CLIMATE CHANGE AND FOOD SAFETY: ASSESSMENT OF THE TEMPERATURE IMPACT ON THE ATTACHMENT OF *ESCHERICHIA COLI* PATHOGROUPS ON CRESS LEAF

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

HİLAL SAMUT

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

JUNE 2022

Approval of the thesis:

CLIMATE CHANGE AND FOOD SAFETY: ASSESSMENT OF THE TEMPERATURE IMPACT ON THE ATTACHMENT OF *ESCHERICHIA COLI* PATHOGROUPS ON CRESS LEAF

submitted by **HİLAL SAMUT** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Food Engineering, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Serpil Şahin
Head of the Department, **Food Engineering** _____

Assoc. Prof. Dr. Yeşim Soyer
Supervisor, **Food Engineering, METU** _____

Examining Committee Members:

Prof. Dr. Hüseyin Erten
Food Engineering, Çukurova University _____

Assoc. Prof. Dr. Yeşim Soyer
Food Engineering, METU _____

Prof. Dr. Behiç Mert
Food Engineering, METU _____

Prof. Dr. Nuray Çömlekçioğlu
Horticulture, Osmangazi University _____

Assoc. Prof. Dr. Cemil Kürekci
Faculty of Veterinary Medicine, Hatay Mustafa Kemal Uni. _____

Date: 02.06.2022

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Last name: Hilal Samut

Signature:

ABSTRACT

CLIMATE CHANGE AND FOOD SAFETY: ASSESSMENT OF THE TEMPERATURE IMPACT ON THE ATTACHMENT OF *ESCHERICHIA COLI* PATHOGROUPS ON CRESS LEAF

Samut, Hilal
Master of Science, Food Engineering
Supervisor: Assoc. Prof. Dr. Yeşim Soyer

June 2022, 129 pages

Climate change and its worldwide effects are recognized all over the world. One of the fields where the effect of climate change is observed due to increased mean ambient temperature is food safety. The temperature increase may affect the survival of foodborne pathogens on fresh produce, and not being processed prior to consumption makes fresh produce pose a huge threat to human health. This study was, therefore, planned to present a comprehensive assessment of the impact of climate change, taking its temperature rise scenarios into consideration, on the attachment and biofilm-forming abilities of different pathogenic *Escherichia coli* serotypes on cress leaves grown under controlled conditions. Enterohemorrhagic *E. coli* O157:H7, Enteroaggregative *E. coli* O104:H4 and Enteropathogenic *E. coli* O26 were inoculated on cress plants at a level of 8 log MPN/ml at different stages during growth to investigate how (i) inoculation time (7, 14, 21 and 28 days post sowing; dps) and (ii) contamination route (seed and leaves) affect the pathogen load on fresh produce under different climate change scenarios (+2, +4, and +6 ºC). This present study revealed that temperature increase designed according to mitigation scenarios for climate change did not cause any significant change in the persistence of pathogens studied here on leaf tissue at 30 dps (~4.5 to 7 log MPN/g). In the plants

contaminated at a later stage (21 and 28 dps), the populations of *E. coli* O157:H7, O104:H4 and O26 were higher for all temperatures studied. The results show that *E. coli* strains used in this study have translocated towards leaf portions from seed and established a significant amount of pathogen load on leaf (~4 to 5.3 log MPN/g). Washing the leaves did not show any significant reduction in the *E. coli* populations on leaf tissue, which means that the inoculated bacteria have tightly bound to leaf (~3.5 to 7 log MPN/g) and cannot be eliminated by washing practices. Lastly, the residing biofilm structures were detected on the leaf crevices as well as stomata where enteric bacteria could use for further colonization of cress by Scanning Electron Microscope (SEM).

Keywords: *Escherichia coli*, Biofilm, Attachment, Fresh Produce, Contaminated Irrigation

ÖZ

İKLİM DEĞİŞİKLİĞİ VE GIDA GÜVENLİĞİ: *ESCHERICHIA COLI* PATOGRUPLARININ TERE YAPRAĞINA TUTUNMASINDA SICAKLIK ETKİSİNİN DEĞERLENDİRİLMESİ

Samut, Hilal
Yüksek Lisans, Gıda Mühendisliği
Tez Yöneticisi: Doç. Dr. Yeşim Soyer

Haziran 2022, 129 sayfa

İklim değişikliği ve dünya çapındaki etkileri tüm dünyada tanınmaktadır. Ortalama ortam sıcaklığının artması nedeniyle iklim değişikliğinin etkisinin gözlemlendiği alanlardan biri de gıda güvenliğidir. Sıcaklık artışı, taze ürünlerde gıda kaynaklı patojenlerin hayatta kalma kabiliyetlerini etkileyebilir ve tüketilmeden önce işlenmemesi, taze ürünlerin insan sağlığı için büyük bir tehdit oluşturmasına neden olmaktadır. Bu nedenle, bu çalışma, kontrollü koşullar altında yetiştirilen tere yapraklarına farklı patojenik *Escherichia coli* serotiplerinin tutunma ve biyofilm oluşturma yetenekleri üzerindeki sıcaklık artış senaryolarını dikkate alarak iklim değişikliğinin etkisinin kapsamlı bir değerlendirmesini sunmak üzere planlanmıştır. Farklı iklim değişikliği senaryoları altında (+2, +4, ve +6 ºC), (i) aşılama süresinin (ekimden 7, 14, 21 ve 28 gün sonra) ve (ii) kontaminasyon yolunun (tohum ve yapraklar) taze ürünlerde patojen yükünü nasıl etkilediğini araştırmak için Enterohemorajik *E. coli* O157:H7, Enteroagregatif *E. coli* O104:H4 ve Enteropatojenik *E. coli* O26 8 log MPN/ml seviyesinde tere bitkilerine inoküle edilmiştir. Bu çalışma, iklim değişikliği için hafifletme senaryolarına göre tasarlanan sıcaklık artışının, burada incelenen patojenlerin ekimden 30 gün sonra (~4.5 ile 7 log MPN/g) yaprak dokusu üzerinde kalıcılığında önemli bir değişikliğe neden olmadığını ortaya koymuştur. Daha sonraki aşamada (ekimden 21 ve 28 gün sonra)

kontamine edilmiş bitkilerde, *E. coli* O157:H7, O104:H4 ve O26 popülasyonlarının, çalışılan tüm sıcaklıklar için daha yüksek olduğu görülmüştür. Sonuçlar, kullanılan *E. coli* suşlarının tohumdan yaprak kısımlarına doğru hareket ettiğini ve yaprak üzerinde önemli miktarda patojen yükü oluşturduğunu (~4 ile 5.3 log MPN/g) göstermektedir. Yaprakların yıkanması, yaprak dokusu üzerindeki *E. coli* popülasyonlarında önemli bir azalma göstermemiştir; bu, inoküle edilen bakterilerin yaprağa sıkıca bağlandığı (~3.5 ile 7 log MPN/g) ve yıkama uygulamalarıyla yok edilemeyeceği anlamına gelmektedir. Son olarak, Taramalı Elektron Mikroskobu (SEM) ile enterik bakterilerin tere bitkisinin kolonizasyonu için kullanabilecekleri stomaların yanı sıra yaprak kıvrımlarında kalıcı biyofilm yapıları oluşturdukları tespit edilmiştir.

Anahtar Kelimeler: *Escherichia coli*, Biyofilm, Tutunma, Taze Ürünler, Kontamine Sulama

To my family,

ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest gratitude to my beloved advisor Assoc. Prof. Dr. Yeşim Soyer for your endless support, guidance, motivation and enthusiasm. None of this would have been possible without your invaluable guidance and support.

I thank my dearest friends in METU: Arda Tuhanioğlu, Aysu Deniz, Ayşe Küpeli, Demet Sönmezler, Diala Konyalı, Emre Koç, Furkan Erdem, Hilmi Eriklioğlu, Mustafa Güzel, Seray Tok, Utku Uysal, and İrem İskender for all the joy we had throughout this journey. You have always been there to listen and comfort me during my toughest times. I will miss our coffee breaks that we had during pandemic. Also, we were into this work together with Şahin Namlı and I am grateful to you for all the support and patience for answering my endless questions. I would also like to thank the previous and current members of Food Safety Laboratory as well as my colleagues in Food Engineering Department.

Last but not least, Mom, my brother Serkan and Dad, I am grateful to have you. Thank you for always believing in me and supporting me on all my journeys.

