CHLORINE TREATMENT BOTH IN-VITRO AND IN MODEL WASH SYSTEM TREATING ROMAINE LETTUCE

Manreet Singh Bhullar 1, Angela Shaw 1,*, Aubrey Mendonca 1, Ana Monge and Lillian Nabwiire 1

1 Department of Food Science and Human Nutrition, Iowa State University, Ames Iowa 50011, USA;
* Correspondence: angelaml@iastate.edu; Tel.: 1-515-294-0868

Modified from a manuscript to be submitted to Foodborne Pathogens and Disease

Abstract

Foodborne outbreaks associated with fresh produce pose a significant public health concern in the United States. From 1998 to 2008, 46% of illnesses and 23% of deaths have been associated with produce outbreaks. Escherichia coli (E. coli) is reported to be a major human pathogen involved in produce outbreaks. The use of sanitizers such as chlorine is a common practice in post-harvest washing of produce. Standards for the use of sanitizers have been developed based on scientific studies determining the inactivation of cells in the stationary stages of growth; however, little is known about sanitizer efficacy in the long-term survival (LTS) stage. The objective of the study was to evaluate the differences in inactivation of stationary phase cells and long-term survival phase cells of E. coli in-vitro and in a lettuce wash model, mimicking real world scenarios. Four lettuce-outbreak strains of E. coli (O145, O26, O121, O157:H7) were used as a cocktail and treated with three different concentrations of free chlorine (0.25 - 40 ppm). The cell cultures were treated for 30 s, and the reaction was neutralized using sodium thiosulfate (5% w/v). Treatments were conducted randomly and in triplicate. The results reported higher resistance of LTS cells in-vitro when compared to stationary phase cells against all chlorine concentration, while in a lettuce model resistance was not shown (p>0.05). The study
concluded that at low doses of free chlorine (10-40 ppm), there exists no difference between inactivation of LTS and stationary cells, with maximum log reduction of < 2 log CFU/mL.

Keywords: long term survival, chlorination, lettuce, produce wash, resistance

**Introduction**

The implication of fresh produce in foodborne outbreaks has been on the rise in the past two decades. The increased consumption of fresh produce with the change in trends towards healthier diets, globalization of food trade, and increased demand for fresh produce has drastically altered the food safety equation (Alegbeleye, Singleton, & Sant’Ana, 2018). The per capita consumption of fresh vegetables increased by 67%, from 86.9 lbs. in 1970 to 145.0 lbs in 2017 (USDA-ERS, 2019). From 2004 to 2012, a total of 377 outbreaks in the US and 198 outbreaks in the European Union were reported to be associated with fresh produce (Callejón et al., 2015). The major foodborne pathogens implicated in these outbreaks include *Escherichia coli* O157:H7, *Salmonella*, and *Listeria* (Alegbeleye et al., 2018; Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). Among fresh produce types linked to outbreaks, leafy greens including lettuce and spinach have been greatly involved in foodborne outbreaks from 1996 to 2010 (CDC, 2019).

Fresh produce is grown in natural environments and is prone to microbial contaminants through diverse potential routes (Alegbeleye et al., 2018; FDA, 2019; Lynch, Tauxe, & Hedberg, 2009). The contamination can occur in the preharvest stage in the field and can spread during post-harvest processing (Macarisin et al., 2017). During the washing of fresh produce, a large quantity of fruits and vegetables is dumped in water in the flumes or the tanks, and the chances of microbial contaminants transferring from one produce item to another are significant (Gombas et al., 2017; Murray, Wu, Shi, Jun Xue, & Warriner, 2017; Uyttendaele et al., 2015). Since fresh produce is commonly eaten raw, post-harvest washing is a critical step for control of cross-
contamination and reducing microbial load. Food safety guidelines recommend that growers use antimicrobials and sanitizers during the washing of fresh produce to prevent cross-contamination (FDA, 2019).