This work is funded by Scientific and Technological Research Council of Turkey under grant numbers TUBITAK 118O673 and 220O073.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

Amp: Ampicillin

BHI: Brain Heart Infusion

BPLS: Brilliant-green Phenol-red Lactose Sucrose

BPW: Buffered Peptone Water

CDC: Centers for Disease Control and Prevention

CFU: Colony Forming Unit

dps: days post sowing

EAEC: Enteroaggregative *E. coli*

EHEC: Enterohemorrhagic *E. coli*

EIEC: Enteroinvasive *E. coli*

EPEC: Enteropathogenic *E. coli*

ETEC: Enterotoxigenic *E. coli*

HC: Hemorrhagic colitis

HUS: Hemolytic uremic syndrome

IPCC: Intergovernmental Panel on Climate Change

LB: Luria-Bertani

MPN: Most Probable Number

Na: Nalidixic acid

NORS: National Outbreak Reporting System

OD: Optical Density

rpm: Revolutions per minute

S: Streptomycin

SEM: Scanning Electron Microscope

STEC: Shiga Toxin producing *E. coli*

Sxt: Trimethoprim-Sulfamethoxazole

Te: Tetracycline

ºC: Degree Celcius

CHAPTER 1

INTRODUCTION

1.1 Foodborne Pathogens and Diseases: *Escherichia coli*

It is estimated that each year, nearly 50 million people get foodborne diseases with nearly 130,000 hospitalizations and 3000 deaths (CDC, 2018a). The reported cases associated with the leafy greens have been increasing in 2000s and this increase has been attributed to the nearly 4.5 % increase in the consumption of fresh fruit and vegetables annually from 1990 to 2004 (Herman et al., 2015). From 1998 until 2008, fruits, nuts, fungi, leafy greens, root vegetables and sprout vegetables have been identified as comprising 46 % of all reported foodborne diseases; however, fresh produce related outbreaks had been reported to be larger than the outbreaks that are related to other food sources such as poultry, eggs and pork from 2005 to 2011 (Turner et al., 2019). This can be attributed to that many fresh produces are being consumed in their raw form or they are only minimally processed, which is not sufficient to remove the pathogenic bacteria. The most common foodstuffs involved in ready to eat produce related outbreaks are presented in Table 1.1 (Machado-Moreira et al., 2019).

Table 1.1 The most common vegetable and vegetable-based foodstuffs that are involved in ready to eat produce related outbreaks from 1980 to 2016 in developed countries. Adapted from Machado-Moreira et al. (2019)

Sources	Cases	Deaths	Outbreaks
Radish	10,126	NR	1
Salad*	9,627	4	202

Table 1.1 (Continued)

Lettuce	6,000	22	84
Fenugreek	4,018	50	1
Tomatoes	3,231	NR	20
Alfalfa	3,116	NR	33

*"salad" represents all the incidents where no information is available for the specific ingredients involved in the outbreak.

NR: not recorded

Salmonella spp. and *Escherichia coli* are common reasons for foodborne diseases, hospitalizations and deaths occurring in the US (de Oliveira Elias et al., 2018; Mercanoglu Taban & Halkman, 2011; Turner et al., 2019). The common foodborne pathogens associated with fresh produce are Hepatitis A, Norovirus, *Bacillus cereus*, *Campylobacter* spp., *Clostridium botulinum*, *E. coli*, *L. monocytogenes*, *S. enterica*, *Shigella* spp., *Staphylococcus* spp., and *Yersinia* spp. (Carstens et al., 2019). The common foodborne pathogens and their number of cases related to ready to eat produce from 1980 to 2016 in the developed countries have been demonstrated in Table 1.2 (Machado-Moreira et al., 2019). Apart from these, diseases associated with Shiga toxin producing *E. coli* O157:H7 are posing crucial health risk and they make nearly 36 % of the 265,000 Shiga Toxin producing *E. coli* (STEC) infections annually occurring in the US (CDC, 2014).

Table 1.2 The bacterial pathogenic bacteria that are involved in ready to eat food related outbreaks from 1980 to 2016 in developed countries. Adapted from Machado-Moreira et al. (2019)

Pathogen	**Cases**	**Deaths**	**Outbreaks**
E. coli	16,416	60	41
Salmonella	15,137	24	128
Shigella	1,139	NR	12
Yersinia	686	1	6
L. monocytogenes	436	83	13

NR: not recorded

The analysis of 12,714 reported foodborne diseases from 1973 until 2012 occurred in the US revealed that 606 of them were related to leafy vegetables, and they ended up with nearly 20,000 illnesses, 1000 hospitalizations and 19 deaths (Herman et al., 2015). The study of Herman et al. (2015) revealed that Norovirus was the most prevalent agent for the 272 confirmed single etiology foodborne outbreaks, which is followed by STEC and *Salmonella* spp. Furthermore, *E. coli* O157 was the most common STEC serogroup involved in fresh produce related outbreaks with 45 outbreaks, while *E. coli* O145 and O121 are the other STEC serogroups causing 2 and 1 outbreaks, respectively, through fresh produce. In addition, the number of reported outbreaks has increased during spring times. Norovirus cases were the highest in all seasons, while its prevalence has decreased during summer and autumn times. STEC cases showed a peak in autumn with 51% of the STEC related outbreaks occurring during autumn times. Furthermore, the findings from the study of Turner et al. (2019) agree with this result. The analysis of foodborne outbreaks related to leafy green vegetables occurring in California from 1996 to 2016 showed that the most foodborne incidents have taken place during autumn times with 21 incidents causing a majority of the illnesses occurring in this period. Also, the second peak has occurred during the summer months with 11 reported incidents in this period (Turner et al., 2019).

According to the analysis of Herman et al. (2015), 18 % of the leafy greens related foodborne outbreaks that occurred in the US from 1973 to 2012 has resulted from STEC. In addition, Turner et al. (2019) demonstrated that Romaine lettuce and spinach are the most prevalent agents for 134 leafy green related foodborne outbreaks that occurred in California from 1996 to 2016. *E. coli* was identified as the most common infection agent with 54 outbreaks and of the 54 outbreaks, *E. coli* O157:H7 was responsible for the infections in 37 outbreaks. Among *E. coli* pathogroups, Enterotoxigenic *E. coli* was the leading outbreak agent with nearly 90 million cases worldwide, which has been followed by Enteropathogenic *E. coli* with 23 million cases and STEC with 1.2 million cases (WHO, 2017).

1.1.1 *E. coli* and *E. coli* related outbreaks due to fresh produce

Commensal *E. coli* strains colonize in human and animal intestines, and therefore are among indicator microorganisms for fecal contamination. Although its optimum growth takes place at 37 °C, there are some *E. coli* strains that can grow between 7 and 46 °C. *E. coli* can tolerate NaCl up to 6 % and pH values above 5.4, with exceptions in some serogroups (Carstens et al., 2019). Characterization of *E. coli* strains are identified according to Kauffman classification scheme: somatic (O), flagellar (H) and capsular (K) surface antigens (Croxen et al., 2013).

Meat can be contaminated due to contact with feces during slaughtering. Meat and meat products are considered as the main routes for foodborne diseases with *E. coli* (Croxen et al., 2013). However, *E. coli* can contaminate and persist in the soil, water and seeds for a long time and subsequently *E. coli* can contaminate the leafy greens and fresh produce through inappropriately handled manure and irrigation water. With the increasing numbers of outbreaks related to leafy greens and fresh produce, these foodstuffs are considered as the vectors for foodborne diseases although plant tissue provides a harsh environment for the survival of enteric pathogens. As Table 1.2 indicates, outbreaks related to *E. coli* and ready to eat food have a high number of infections with nearly 17,000 reported infections (Machado-Moreira et al., 2019). In addition, fresh produce related foodborne outbreaks are more and more reported

to be related to STEC (WHO, 2017). According to data collected from CDC's National Outbreak Reporting System (NORS) (CDC NORS Dashboard, 2018), there are 33 multistate and 26 single state fresh produce related outbreaks with *E. coli* etiology occurring from 2009 to 2018 in the US. The numbers of reported illnesses, hospitalizations and deaths resulting from these outbreaks were 1517, 445 and 10, respectively, in total in this period, and 977 of illnesses, 332 of hospitalizations and 7 of the deaths belong to outbreaks occurred in multistate. Analyzing the outbreaks by classifying them into single state and multistate is important. According to Herman et al. (2015), the large number of outbreaks that occur in more than one state suggests that contamination of leafy greens and fresh produce with enteric pathogens such as *E. coli* and *Salmonella* could happen in the early stages of the production, and underlines the importance of contamination in the field although contamination of fresh produce could happen at any time and point in the farm to fork chain.

Table 1.3 shows the number of serotypes and food vehicles that are responsible for 33 multistate and 26 single state outbreaks occurred due to *E. coli* and fresh produce in 2009-2018 period in the US. According to data obtained from NORS database (CDC NORS Dashboard, 2018), STEC O157:H7 is the most dominant serotype among others for both multistate and single state outbreaks. Six serotypes have been identified in multistate outbreaks, and all of them belong to STEC pathogroup. Nine serotypes are involved in single state outbreaks, and STEC group is the dominant group followed by EAEC group with 2 outbreaks. In addition, lettuce and lettuce related foodstuffs are the most common reasons for both multistate and single state outbreaks. There were reported 10 multistate and 3 single state outbreaks due to *E. coli* infection with Romaine lettuce between 2009 and 2018 (Table 1.3). Nine of these multistate outbreaks were related to *E. coli* O157:H7, and one was due to *E. coli* O145. It is worth to mention that there was no fruit-based outbreak in multistate outbreaks. Moreover, in multistate outbreaks, three outbreaks have resulted from sprouts (clover and alfalfa) with 59 illnesses and 14 hospitalizations. Since growth conditions of sprouts require higher humid environments, they pose a higher risk, and their growth is being conducted under more controlled environments (Xiao et al., 2014).

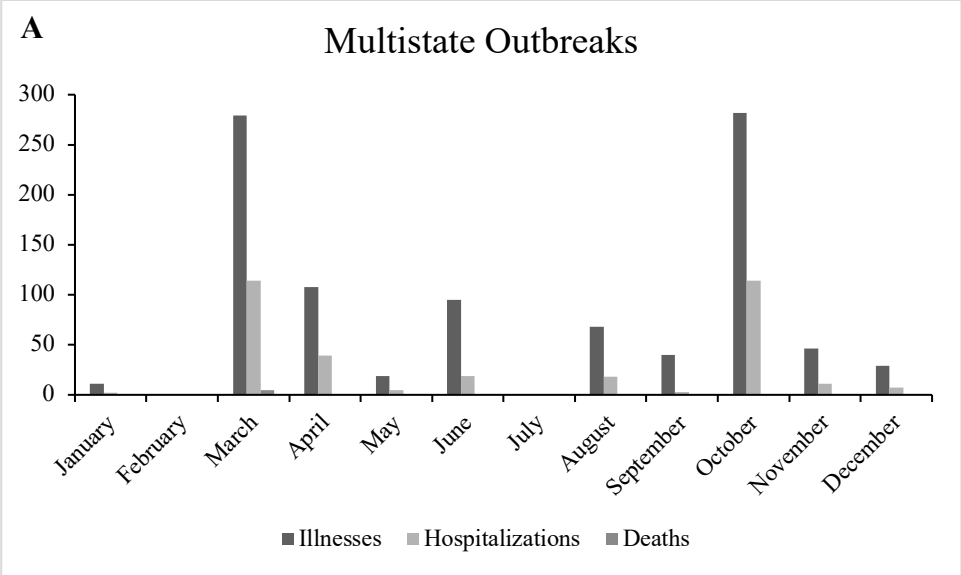
Table 1.3 The number of serotypes and food vehicles that are responsible for the multistate (n=33) and single state (n=26) *E. coli* and fresh produce related outbreaks in 2009-2018 period in the US. Data were obtained from NORS database on October 10, 2021. The numbers are presented as [total number (% of total outbreaks)]

Multistate		**Single State**	
Serotype	**Number**	**Serotype**	**Number**
STEC O157:H7	23 (69.70%)	STEC O157:H7	16 (61.54%)
STEC O145	3 (9.09%)	STEC O111:NM	1 (3.85%)
STEC O26	3 (9.09%)	EAEC O126	1 (3.85%)
STEC O157:NM (H-)	2 (6.06%)	STEC O145	1 (3.85%)
STEC O111	1 (3.03%)	STEC O157	1 (3.85%)
STEC O121	1 (3.03%)	STEC O103	1 (3.85%)
		STEC O157:NM (H-)	1 (3.85%)
		STEC O45	1 (3.85%)
		ETEC O6:H16	1 (3.85%)
		unknown	2 (7.69%)
Food Vehicle	**Number**	**Food Vehicle**	**Number**
romaine lettuce	10 (30.30%)	lettuce	5 (19.23%)
lettuce	3 (9.09%)	romaine lettuce	3 (11.54%)
prepackaged leafy greens	3 (9.09%)	apple	3 (11.54%)
spinach	3 (9.09%)	cilantro	1 (3.85%)
clover sprouts	2 (6.06%)	fruit	1 (3.85%)
iceberg lettuce	2 (6.06%)	green beans	1 (3.85%)
leafy greens	2 (6.06%)	tomato	1 (3.85%)
pre-packaged salad	2 (6.06%)	kale	1 (3.85%)
lettuce; ambrosia	1 (3.03%)	leaf lettuce	1 (3.85%)
cabbage	1 (3.03%)	green leaf lettuce	1 (3.85%)
alfalfa sprouts	1 (3.03%)	lettuce-based salads	1 (3.85%)
salad	1 (3.03%)	arugula	1 (3.85%)
leaf lettuce	1 (3.03%)	mixed green salad	1 (3.85%)

Table 1.3 (Continued)

spring salad; baby leaf, unspecified; mizuna	1 (3.03%)	celery	1 (3.85%)
		cucumber	1 (3.85%)
		strawberries	1 (3.85%)
		salad; vegetable tray; cheese dish; dips; fruit	1 (3.85%)
		tomato; cucumber; lettuce	1 (3.85%)

From Figure 1.1 and Figure 1.2, it could be deduced that the season might affect the number of illnesses. Multistate outbreaks show peaks in March and October for outbreaks that occurred due to *E. coli* and fresh produce from 2009 to 2018 in the US (Figure 1.1-A). When seasonality effect are analyzed, a similar trend is observed for multistate outbreaks with peaks in spring and autumn (Figure 1.1-B). In addition, the biggest peak has been obtained in July in single state outbreaks (Figure 1.2-A), and similarly, the highest number of illnesses were observed in the summer months (Figure 1.2-B).



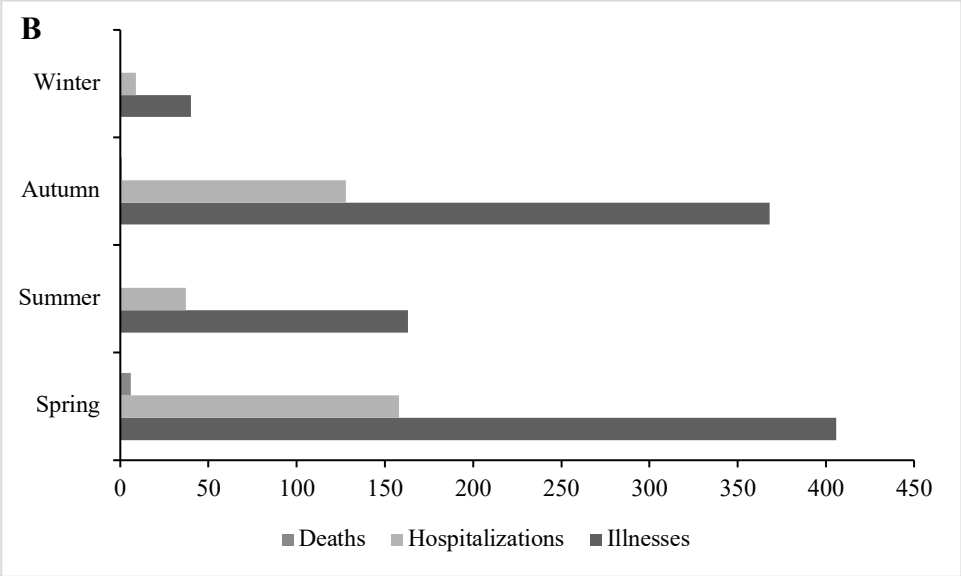


Figure 1.1 The number of illnesses, hospitalizations and deaths occurred due to multistate outbreaks related to *E. coli* and fresh produce in the period 2009 to 2018 in the US. Data were collected from NORS database on October 10, 2021. (A) shows the illness, hospitalization and death data with respect to months, while (B) shows with respect to seasons. (Seasons: spring, March-May; summer, June-August; autumn, September-November; winter, December-February).

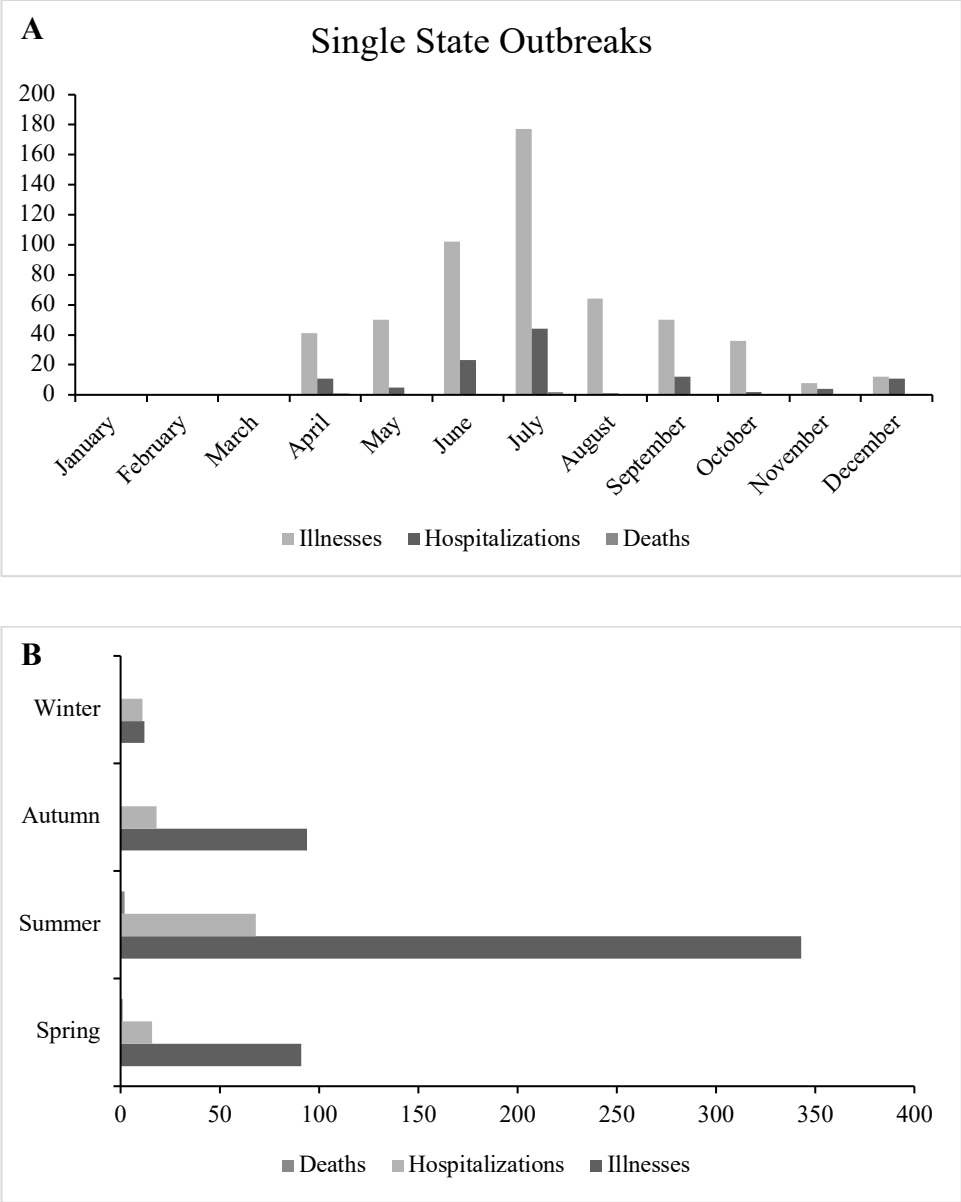


Figure 1.2 The number of illnesses, hospitalizations and deaths occurred due to single state outbreaks related to *E. coli* and fresh produce in the period 2009 to 2018 in the US. Data were collected from NORS database on October 10, 2021. (A) shows the illness, hospitalization and death data with respect to months, while (B) shows with respect to seasons. (Seasons: spring, March-May; summer, June-August; autumn, September-November; winter, December-February).