Chlorination is a commonly used post-harvest treatment in the produce industry. Disinfection by chlorine has been in use for decades and is often applied in the production, harvest, and post-harvest handling of fresh fruits and vegetables (Hough & Kellerman, 1971; Rabin, 1986; Winston & Johnson, 1953). For several decades, chlorine in the form of sodium or calcium hypochlorite has been an integral part of horticulture sanitation programs (Suslow, 2000). The addition of sodium hypochlorite into water creates free chlorine in different forms, with hypochlorous acid (HOCl) being the most effective disinfectant due to 90% ion concentration at pH of 6.5 (Suslow, 2000). The benefits of chlorination include its high efficacy, low cost, ready availability, and that it can be used for any scale of operation. However, the pitting of the equipment at lower pH use (<6.5) and formation of disinfection-by-products (mainly chloramines) are the major limiting factors for the use of chlorine (Goslan et al., 2009; Nou & Luo, 2010; Tirpanalan, Zunabovic, Domig, & Kneifel, 2011).

Most microbiological studies are conducted using in-vitro conditions and surrogate cells of foodborne pathogens at the stationary cell growth stage. However, few studies in the literature report the fifth growth stage called long term survival phase (Finkel, 2006; Steinhaus & Birkeland, 1939; Wang et al., 2018; Wen, Anantheswaran, & Knabel, 2009). Under starved conditions after the death phase, cells transition into a long term survival phase (Djebbi-Simmons, Xu, Janes, & King, 2019; Wang et al., 2018). During this phase, the cells may show increased resistance to secondary stress and higher pathogenicity (Lisle et al., 1998). Literature reports that LTS cells hold significant viability and higher resistance to antimicrobial treatments.
and exhibit rapid germination to vegetative cells when placed in fresh nutrient media (heat, high pressure, UV) (Wang et al., 2018; Wen et al., 2009). Thus, studies based on stationary results may report an overestimation of the efficacy of particular antimicrobials against microbial cells. The ability of LTS cells to persist in the natural environment poses a critical challenge to the mitigation of food safety risks associated with growing and processing fresh produce.

Under realistic produce field conditions, the possibility of having a bacterial cell in the fifth phase is very common (Djebbi-Simmons et al., 2019; Gurresch, Gerner, Pin, Wagner, & Hein, 2016; Wang et al., 2018). Therefore, fresh produce grown in fields is highly likely to become contaminated with LTS cells. There has been limited peer-reviewed literature studying LTS cells in fresh produce and the effects of chlorine on LTS cells in fresh produce processing. The present study would be the first paper that focuses on addressing this knowledge gap by providing information on the survival and extent of LTS cell inactivation on romaine lettuce treated with chlorine solution. Also, the paper reports critical information regarding sensitivity of stationary and LTS cells against chlorine treatment.

**Materials and Methods**

**Bacterial Cultures and Culture Conditions**

Four lettuce-outbreak strains of *E. coli* (O145, O26, O121, O157:H7) were obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University, Ames. The stock cultures were maintained at -80 °C in Brain Heart Infusion (BHI) broth (Difco, Becto Dickinson, Sparks, MD) using 10% (v/v) glycerol.

**Preparation of Inoculum**

For the overnight culture of stationary phase cells, the frozen cultures were thawed and inoculated in Tryptic Soy Broth (Difco, Becto Dickinson, Sparks, MD) supplemented with 0.6% (w/v) yeast extract for 24 hours at 35 °C. Two consecutive 24-hour transfers were made to grow
a healthy population of cells for all the *E. coli* strains and were used as stationary cells. To prepare LTS phase cells, 1 mL of each bacterial strain from the second subculture was transferred to 99 mL of TSBYE in 100 mL sterile bottles, incubated at 35 °C. The LTS cells were harvested after 21 days. 10 mL of each strain for both stationary and LTS cells were combined to obtain a 4-strain mixture. Cells were harvested by centrifugation using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., Eat Lyme, CT) at 10,000 rpm for 10 mins and at 22 °C. The harvested cells were washed twice in 40 mL Dubelco’s Phosphate Buffer Saline (DPBS) (GE Healthcare, Pittsburg, PA, USA) and resuspended in DPBS to make bacterial suspension with the target population around 8 log colony forming units (CFU)/mL. The growth of LTS cells was measured at 1, 4, 12, 28, and 50 days to determine the growth curve (see Figure 5.1).

![Figure 5.1. Growth curve of long-term survival cells incubated at 35 °C for 50 days.](image)

**Minimum Bactericidal Concentration (MBC) of Chlorine**

Sodium hypochlorite (reagent grade) was purchased from Sigma-Aldrich, Milwaukee, WI. The MBC for chlorine against stationary and LTS cells was determined using the broth
dilution method as described by Vigil, Palou, Parish, & Davidson (2005). However, sterilized distilled water was used as a medium to determine MBC values as the organic ingredients in the broth would have altered the free chlorine concentration. Serial dilutions (two-fold) of the stock solution of free chlorine (100 ppm, pH= 6.5) were made in sterilized distilled water (pH = 6.5) to obtain concentrations of 0.00975 – 5 ppm. Each tube was inoculated with 50 uL of LTS cells and incubated at 35 °C for 24 hours. After incubation, 10 uL aliquots from all the dilution tubes were plated onto nonselective agar (Tryptic Soy Agar (Difco, Becto Dickinson, Sparks, MD) with yeast extract (TSAYE)). The MBC for both stationary and LTS cells was determined to know the minimum concentration of free chlorine that inactivated bacterial population by a minimum of 3-log reduction (99.9% reduction). Tests for MBC were conducted in triplicate.

**Preparation of Chlorine Solutions and Treatment**

**In-vitro model**

Sodium chloride (NaOCl, reagent grade) was used to make chlorine solutions for the treatment using sterilized distilled water (pH=6.5). Different concentrations of free chlorine were freshly prepared for treating stationary and LTS cells (see Table 5.1). The concentrations of free chlorine were measured before the treatment using a Hach Calorimeter (DR300 Model) which employed the DPD (N,N Diethyl-1,4 Phenylenediamine Sulfate) reagent method. The pH of the chlorine solution was adjusted to 6.5 using hydrochloric acid (1 M) and sodium hydroxide (1 M). 10 mL of each concentration of chlorine solution was transferred into 15 mL sterile centrifuge tubes for the treatment. 1 mL of the bacterial suspension was added to a randomly selected tube. The tubes were vortexed immediately after addition of the cells, and the reaction was neutralized at 30 seconds using sodium thiosulfate (500 ppm) (Fisher Scientific, Hampton, NH). The treatment with the highest concentration was tested for presence of free chlorine after the addition of sodium thiosulfate to confirm the neutralizing effect.
Table 5.1. Free chlorine doses for different treatments.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Setup</th>
<th>Chlorine Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary</td>
<td>In-vitro</td>
<td>0.25, 0.5, 1.0</td>
</tr>
<tr>
<td>Long Term Survival</td>
<td>In-vitro</td>
<td>2.5, 5.0, 10.0</td>
</tr>
<tr>
<td>Stationary</td>
<td>Lettuce model</td>
<td>10.0, 20.0, 40.0</td>
</tr>
<tr>
<td>Long Term Survival</td>
<td>Lettuce model</td>
<td>10.0, 20.0, 40.0</td>
</tr>
</tbody>
</table>

**Lettuce wash model**

Romaine lettuce heads were procured from a local grocery store (Ames, IA) and stored in a refrigerator (4 °C) until further use. The leaves were detached from the head and a 6.15 cm² leaf area was cored to make coupons. 50 uL of stationary and LTS cells were inoculated on the dry surface of lettuce leaf coupons and left overnight in sterile Petri dishes under the biosafety cabinet for drying and attachment of cells to the leaf surface (See Figure 5.2). A similar procedure as described above was used to obtain chlorine solutions for different concentrations but using sterilized tap water, mimicking field situations. In this case, 30 mL of free chlorine solution was prepared in a sterile whirl-pak bag and three leaf coupons were added to the treatment solution immediately. The bag was manually shaken to mimic washing of lettuce. After 30 seconds, 3 mL of sodium thiosulfate (500 ppm) was added to neutralize the reaction. The leaf coupons were hand massaged to resuspend attached cells in the solution before sampling.