1.1.2 Diarrheagenic *E. coli* Pathogroups

In general, pathogenic *E. coli* can cause infections in urinary tract, bloodstream and central nervous system besides gastrointestinal diseases (Croxen et al., 2013). Diarrhea causing *E. coli* are investigated under five major pathogroups, which are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and enterotoxigenic *E. coli* (ETEC).

1.1.2.1 Enteropathogenic *E. coli*

EPEC pathogroup is the first group that has been identified among other diarrheagenic *E. coli* groups. The strains in this group are best known for their attaching and effacing (A/E) ability that can enable them to form characteristic lesions on epithelial cells on the intestines (Croxen et al., 2013). EPEC strains do not produce any heat sensitive or resistant enterotoxins. It is associated with infantile diarrhea and watery diarrhea with higher frequencies in developing countries (Nataro & Kaper, 1998). Fecal-oral route is an important transmission route for EPEC strains (Levine & Edelman, 1984). Although no specific food source has been linked to cause EPEC related outbreaks, it has been found that most of the EPEC originated outbreaks have resulted from contaminated water resources (Feng, 2017). The reported infectious dose in adults is between 10^8 and 10^{10} CFU/ml or gram food (Bieber et al., 1998). Additionally, there is a seasonal effect on the EPEC outbreaks with higher incidence in warmer seasons (Afset et al., 2004; Behiry et al., 2011). When the outbreaks that are originated from EPEC group have been analyzed, it has been seen that there is an outbreak caused by EPEC and STEC pathogroups, which affected 237 people in Helsinki, Finland, in August 2016 (Kinnula et al., 2018). Although EPEC originated outbreaks are mostly seen in developing countries, it is still possible to observe them in developed countries (Croxen et al., 2013).

1.1.2.2 Shiga toxin Producing *E. coli*

The presence of Shiga toxin producing genes, stx_1 or stx_2, in an *E. coli* isolate identifies it as a Shiga toxin producing *E. coli* (STEC). Although there are many STEC strains, only some of them causes diseases in humans, and Table 1.4 summarizes the common STEC serotypes that are linked to human diseases and their characteristics. Enterohemorrhagic *E. coli* (EHEC) is classified under STEC group. The main difference between the EHEC serotypes and non-EHEC STEC serotypes is that all wild type serogroups of EHEC are LEE positive and can form A/E lesions (Croxen et al., 2013).

Table 1.4 Common STEC serotypes with O serogroups and H antigens that they can possibly have and their characteristics. Adapted from Croxen et al. (2013)

O serogroup	**H antigen(s)***	***eae***	**HC****	**HUS****
O26	NM, H11	+	+	+
O45	NM	+	+	-
O91	NM, H21	-	+	+
O103	NM, H2	+	+	+
O111	NM, H8	+	+	+
O113	H21	-	+	+
O121	H19	+	+	+
O145	NM, H25, H28	+	+	+
O157	NM, H7***	+	+	+

*NM: non-motile

**HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome.

***can ferment sorbitol

Similar to other *E. coli* pathogroups, STEC is also transmitted through fecal-oral route as well as foodborne, person-to-person, animal contact and consuming

contaminated water (Carstens et al., 2019). Furthermore, STEC can tolerate high acidity. Therefore, it can survive and resist the high acidic environment of the stomach, and even a low number of cells can cause the development of infection (Hong et al., 2012). Thus, the infectious dose is considered to be as low as 100 to 1000 CFU (Croxen et al., 2013). Symptoms that are involved with STEC infection are mild gastroenteritis, bloody diarrhea, and hemolytic uremic syndrome (HUS).

There is a wide variety in the STEC group in terms of serotypes and virulence factors. Shiga toxins are classified into two groups: Stx1 and Stx2. Stx1 has three subtypes: a, c and d, while Stx2 has seven subtypes: a to g. STEC can have either one of the genes encoding for Shiga toxin (*stx*$_1$ or *stx*$_2$) or both of them. *stx*$_1$ containing STEC can cause the development of HUS. However, the presence of *stx*$_2$ has been reported to cause more severe human diseases (Croxen et al., 2013). Shiga toxin encoding genes are located on prophages inserted in the chromosome. It is thought that these prophages become lytic during stress and during lysis, the Shiga toxin is released from the cell material into the tissue (Neely & Friedman, 1998). EHEC hemolysin (*hlyA*) is a pore-forming toxin, and it has cytotoxic effect on the endothelial cells contributing to HUS development. There are various adhesins encoded in the LEE region in LEE-positive STEC that are important in the attachment and colonization of epithelium cells in the intestines as well as other surfaces like leaf surface (Croxen et al., 2013).

Foodstuffs that have been associated with STEC infections in human are uncooked hamburger, sausage, raw milk, dairy products, apple cider, lettuce, spinach and sprouts (Croxen et al., 2013). Previously, it has been shown that STEC O157:H7 and O26:H11/NM attached to spinach with their EspA virulence factor; therefore, the efficacy of washing on the elimination of bacteria has reduced (Shaw et al., 2008; Wright & Holden, 2018). Also, internalization inside the leaf tissue with these pathogens has been reported (Saldaña et al., 2011). Furthermore, intact structure of leaf tissue is also important in terms of preventing attachment of STEC on the fresh produce. It has been shown that the damaged leaf tissue (bruised or shredded) is more vulnerable to be contaminated by STEC O157:H7 strains than the intact leaf surfaces (Hartmann et al., 2017).

There are many outbreaks that occurred due to EHEC O157:H7 in the past affecting many people. Nearly 112,000 cases of non-O157 STEC related foodborne outbreaks occur annually with a 12.8 hospitalization rate in the US, while approximately 65,000 foodborne cases occur due to STEC O157 with a hospitalization rate of 46.2% each year in the US (Carstens et al., 2019).

One of the devastating outbreaks related to raw sprouts occurred in Japan in 1996 and it has been known as the largest reported *E. coli* O157:H7 related outbreak in the world (Taormina et al., 1999). 6000 people were affected in this EHEC O157:H7 related outbreak due to white radish sprout consumption. Seeds were investigated for the presence of *E. coli* O157:H7. Although seeds were negative for *E. coli* O157:H7, 126 people have been affected with *E. coli* O157:H7 infection through white radish sprouts next year.

In addition, between 2009-2018 period, the highest illnesses and deaths have been reported in the Romaine lettuce related outbreak that started in March 2018 affecting 36 states in the US (CDC, 2018b). This outbreak has reported to lead 210 illnesses and 96 hospitalizations. Also, 27 people have developed kidney failure with HUS, and five deaths were reported. The traceback analysis with the epidemiologic and laboratory findings have showed that the most probable source for this outbreak is Romaine lettuce grown in the Yuma region, Arizona. *E. coli* O157:H7 was detected in the water samples that are sampled from the Yuma growing region, and it was genetically related to the strains isolated from infected people.

Another deathliest fresh produce related outbreak occurred in 2006 in the US. The analysis of DNA fingerprints showed that the *E. coli* O157 was responsible for this outbreak and it was isolated from 13 spinach packages that are collected from 10 states. Nearly 200 people were affected, for which 116 (56 %) were hospitalized and 39 (19 %) developed HUS while 5 (2 %) died (Sharapov et al., 2016). 72 % of the cases were female, and the highest proportion for the HUS cases was observed in children who are less than 5 years with 35 %. Of 5 deaths, 3 were related to HUS development (Sharapov et al., 2016).

Another STEC related foodborne outbreak occurred in 2020 was due to the consumption of raw clover sprouts affecting ten states in the US. There were 51 reported cases with three hospitalizations. No death was reported. *E. coli* O103 was detected as the probable contaminating agent. The age range was between 1 and 79, with a median of 29, and 55 % of infected people were reported as female. Although there are more strict regulations for the production of sprouts due to being grown under high humid environments, sprout related foodborne outbreaks still continue to occur and they affect mostly young people and females (CDC, 2020a).

1.1.2.3 Enteroinvasive *E. coli*

The transmission of EIEC takes place by fecal-oral route, and the contaminated food and water sources take a role in the transmission as well as direct person to person dissemination (Harris et al., 1985). EIEC require 10^6 to 10^{10} cells to start an infection. EIEC can rapidly adapt to variations in the environment including low pH of the stomach, temperature and availability of oxygen. As an invasive pathogen, EIEC uses intestinal epithelial cells in large intestines for replication site (Croxen et al., 2013).

EIEC causes less severe clinical symptoms that are clinically reported. Most of the time, EIEC infection causes self-limiting watery diarrhea. There is no reported EIEC outbreak or cases in the US and Europe (Croxen et al., 2013).

1.1.2.4 Enteroaggregative *E. coli*

After its identification, EAEC has been detected in many diarrheal diseases around the globe, and it has been identified as the most common bacterial pathogen in the diarrheal fecal samples (Croxen et al., 2013). There are many EAEC related outbreaks causing gastroenteritis and affecting many adults and children in developing and developed countries. It leads to the development of persistent diarrhea in children and people infected with HIV. The main concern with the EAEC infection is malnourishment that can be developed in children in developing

countries. Persistent diarrhea may cause chronic inflammation by damaging epithelium cells in the intestines and subsequently it inhibits the ability to absorb nutrients in the gut. It can cause traveler's diarrhea. It spreads through contaminated water and food. Also, food handlers have a role in EAEC spread underlining the importance of following sanitary practices during food preparation. There are different infectious doses identified for different EAEC serogroups in the epidemiologic studies changing from 10^8 to 10^{10} CFU/ml (Hebbelstrup Jensen et al., 2014). Furthermore, biofilm formation holds vital role in the pathogenesis of EAEC group. To initiate the infection, the EAEC strains firstly adhere to the intestines. Fimbria or the aggregative adherence fimbria (AAF) is associated with the first adherence as well as *aggR* gene. After first adherence, EAEC strains start to form biofilms which later on inhibit the uptake of antibiotics or any other antimicrobial by the cells and let infections lead for longer times (Hebbelstrup Jensen et al., 2014).

There are many outbreaks related to EAEC strains. In 1993, nearly 2,700 children were affected by food poisoning in Tajimi, Japan. It is thought that the school lunch was the probable reason for the infection, and this is the second largest EAEC related outbreak after the 2011 German O104:H4 outbreak. The stool samples from 30 children with severe diarrhea showed aligned cells in stacked-brick form on Hep-2 cells, which is an attribute belonging to strains in EAEC group (Itoh et al., 1997).

EAEC group is not usually known for their ability to produce Shiga toxin. However, the presence of hybrid strains of EAEC and STEC has been reported. For example, it has been detected that EAEC O104:H4 serogroup has attained the ability to produce Shiga toxin by acquiring a prophage that encodes for the Shiga toxin 2 gene (*stx*$_2$) in an outbreak that occurred in 2011 in Germany (Rasko et al., 2011). This is the largest outbreak caused by EAEC group that has been reported so far. In this outbreak, 4075 people were affected, and 855 (22 %) had developed HUS and 50 (1.4 %) died. Of the deaths, 35 of them were related to HUS development (Robert Koch Institute, 2011). The most probable source was identified as the fenugreek seeds imported from Egypt and grown in Germany and France. The isolated outbreak strain has shown stacked-brick pattern on Hep-2 cells, and the main EAEC virulence genes were present in the genome of the isolate. The genome analysis also showed

the presence of the *stx₂* gene, which is normally not found in the genome of EAEC strains. HUS cases and deaths reported in this outbreak were more than a total of 350 outbreaks that occurred due to *E. coli* O157:H7 between 1982 and 2001 in the US (Safadi et al., 2012).

Mellmann et al. (2011) suggested that the relatedness of 2011-German outbreak strain (LB226692) and previously isolated *E. coli* O104:H4 strains (enterohemorrhagic *E. coli* O104:H4 strain 01-09591 isolated from HUS patient in Germany in 2001 and enteroaggregative *E. coli* O104:H4 strain 55989 isolated in Central Afrika in the late 1990s) can be explained by two evolutionary models: (i); common ancestor model and (ii); linear ancestry model. The former accepts that there is a hypothetical Shiga-toxin producing EAEC O104:H4 ancestor leading to the evolution of LB226692 and 01-09591, whilst the latter asserts all the EHEC O104:H4 (e.g., 01-09591) are derived from EAEC 55989 strain. For 2011-German outbreak, the common ancestor model was thought to describe the evolution of this outbreak strain and previously isolated strains. According to this model, apart from LB226692 and 01-09591, EAEC 55989 might also be evolved from a hypothetical Shiga-toxin producing EAEC O104:H4 ancestor by losing its *stx2* gene. The visualization of the evolutionary model is given in Figure 1.3, which is adapted from the study of Mellmann et al. (2011).

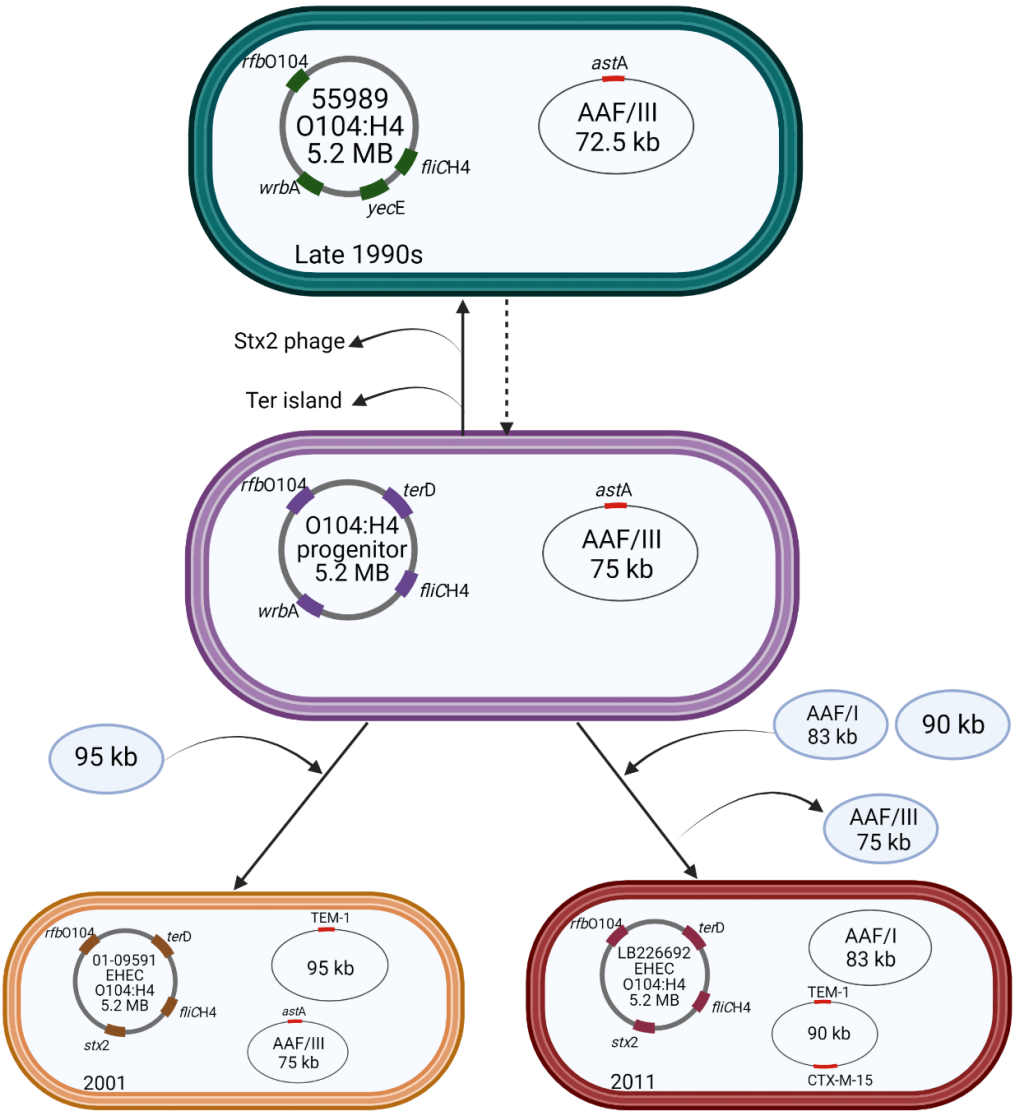


Figure 1.3 An evolutionary model for both EHEC and EAEC O104:H4. Numbers on the lines are the number of loci differing between the strains. Since the exact chronological sequence is not known for the loss and acquisition of plasmids, they were chosen randomly in the diagram. Adapted from Mellmann et al. (2011).

Similarly, the existence of EAEC strains that have previously acquired the ability to produce Shiga toxin has also been detected (Hebbelstrup Jensen et al., 2014):

- HUS cases seen in ten kids in France in 1992 were found to be resulted from the EAEC O111:H2 serotype carrying *stx2* gene

- A 3-year-old child diagnosed with HUS in Japan in 1999 was found to be affected by the EAEC O86:HNM serotype, which also carries the *stx2* gene

- Another EAEC O111:H21 serotype carrying *stx2* gene was associated with a HUS outbreak in an Irish hospital in 2012.

All of these outbreaks highlight the importance of surveillance, showing that the EAEC group is prone to acquire additional virulence factors through horizontal gene transfers (Hebbelstrup Jensen et al., 2014).

1.1.2.5 Enterotoxigenic *E. coli*

The transmission route for ETEC infections is mainly fecal-oral route. Contaminated food and drinking water, especially surface water in developing countries, are the sources of ETEC. In freshwater, ETEC was reported to persist three months (Lothigius et al., 2010). The infectious dose is relatively high, and 10^6 to 10^8 organisms are required to start an infection. Incubation time is generally between 5 hours and 1 to 2 days upon digestion. Diarrhea can generally persist about 3 or 5 days, and if the required treatment is provided, the mortality rates are so low (Croxen et al., 2013).