Figure 5.2. (a) Lettuce leaf coupon (6.15 cm²), (b) Inoculation of *E. coli* (50 uL) on coupons
Determination of Sublethal Injury

The plate counts from the selective media (SMAC) and non-selective media (TSAYE) were used to calculate the sublethal injury in the surviving populations of *E. coli* strain mixtures. The following equation was used to calculate the sublethal injury as described by Lan, Zhang, Zhang, & Shi, (2019) and Tian, Yu, Shao, Li, & Dai, (2018), and plate count data was based on mean values obtained from three independent experiments.

\[
\% \text{ Injury} = \left( \frac{\text{CFU}_\text{mL} (\text{TSAYE})}{\text{CFU}_\text{mL} (\text{SMAC})} - 1 \right) \times 100
\]

Microbiological Analysis

Treated samples were serially diluted as appropriate using 0.1% buffered peptone water (Difco, Becto Dickinson, Sparks, MD) and plated on selective (Sorbitol MacConkey Agar (SMAC), Oxoid, Ltd., Basingstoke, Hampshire, England) and nonselective (TSAYE) media. To ensure the true zeroes for samples that showed no growth on plate counts, 1 mL aliquot of treated sample was inoculated into 9 mL of TSBYE and incubated for 24 hours at 35 °C. The enriched samples were incubated for 48 hours at 35 °C and streak plated onto selective media (SMAC) to detect the presence of *E. coli*.

Scanning Electron Microscopy

Leaf samples were fixed with 1% paraformaldehyde and 3% glutaraldehyde in cacodylate buffer (0.1M) at pH 7.2 for at least 1 hour / 4°C. After fixation, samples were rinsed 3x (15 m each) in cacodylate buffer (0.1M). Then samples were post-fixed in 1% osmium tetroxide in cacodylate buffer (0.1M) for 1 hour. After several washes with deionized water, samples were dehydrated twice through graded ethanol series (25%, 50, 70, 85, 95, 100) for 15 minutes each. Samples were critical point dried using a Denton Vacuum, Inc. drying apparatus, Model DCP-1.
(Denton Vacuum, Moorestown, NJ). Dried materials were mounted on aluminum stubs with carbon tape and colloidal silver paint and sputter-coated with platinum using a Cressington HR208 Sputter Coater (cressington.com). Images were captured using a Hitachi SU-4800 field emission scanning electron microscope at 10 kV (hitachi-hightech.com/us/).

**Statistical Analysis**

Log inactivation data for stationary and LTS phase cells against different chlorine concentrations were recorded as log$_{10}$ CFU/mL. The data were log-transformed to meet the assumptions of statistical analysis. Data were analyzed using Microsoft Excel and statistical analysis software (SAS) version 9.4 (SAS Institute, Inc., Cary, NC). Assuming normality, data were analyzed using two-way ANOVA, and level of significance was developed using a p-value of 0.05. All the treatments were run in triplicate to reduce variation and standard deviation.

**Results and Discussion**

**Minimum Bactericidal Concentration**

The average MBC for stationary and LTS cells was reported as 0.0391 ppm and 0.078 ppm, respectively. The results are consistent with reported literature stating higher resistance of LTS cells when compared to stationary cells (Wang et al., 2018; Wen et al., 2009). However, the exact mechanism of protection to LTS cells is unknown.

**Chlorine Treatment of LTS and Stationary Cells – *In-vitro***

The starting concentration of *E. coli* stationary cells was 7.84 CFU/mL and was reduced by 0.19, 1.26, and 5.14 log CFU/mL when treated with free chlorine concentrations of 0.25, 0.5, and 1 ppm, respectively (Figure 5.3). The inactivation curve follows first-order kinetics with R$^2$ value of 0.91 (Figure 5.4). However, for LTS cells of *E. coli*, the starting concentration was 7.04 CFU/mL, and log reductions of 2.07, 3.46, and 4.63 log CFU/mL were achieved with chlorine treatment of 2.5, 5.0, and 10.0 ppm, respectively (Figure 5.3). The inactivation curve follows
polynomial fit with $R^2$ value of 0.99 (Figure 5.4). When lower concentrations (0.25, 0.5 and 1 ppm) of free chlorine were used in preliminary studies, no LTS cells of *E. coli* were killed. On average, LTS cells required approximately 10 times more free chlorine concentration to inactivate similar a log population as stationary cells. Also, the formation of tailing at higher doses for LTS cells supports the literature for higher resistance of LTS cells, i.e., with an increase in free chlorine concentration, there is lower log reduction. Also, preliminary data has consistently shown that stationary cells are more sensitive than the LTS at the same dose (data not shown). At 5 ppm chlorine, LTS cells survived in good numbers; however, stationary cells were not detected.

![Graph showing log reduction of colony forming units per milliliter (CFU/mL) LTS and stationary cells of *E. coli* (Starting population of 7.04 and 7.84 CFU/mL) at different chlorine concentrations in-vitro.](image)

Figure 5.3. Log reduction of colony forming units per milliliter (CFU/mL) LTS and stationary cells of *E. coli* (Starting population of 7.04 and 7.84 CFU/mL) at different chlorine concentrations in-vitro.
Figure 5.4. Inactivation curves for stationary and LTS cells against chlorine treatment.

**Chlorine Treatment of LTS and Stationary cells – Lettuce Model**

The control population of stationary and LTS cells on the leaf surface was 6.96 and 5.49 CFU/mL/cm². The inoculated leaf coupons were treated with a free chlorine concentration of 10, 20, and 40 ppm in triplicate. At these treatment levels, log inactivation for stationary cells were observed to be 1.09, 1.61, and 1.35 log CFU/mL; however, for LTS cells, log reductions of 0.92, 1.71, and 0.75 log CFU/mL were achieved. The statistical analysis reports insignificant differences in log inactivation (p>0.05) of stationary and LTS cells achieved at the same chlorine treatment levels. This could be due to various factors that may have affected the efficacy of chlorination. Literature reports that the rate of microbial inactivation in produce washing highly depends upon sanitizer type, target pathogens, and organic matter (Cangliang Shen, Luo, Nou, Wang, & Millner, 2013; Zhang, Ma, Phelan, & Doyle, 2009).

At a free chlorine concentration of 40 ppm, the inactivation of *E. coli* stationary cells attached to romaine lettuce is low when compared to previous literature studies. In contrast to the results with the in-vitro study, there is a large difference in free chlorine concentration that
inactivated more than 5 log CFU/mL of *E. coli* at a 1 ppm dose. A recent study suggested a minimum free chlorine level of 10 mg/L as the lowest effective dose for the washing of industrial leafy greens (Luo et al., 2018a). Another study reported undetectable levels of *E. coli* O157:H7 when treated with 0.5 ppm of free chlorine for 5 seconds (Cangliang Shen et al., 2013). Similar findings were reported by Swanson & Fu (2017) showing a log reduction of 6 log CFU/mL after treating *E. coli* O157:H7 for 20 seconds with 1 ppm free chlorine.

However, in the present study, various factors could have affected the efficacy of chlorination in lettuce wash. Literature reports that the inactivation of pathogens suspended in the wash water is easier than pathogens attached to the leaves (Davidson, Kaminski, & Ryser, 2014; Fu, Li, Awad, Zhou, & Liu, 2018; C Shen, 2014). Interestingly, it was found that the lettuce juice is reported to consume free chlorine and lessened the antimicrobial effect (Fu et al., 2018). A study evaluating the chlorine demand of different produce items, including lettuce, carrots, and spinach, reported the highest chlorine demand for lettuce produce wash water (Gil, López-Gálvez, Andújar, Moreno, & Allende, 2019). Other factors that were not controlled during this study but have been reported to affect the efficacy of chlorination are total organic content, chemical oxygen demand, total dissolved and suspended solids, residual free chlorine, oxidation-reduction potential, Brix, and conductivity (Gombas et al., 2017; Luo et al., 2018b).