ETEC cases are mostly seen in underdeveloped countries. Basically, ETEC causes traveler's diarrhea in children leading to significant rates of mortality in children in developing countries (Croxen et al., 2013). Nearly 840 million people are affected by ETEC in these countries each year, and 280 million of them occur in children between 0 and 4 years old (Wennerås et al., 2004).

1.2 Contamination Sources for Fresh Produce

Fruits and vegetables are being consumed more and more due to their beneficial health effects with their substantial essential vitamin and mineral content as a result

of changes in lifestyle and health concerns. In the countries with higher income, the consumer behavior has shifted towards consuming more fresh produce recently with the recommendations of the World Health Organization for consuming at least 400-gram fruits and vegetables in a day (WHO, 2003). In a WHO report published in 2003, it was clearly stated that having a fresh produce-rich diet is crucial to prevent cardiovascular diseases, diabetes, obesity and cancer. In 2018, fresh vegetable consumption was around 67 kg per capita in the US (Food Availability (Per Capita) Data System. Summary Findings USDA, 2020).

The fresh produce, especially leafy green vegetables, sprouts and microgreens, are consumed in their raw or minimally processed forms in ready-to-eat foods. The microflora of fresh produce is quite rich in mesophilic bacteria, yeast and molds. The microbial load of flora could change from 10^3 to 10^9 CFU/g depending on the processing levels (Carstens et al., 2019). Although they contain high microflora content, the main concern with fresh produce is contamination with the pathogens. Since fresh produce is not processed to decrease the microbial load on them, they can play a role in the development of foodborne illnesses with foodborne pathogens; viruses, parasites, and bacteria. The rise in consuming more and more fruits and vegetables could also have the potential to increase the probability of fresh produce related outbreaks and illnesses (Roy & Melotto, 2019). The fresh produce is prone to get easily contaminated during both pre- and post-harvest stages. Pre-harvest contamination sources which are manure, fertilizer, soil, the water used for irrigation purposes and wildlife including insects or post-harvest contamination sources that are wash water, farmworkers and equipment used for harvest could be the sources of contamination of fresh produce in the field (Carstens et al., 2019; Liu et al., 2013; Thao et al., 2019). With globalization, trade across continents has become available and the inappropriate transportation conditions could also lead to the growth or survivability of undesired foodborne pathogens on the fresh produce (Carstens et al., 2019).

The contamination of fresh produce could be influenced by animal and environmental intervenes. As the globalization and close contact increase with the animals and their close environments, the likelihood of contamination rises. It is

important to outline all the possible outcomes from the increased likelihood of contamination. “One Health” approach is an effective tool to present a safer human-animal-environment interface (CDC, 2022). With the combination of scientific opinion over environment, human health, plant health, and animal health, One Health aims to use an interdisciplinary approach to control the public health threats by collaboration of scientists from different fields (Destoumieux-Garzón et al., 2018). By doing so, this approach could prevent the zoonotic diseases, improve food safety as well as food security, and decrease the dissemination of antibiotic resistance by improving the human and animal health. One Health approach improves food safety by predicting the behavior of human enteric pathogens in environment and how the pathogens are affected by the changes in environment, and it proposes a comprehensive solution to improve food safety.

The main bacterial pathogens causing foodborne outbreaks through the consumption of fresh produce are determined to be *E. coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* (de Oliveira Elias et al., 2018; Mercanoglu & Halkman, 2011; Turner et al., 2019). In short, ruminants such as cattle and sheep are the main reservoir for *E. coli*, and *E. coli* O157:H7 is shed in the feces of these animals to the environment, later contaminating the water sources and soil. The whole mechanism for the spread of pathogenic bacteria and their colonization on the fresh produce is discussed in detail in the following sections, and it was summarized in Figure 1.4.

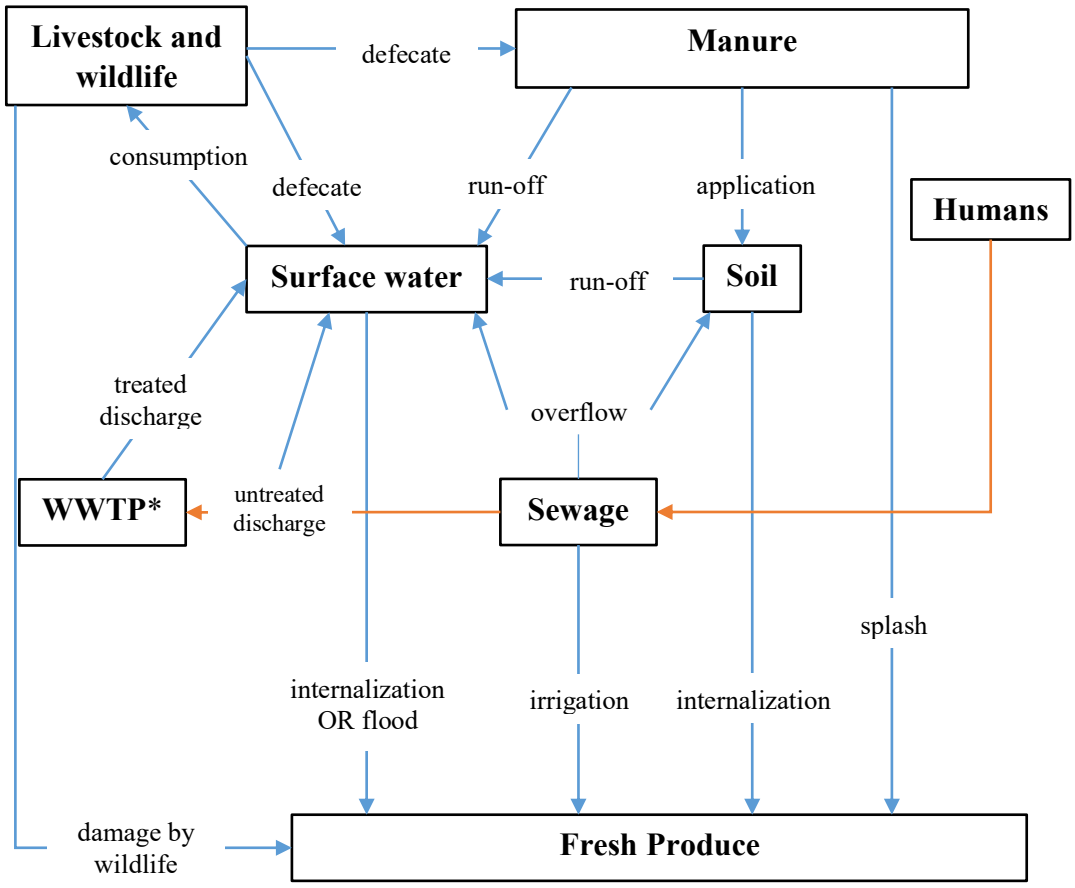


Figure 1.4 The main pathways and reservoirs for pathogenic bacteria to spread and colonize the fresh produce. Boxes represent the reservoirs for the pathogenic bacteria spread, while arrows show how bacteria spread from main reservoirs and colonize fresh produce in the field. *WWTP represents wastewater treatment plants. Adapted from Liu et al. (2013).

1.2.1 Irrigation Water

The higher demand for fresh water is leading to new challenges for supplying both microbiologically and chemically safe potable water in the world (Mukherjee et al., 2018). The increasing globalization, industrialization and changes in climate are introducing more challenges for the supply of fresh water. In the US, there were

nearly 40 identified waterborne outbreaks in one-year interval between 1999 and 2000 (CDC, 2002).

Water that is used in agricultural practices is considered as one of the most important routes for the contamination of fresh produce (Van der Linden et al., 2014). Particularly, irrigation water is an important source for the contamination of both microgreens and sprouts which can be cultivated in water-based agriculture, i.e., hydroponically (Riggio et al., 2019). In water, the foodborne pathogens, *Yersinia enterocolitica, E. coli, Salmonella* spp. and *Shigella* spp. have been identified and they can persist in water (Simmons et al., 2001). Surface water; i.e., rivers, lakes, streams and creeks; harvested rain water, wastewater and groundwater could be the sources for irrigation water.

1.2.1.1 Irrigation Water Sources

1.2.1.1.1 Surface Water

Water from lakes, rivers, ponds and creeks constitutes surface water. They could be used for drinking and irrigation purposes. However, they can be easily contaminated with sewage overflow and animal feces. Yet, the lake has been found to be microbiologically safer rather than the river, which could be easily contaminated with upstreams (Uyttendaele et al., 2015).

1.2.1.1.2 Groundwater

It is estimated that nearly half of water used for agricultural purposes is supplied through groundwater, and it is mostly considered as high-quality water (Taylor et al., 2013). However, due to increased global activities, the sources are getting lower, which makes usage of surface waters to increase. Though, this event can pose a threat by increasing the odds of contamination through foodborne pathogens (Riggio et al., 2019).

1.2.1.1.3 Wastewater

Wastewater has very poor microbiological quality. It can be categorized as blackwater and greywater. Blackwater is used water that is contaminated with fecal material besides toxic chemicals and grease (Jongman & Korsten, 2018). Since fecal content can harbor pathogens, it can be considered highly contaminated. It is mostly called sewage. Greywater is also used water that is collected from sinks and showers. Compared to blackwater, the contamination levels are lower in the latter. However, greywater can also contain pathogens that could result in foodborne and waterborne diseases (United States Environmental Protection Agency (EPA), 2011). Therefore, the wastewater needs to be treated thoroughly to decontaminate it microbiologically and chemically. Without treatment, wastewater is not recommended for irrigation. However, if thoroughly treated, recycled greywater can be used for irrigation of freshly consumed commodities. In addition, wastewater is not only cheap but also contains micronutrients, such as nitrogen and phosphorus that can be a valuable source for plant growth (Liu et al., 2013). In developing countries and places where water scarcity is present, blackwater and greywater are being used for irrigation in agricultural fields (Amoah et al., 2005). Yet, there is a negative perspective towards irrigation of fresh produce with recycled water since inappropriately or inadequately treated wastewater could still contain microorganisms of health concerns (Liu et al., 2013).