**Sublethal Injury**

**In-vitro model**

Tables 5.2 and 5.3 show the average values of the percentage sublethal injury for *E. coli* survivors following exposure to free chlorine concentrations in-vitro. The average sublethal injuries of 18.53, 37.61, and 12.23% were observed with chlorine treatments of 0.25, 0.5, and 1 ppm, respectively. However, for LTS cells, the average sublethal injury for chlorine concentrations of 2.5, 5.0, and 10.0 ppm was 11.41, 11.91, and zero percent, respectively. The
average sublethal injury percentage is more for stationary cells as compared to the LTS cells for similar log inactivation. Sublethal injury is the percentage of the surviving population that has been damaged but is not dead (Russell, 1984). Damage is induced by physical or chemical treatment that is not sufficient enough to inactivate the cell (Han et al., 2018). Injured cells fail to grow on selective media and can fully resuscitate under favorable conditions, posing a significant risk to food safety (Lv et al., 2019). Therefore, with lower sublethal injury comes lower damaged cells, stating a higher resistance to the antimicrobial treatment of the LTS cells in the surviving population.

Table 5.2. Sublethal injury (%) and cell death (%) for stationary cells of *E. coli* (In-vitro).

<table>
<thead>
<tr>
<th>Free Chlorine (ppm)</th>
<th>Average</th>
<th>Cell Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>18.53 ± 11.81</td>
<td>2.48 ± 0.76</td>
</tr>
<tr>
<td>0.5</td>
<td>37.61 ± 14.94</td>
<td>16.03 ± 8.72</td>
</tr>
<tr>
<td>1</td>
<td>12.23 ± 13.49</td>
<td>65.53 ± 13.17</td>
</tr>
</tbody>
</table>

Table 5.3. Sublethal injury (%) and cell death (%) for LTS cells of *E. coli* (In-vitro).

<table>
<thead>
<tr>
<th>Free Chlorine (ppm)</th>
<th>Sublethal injury</th>
<th>Cell Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>11.41 ± 2.84</td>
<td>28.93 ± 3.04</td>
</tr>
<tr>
<td>5</td>
<td>11.91 ± 8.86</td>
<td>48.26 ± 3.16</td>
</tr>
<tr>
<td>10</td>
<td>0 ± 21.91</td>
<td>64.50 ± 1.92</td>
</tr>
</tbody>
</table>

**Lettuce Model**

The percentage sublethal injury for stationary and LTS cells in the lettuce model is listed in Table 5.4. At a dose of 10, 20, and 40 ppm, stationary cells had an average sublethal injury of 10.05, 13.77, and 9.02 %, respectively. In contrast, LTS cells had average sublethal injuries of 6.09, 0, and 8.82 % at the same chlorine treatments. However, the statistical difference in sublethal injury between the two types of cells is insignificant (p>0.05). When compared with stationary cells, the lower injury percentage for LTS cells at all doses of free chlorine provides
information about the hard to inactivate nature of LTS cells. It is reported in literature that the LTS cells possess decreased surface area and form coccoid shaped cells from rod-shaped cells in the stationary phase, and they possess higher resistance to unfavorable environments (Wen et al., 2009). The exact mechanism of LTS cell resistance to chlorination is unknown. However, the exposure of pathogenic bacteria to adverse conditions helps to survive under sublethal injury states and pose significant food safety risks to public health (Dhakal et al., 2018).

Table 5.4. Sublethal injury (%) in stationary and LTS cells (Lettuce model).

<table>
<thead>
<tr>
<th>Free Chlorine (ppm)</th>
<th>Stationary Cells</th>
<th>LTS cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10.05 ± 14.14</td>
<td>6.09 ± 12.04</td>
</tr>
<tr>
<td>20</td>
<td>13.78 ± 13.63</td>
<td>0 ± 15.71</td>
</tr>
<tr>
<td>40</td>
<td>9.02 ± 6.35</td>
<td>8.82 ± 9.71</td>
</tr>
</tbody>
</table>

**Scanning Electron Microscopy**

The imaging of lettuce leaves provided critical information about the attachment and localization of *E. coli* cells on the lettuce leaf surface. The images for control show no microbial population present on the leaf surface, ensuring no background microbial flora on the lettuce leaves. In Figure 5, the inoculated leaves show that both stationary and LTS cells lie around the stomata and even translocate into the stomata. The internalization of microbial cells in the lettuce plants is well reported in the literature (Deering, Mauer, & Pruitt, 2012; Ge, Lee, & Lee, 2012; Kroupitski, Gollop, Belausov, Pinto, & Sela, 2019; Zhang, Ma, Beuchat, et al., 2009). Also, studies have reported that LTS cells are not capable of forming biofilms due to their dormant stage; however, stationary cells of gram-negative bacteria such as *E. coli* O157:H7 are widely known for their ability to form biofilms on surfaces (Chen, Zhao, & Doyle, 2015; Kim, Pitts, Stewart, Camper, & Yoon, 2008; Marin, Hernandiz, & Lainez, 2009).
The formation of biofilms is reported to protect the cells from chemical sanitizers (Joseph, Otta, Karunasagar, & Karunasagar, 2001; Starkey, Parsek, Gray, & Chang, 2004). A study reported that chlorine treatment at 200 ppm was effective against water suspended planktonic cells, though it was ineffective when biofilm-associated *E. coli* O157:H7 was treated (Ryu & Beuchat, 2005). Fish & Boxall (2018) reported that high chlorine concentration (0.80mgL\(^{-1}\)) did not prevent natural biofilm formation in drinking water distribution system loops and rather resulted in increased bacterial growth during the re-growth phase, potentially increasing the risk of contamination.

On combined inoculation of both LTS and stationary cells, it was observed that stationary cells formed extensive biofilms trapping the LTS cells, which can be hypothesized as mimicking real environmental conditions in the produce fields. The persistence of human pathogens can occur in different growth phases in the natural environment, and the possibility of having stationary and LTS cells at one locus is not uncommon. Interestingly, the cells were observed translocating through the leaf on the opposite side of inoculation (see Figure 5) and significantly challenged inactivating cells hiding inside the leaf surface. The authors hypothesize that the LTS cells in combination with stationary cells exaggerated the food safety risks with enhanced attachment, more translocation within the leaf surface, and increased biofilm formation.

The SEM images also support the hypothesis that the organic matter, including debris and cuticular wax on the lettuce leaves, reduced the efficacy of free chlorine, and protected the inactivation of cells. Low dose, short-time treatment, organic matter, and other factors associated with lettuce may have a combined effect in shielding the cells and adding impracticability to identify differences in chlorine sensitivity between the stationary and LTS phase cells.
Figure 5.5. SEM images of stationary and LTS cells on control and inoculated leaves. (a)(b) SEM images of control romaine lettuce leaves with open and closed stomata, (c)(d) SEM images of *E. coli* stationary cells, localized inside the stomata, and half-open stomata with cuticular wax and debris on the leaf surface; (e)(f) SEM images of LTS cells of *E. coli* on the stomata walls and internalized inside the stomata, (g)(h) SEM images of stationary and LTS cells inoculated together on the lettuce leaf, showing formation of biofilm, translocation through the leaf surface to opposite side of inoculation, and forming clumps.
In conclusion, the present study reported that LTS cells possess higher resistance to chlorine treatment than stationary cells in-vitro and insignificant differences in the lettuce wash model. The lettuce wash results were not in agreement with previous research reporting higher resistance of LTS cells against antimicrobial treatments (Ultraviolet irradiation and High-Pressure Processing). Diverse factors including organic content, pathogen load, and produce type may have significantly affected the efficacy of chlorine treatment. In this study, there were limiting factors to control the chlorine treatment that could have been critical in evaluating the efficacy of the treatment. Further research with higher doses of chlorine and other sanitizers is suggested to understand the resistant behavior of LTS cells in the food supply chain, optimize industrial treatment processes, and minimize food safety risks. Continuous real-time monitoring of various chlorine treatment parameters is needed to ensure a higher efficacy of pathogen inactivation while minimizing cross-contamination in commercial post-harvest washing of produce.

**References**


CHAPTER 6. GENERAL CONCLUSION

Fresh produce has been established as known vehicle for foodborne diseases. The complexity of diverse routes of contamination has been studied and further research is underway to develop advanced interventions aiming at reducing the food safety risks. Fresh produce can harbor and sustain pathogenic contaminants at any point throughout the supply chain. Among different factors that affect fresh produce safety, agricultural water poses a significant threat. Based on scientific studies, FSMA Produce Safety Rule mandates pre-harvest and post-harvest water management practices are in place to protect the safety of the agricultural water. The implementation of Water Rule has been challenging and feedback from the produce industry stakeholders has insisted FDA to reconsider the water standards and add modifications aiming user friendly testing and easy monitoring of water quality. One of the challenges included the short timeframe for sample holding as a method requirement for all quantitative and qualitative water testing methods in the Produce Safety Rule.