Besides being used as irrigation water, wastewater and sewage are causing another concern, which is sewage overflow due to extreme weather events, such as flood and heavy rain (Decol et al., 2017). Sewage overflow could lead to the spread of pathogenic *E. coli* species and *Salmonella* spp. to surface water, soil and seeds. Through soil and seed contamination, the pathogens present can translocate towards the edible portions of the fresh produce (Kroupitski et al., 2009, 2019; Zarkani et al., 2019). Likewise, *E. coli* O157:H7 and *Salmonella* spp. present in irrigation water could internalize inside plant tissue through an open stoma and vascular tissues (Roy & Melotto, 2019).

1.2.1.1.4 Harvested Rainwater

Harvested rainwater is considered as an alternative to freshwater that could supply part of the demand for freshwater, and they can be used for drinking and irrigation purposes. Rain water could be harvested by roof-harvesting or ground surface-harvesting (Jongman & Korsten, 2018). In these systems, water is harvested from either rooftops or leaks from surface runoffs. However, animal droppings, insects and pests could contaminate the rain water harvested from roof, so the wildlife could contaminate these types of water (Ahmed et al., 2011). Sánchez et al. (2015) reported that harvested rainwater could contain *Salmonella* spp. and *Campylobacter* spp.

1.2.1.2 Legislations and Guidelines for Water

Although the interaction between foodborne pathogens and freshly consumed leafy greens has been known for nearly 30 years, the minimum requirements for the quality of irrigation water were set in near past (Allende & Monaghan, 2015). The European Commission Regulation No 852/2004 for food stuff hygiene does not specifically define the criteria for the microbiological quality of potable water. However, it forces the potable water to meet the minimum requirements that are set for the water intended for human consumption (European Commission (EC), 2004).

The Codex Alimentarius General Principles of Food Hygiene-CXC 1–1969 (Codex Alimentarius Commission, 2020) states that the water used in the pre-harvest conditions of fresh produce should have adequate quality. The chemical and microbiological quality of water should be identified by growers. Although there are remarks for the precautions to reduce the possibility of fresh produce contamination in the field, such as preferring drip irrigation instead of overhead irrigation and paying attention to wetting duration, there is no exact criteria for the microbiological requirements for the irrigation water. It has been stated that special care should be taken in irrigation for fresh produce that will not be further processed or treated for post-harvest washing before transportation stages. However, again there are no certain criteria for water used to irrigate fresh crops in the field. Moreover, the “Fresh

Leafy Vegetables" Annex of The Codex Alimentarius Code of Hygienic Practice for Fresh Fruits and Vegetables CXC 53-2003 (Codex Alimentarius Commission, 2013) does not also state the exact requirements for irrigation, but it delivers that to reduce the odds of contact with pathogens, the water that will contact the edible parts should have the standards for potable water, which is identified by the WHO Guidelines as water that does not ruin the food safety.

However, FDA (2019) sets the minimum requirements for irrigation water in compliance with the Food Safety Modernization Act (FSMA), becoming the US law in 2011. Except for sprouts, the quality of water that will be in direct contact with fresh produce should contain less than 126 CFU generic *E. coli* in 100 ml water and the microbiological quality of 10 % of the samples collected should not exceed 410 CFU generic *E. coli* in 100 ml water. If the mentioned requirements cannot be met, then the corrective measures should be taken, such as water treatment and waiting for some time to allow harmful microorganisms to die.

1.2.1.3 Importance of Water in Terms of Food Safety

In terms of food safety, the quality of irrigation water is a critical control point that can stop the spread of enteric pathogens through freshly consumed leafy greens. It has been previously shown that *E. coli* O157:H7 and *Salmonella* spp. can colonize the edible portions of fresh produce through stoma when contaminated water is used for irrigation purposes. Norovirus-infected irrigation water is also shown to be adhering to fresh vegetables and fruits (Alum et al., 2011). In addition, if there are bruises or cuts on the fresh produce, the colonization and internalization of enteric pathogens inside apoplastic regions get easier upon contamination with irrigation water (Hartmann et al., 2017). However, the high dosages of *E. coli* O157:H7 are required for internalization and survival (Erickson et al., 2014).

Many foodborne outbreaks have been traced back to contaminated water. The contaminating agent for the prepacked spinach outbreak in 2006 in the US that affected nearly 200 people was detected as *E. coli* O157:H7 which contaminated the surface through shedding by cow and pig feces. Another *Salmonella* Litchfield

related outbreak that is originated from contaminated wash water of papayas occurred in 2012 in Austria, and 26 people were infected (Gelting, 2007). Table 1.5 shows the microbial quality of irrigation water used in fresh produce in different countries.

Table 1.5 Prevalence or microbial load of indicator and foodborne microorganisms in irrigation water that is used in fresh produce production

Country	**Field**	**Water source**	**Microorganisms**	**Prevalence or Microbial load (CFU/100ml)**	**Reference**
Southern of Brazil	Whole lettuce	Natural ponds	*E. coli* spp.	2.1 to 5.4 log CFU/100 ml	(Decol et al., 2017)
			E. coli O157:H7	9/50	
		Streams	*E. coli* spp.	2.6 to 4.3 log CFU/100 ml	
			E. coli O157:H7	4/12	
South-east of Spain	Iceberg lettuce – where flood events happened	Irrigation systems – 1-week after the flood events	*L. monocytogenes*	Not Detected	(Castro-Ibáñez, Gil, Tudela, & Allende, 2015)
			Salmonella	2/4	
			E. coli O157:H7	0/4	
			E. coli O26, O103, O111, and O145	0/4	
			E. coli spp.	~4 log CFU/100 ml	

Table 1.5 (Continued)

South-east of Spain	Baby spinach	Irrigation water from ponds	*Salmonella*	1/50	(Castro-Ibáñez, Gil, Tudela, Ivanek, et al., 2015)
			E. coli O157:H7	0/50	
			E. coli O26, O103, O111, and O145	0/50	
			E. coli spp.	0 to 2 log CFU/100 ml	
Spain	Tomatoes	Surface water	*E. coli*	0 to 1.5 log CFU/100 ml	(Lopez-Galvez et al., 2014)
			L. monocytogenes	0 to 3.5 log CFU/100 ml	
		Reclaimed water	*E. coli*	0 to 2 log CFU/100 ml	
			L. monocytogenes	0 to 3.5 log CFU/100 ml	
Belgium	Lettuce	Collected rainfall	EHEC	6/120	(Holvoet et al., 2014)
			Campylobacter spp.	23/120	
Italy	Tomatoes	Tap water	*E. coli* spp.	0/30	(Forslund et al., 2012)
		Reclaimed water	*E. coli* spp.	11/30	

To provide the minimum requirements for irrigation water, there are a few recommendations that are set by the authorities. The Codex Alimentarius General Principles of Food Hygiene-CXC 1–1969 (Codex Alimentarius Commission, 2020) states that the water that is in direct contact with the edible portions of leafy green should be clean and potable. Also, irrigation types could impact the contamination levels. Drip irrigation is applied to soil, and therefore it reduces the contact with edible parts. As a result, drip irrigation is recommended instead of overhead irrigation, in which the water directly faces with the edible parts and increases the contamination risk (Allende et al., 2017; Riggio et al., 2019). Moreover, of course, the quality and source of water are crucial. In microgreen production, municipal water usage is likely more common (Riggio et al., 2019). However, it is not always sustainable to use municipal water in production systems although it has the best quality. Uyttendaele et al. (2015) summarized that municipal water is followed by groundwater, gray water and harvested rainwater in terms of quality.

1.2.2 Seeds

Especially in sprout production, the contamination of seeds is one of the most frequent problems that is encountered (Cui et al., 2017; Riggio et al., 2019). Devastating outbreaks related to sprouts occurred in the past affecting thousands of people. For instance, one of the biggest *E. coli* O157:H7 related outbreak which occurred in Japan in 1996 was associated with contaminated radish sprouts and above 6000 people were affected in this outbreak (Taormina et al., 1999). In addition, another large outbreak is the 2011-German outbreak affecting more than 3000 healthy people, and the probable contaminating agent for this outbreak was fenugreek sprouts (Robert Koch Institute, 2011). However, in general, the common practice to cultivate sprouts is growing in either greenhouses or controlled chambers. Therefore, the interaction with wildlife, livestock and sewage is limited in these systems. In addition, thanks to their short growing periods (days – most of the time), sprouts do not require the usage of fertilizer or manure. Therefore, the probable sources for contamination of sprouts could be irrigation water and primarily seeds (Machado-Moreira et al., 2019). The same conditions are also applicable for

microgreen and other leafy green productions. However, unlike microgreen and leafy green production, sprout cultivation is performed under high humidity, relatively high temperatures and dark conditions, which might enhance bacterial growth (Xiao et al., 2014). Once the seeds are contaminated, they can internalize inside the plant tissue and translocate towards the phyllosphere where it can get pretty hard to decontaminate from the plant tissue. van Overbeek et al. (2020) showed that when pea seeds contaminated with *E. coli* O104:H4 are germinated, a substantial pathogen load of around 6 log CFU/g plant has obtained at 8 days post-sowing. Thus, contaminated seed usage can result in a substantial microorganism load on the edible parts of fresh produce.