Sample holding time is the interval between the collection of water sample and the start of the analysis. For all water testing methods mentioned in the Produce Safety Rule, the sample holding time is 8 hours (which includes the laboratory processing time), which is not user-friendly requisite for many small-scale growers. One of the studies focused on extending the sample holding time for the Method EPA 1603, from 6 hours to 24 hours. The results reported that there was an insignificant difference ($p<0.05$) in 6-hour and 24-hour generic $E. coli$ counts per 100 mL of the water sample. The extension in the holding time would potentially bring more fresh produce growers under compliance and would minimize food safety risks on their farm. The study also provides scientific basis for further research to extend the holding time for other water testing methods mentioned in the Produce Safety Rule. Future research is needed to test
other approved water testing methods at longer sample holding times to find commonality in bacterial survival in water samples. Rigorous data collected from these experiments may provide FDA and EPA with scientific evidence and requesting modifications in the current water testing sample holding times. These modifications will bring enhanced food safety measures to protection of fresh produce resulting from use of contaminated agricultural water.

Attempts have been made to understand the growth phases of pathogenic bacterial cells, the morphological changes during growth, and development of resistance to antimicrobial treatments. Literature reported that after the death phase, there is existence of viable and dormant cells in the long-term survival phase, with enhanced tolerance to unfavorable conditions. The study focused on characterizing the cells of four outbreak strains of *E. coli*, and visualized cells under light microscopy and Scanning Electron Microscopy (SEM). The microphotographs illustrated significant differences in cell structures and are in agreement with previous literature reporting similar increase in antimicrobial tolerance in other bacterial organisms. The study provides scientific proof of concept of enhanced resistance in LTS cells and further research is subjected to identifying mechanisms of transition of stationary cells to LTS phase, which are unclear as of today. Also, these findings would assist in developing adequate interventions using the hardest cells to protect the food from contamination, adding extra margin of safety and ultimately enhancing consumer health.

The existence of these LTS cells in natural fruits and vegetable production environments is common and hypothesizing its presence in lettuce produce and washing it with chlorine treatment, provided critical information about potential food safety risks associated with it. The study focused on inactivating both stationary and LTS cells of *E. coli* in-vitro and in a lettuce wash model. Findings reported that significant difference exists in the sensitivity of stationary
and LTS cells in-vitro with a maximum of 5 log reduction; however, statistically insignificant differences were observed in the lettuce model with a maximum of 1.91 log reduction. The efficacy of antimicrobial treatments is always higher in planktonic cells as compared to surface adhered cells. Multiple other factors including organic matter, high chlorine demand of lettuce, formation of biofilms on the lettuce surface and internalization of cells inside the stomata could have played critical role affecting antimicrobial treatment of chlorine. Continuous real-time monitoring of various chlorine treatment parameters such as residual free chlorine levels is needed to ensure higher efficacy of pathogen inactivation while minimizing cross-contamination in commercial post-harvest washing of produce.

The facts gathered from these studies provide resourceful information about the behavior of bacterial cells under different environmental conditions. There is limited information about the LTS cells that illustrate the contamination and persistence of these pathogens on fresh produce. In light of the available evidence, continuous research efforts are needed to efficiently identify diverse routes of contamination, pathogen survival in the produce supply chain and recent trends in the occurrence of foodborne diseases associated with the consumption of fruits and vegetables. Human pathogens such as *Listeria* spp. and *Salmonella* spp. can survive for longer times in the natural environment and there is a good possibility that they transition to LTS phase. Future research is needed to identify modes of survival of LTS cells in the natural food production environment, mechanisms of resistance in LTS cells to antimicrobial treatments and developing interventions capable of inactivating LTS cells in the food supply chain. These findings would add significant advances to the food safety standards and treatment validation programs protecting the food supply chain and the public health.