It has been reported that most of the cases that are related to sprouts had been resulted from contaminated seed before sprouting. Van der Linden et al. (2013) reported that foodborne pathogens, namely *S.* Typhimurium and *E. coli* O157:H7, can survive on the butterhead seeds for up to two years. However, there is another problem with seed contamination, which is uneven distribution of pathogens in a whole batch. Therefore, this could lead to the inability to detect the pathogen presence during routine tests (Cui et al., 2017). The interaction of pathogens with seeds is dependent on the characteristics of the seed surface. The roughness of the seed surface is one of the primary factors that affect the pathogen attachment to the seed. Cui et al. (2017) reported that a lower attachment had occurred on the alfalfa seeds compared to lettuce and tomato. This has basically resulted from the smooth surface structures of alfalfa seeds. The surface structures are also important for seed decontamination. If the surface has lots of crevices and wrinkles, it will get much harder to decontaminate the seeds from undesired pathogenic microorganisms.

Seeds may get contaminated as a result of inappropriately-handled manure fertilization or usage of contaminated irrigation water (Machado-Moreira et al., 2019). All the seeds are not produced for sprouting purposes. Thus, different hygiene practices might be employed for seeds that will be used for other purposes. With mass distribution all over the world, seed batches can be composed of seeds that are produced for different purposes. Thus, it sometimes gets quite hard to trace back the seeds when an outbreak or undesired event occurs. However, EC Regulation

208/2013 states that the seeds imported from non-EU countries should comply with the hygiene standards (European Commission (EC), 2013).

There are different decontamination techniques available for seeds: chemical, physical and biological (Machado-Moreira et al., 2019). Chemical interventions include the usage of organic acids, ozone treatment, chlorine and chlorine-based compounds, or cold atmospheric plasma. Physical decontamination methods are heat treatments, UV treatment and irradiation. As biological methods, the usage of bacteriocins and bacteriophage has been introduced. Although there are no certain criteria for seed decontamination, Produce Safety Rule of FSMA states that scientific methods should be followed, and usage of biocides on seeds should be limited according to the manufacturer's instructions in the US (FDA, 2019).

1.2.3 Soil

During growing, one of the contamination routes for fresh produce is soil. Manure and water due to sewage overflow or heavy rains might contaminate the soil (Zarkani et al., 2019). When soil that has been contaminated with manure and feces is used in the production of fresh produce, it has been reported that *E. coli* O157 and *Salmonella* spp. can persist in soil for some months (Eelco Franz et al., 2008). According to the findings of Chitarra et al. (2014), *E. coli* O157:H7 can persist in soils that are cultivated for lettuce, corn salad and rocket up to 100 days post-inoculation and the survival ability of *E. coli* O157:H7 decreases as the ambient temperature increases. In addition, they have shown that in basil-grown soils, *E. coli* O157:H7 survival could not be observed 60 days post-inoculation. Zarkani et al. (2019) also reported persistence of *S.* Typhimurium in soil up to 49 days with a level of 4 log CFU/g soil. Furthermore, they have also revealed that *Salmonella* can translocate from the rhizosphere to the phyllosphere of the plant and it has been shown that 13 to 4 % of tomato grown in contaminated soils was colonized by *S.* Typhimurium at 7 and 14 days post-inoculation of soil.

Colonization of plant tissues is dependent on the plant, environmental conditions and the genetical features of the pathogen. To colonize inside the plant tissue, the bacteria

present in soil should primarily attach to the root tissue. Then, chemotaxis takes place by bacteria towards the nutrients available, such as carbon sources, on the root exudates. After that, human enteric pathogens prefer to reside inside the apoplastic region where they can easily access nutrients so that they can outcompete the natural microflora of the plant. Through this apoplastic region, they can easily translocate through the phyllosphere or edible parts of the plant tissue via the vascular systems (Chitarra et al., 2014; Cooley et al., 2003; Gu et al., 2011).

1.2.4 Manure

Fertilizers are used as soil amendments to improve the nutritional value of the growth medium for fresh produce in both organic and conventional agricultural practices (Islam et al., 2004). The main constitutes of fertilizers are nitrogen (N), potassium (K) and phosphorus (P). To promote the growth of plant materials, they are largely utilized in the field. They can be mainly classified as commercial and organic fertilizers (Green, 2015). In short, the commercial ones are generally obtained from natural sources, and they are mostly designed to contain main nutrients such as N, P and K. In addition, their nutrient content is identified on the product. However, organic fertilizers, i.e., manure and compost, are obtained from animal feces or decomposed materials from animals and plants. The nutrient value of organic fertilizers may change with respect to the diet of the animal (Green, 2015). The other crucial point for the use of manure is that *Salmonella* and *E. coli* are shed through animal feces to the environment. Even healthy ruminants carry *E. coli* O157:H7 in their gastrointestinal tract and they do not show any symptoms of infection (Islam et al., 2005). Therefore, inappropriately or inadequately handled manure and compost could be harboring human enteric pathogens, and subsequently the use of contaminated manure could end up with contamination of fresh produce in the field.

The analysis for the prevalence of *E. coli* O157 in cattle pens in the US was found to be around 60 % in the time intervals between June and August in 2014 (Cull et al., 2017). In addition, the seasonal changes have an effect over the prevalence of *E. coli* O157:H7 in feces and it is reported that fecal shedding for *E. coli* O157:H7 is

increasing during summer times while it follows a decreasing trend in colder times of the year (Barkocy-Gallagher et al., 2003; Berends et al., 2008). The persistence and survival of enteric bacteria seem to favor the higher temperatures and they are more prone to replicate themselves in the processing facilities (Gautam et al., 2011).

It has been reported that human enteric pathogens can survive for a long time in soils containing contaminated manure. Islam et al. (2005) have shown that *E. coli* O157:H7 can survive in soil samples that are amended with contaminated manure. According to this study, *E. coli* O157:H7 was detectable in soil samples at 126 days post-inoculation with 1 log CFU/g microbial load, which is a quite crucial level for infection with *E. coli* O157:H7 due to being resistant to low pH of the stomach. Moreover, it has been shown that the human enteric bacteria inside manure have the ability to translocate towards edible parts of fresh produce. *E. coli* O157:H7 was still detectable in lettuce samples grown using contaminated manure at a level of 7 log CFU/g at 70 days post-inoculation with the pathogen concentration of around 1 log CFU/g.

In fact, there are a number of different treatments applied on manure to reduce the pathogen load in manure, which are chemical methods such as treating with hydrogen peroxide, physical methods which are pasteurization, drying and irradiation treatments and biological methods which are anaerobic storage (aging), composting and aerobic and anaerobic digestion (Manyi-Loh et al., 2016). However, it has been thoroughly established that the inappropriately processed manure usage could harbor substantial microbial load, and it could subsequently lead to important consequences contaminating the fresh produce (Eelco Franz & Van Bruggen, 2008).

1.2.5 Wildlife

Insects, pests, birds and mammals might shed pathogenic bacteria since they harbor pathogens in their intestines, lungs, feathers and skin. They might contaminate surface water and manure on land by fecal content (Liu et al., 2013; Riggio et al., 2019). In addition, birds and insects could harm the leaves. Pathogens require natural openings present on leaf tissue or damages that have occurred on leaf surface to

internalize inside the nutrient-rich regions of the plant tissue. Therefore, damage on the leaf surface could increase the entry regions for pathogens to internalize, and access to increased nutrients inside the plant tissue could enhance the persistence of pathogens (Orozco et al., 2008). However, although the risks that can be introduced by wildlife are known, it is quite tricky to predict the contamination through wildlife since their behavior in nature is arbitrary and is not known exactly right now (Liu et al., 2013). As a result, the precise effect of wildlife on leafy green contamination is not quite understood and it still requires to be discovered. However, since wildlife could harm the leaves, it might be beneficial to intervene in the relationship between wildlife and freshly consumed commodities by proper fencing and other strategies to prevent the contamination of leafy greens in the field.

1.2.6 Postharvest Contamination Routes

Transport, storage and processing conditions might lead to contamination of fresh produce. Poor hygiene practices followed by farmworkers could also lead to cross-contamination of fresh produce. Due to cross-contamination, the equipment used for harvesting could impair the microbiological quality of fresh produce in the field. It has been found that processing equipment used for the conveyor system has led to cross-contamination of melons, and nearly in 40 % of the cases, *E. coli* contamination has occurred due to this processing equipment in Texas (Castillo et al., 2004). Also, washing or fluming in a tray has resulted in cross-contamination of spinach and Romine lettuce with *Salmonella* and *E. coli* O157:H7 (Smolinski et al., 2018). Even after packaging, contamination could occur due to contaminated carrying equipment usage during storage (Castillo et al., 2004).

After distribution, contamination could occur at the household level or retail level. For ready to eat food and salads, fresh produce is not further processed. From storage to food preparation steps, washing and cutting, food can get contaminated. Thus, to prevent cross-contamination, regular hygiene practices should be strictly followed. Before handling the freshly consumed commodities, all the equipment, such as knives and cutting board, should be cleaned thoroughly. Raw meat products and

fresh produce should be stored separately. The storage conditions should be proper since the higher temperatures could enhance microbial growth. Temperatures between 5 and 60ºC is known as "Temperature Danger Zone" for food products and keeping fresh produce at this interval could result in enhanced growth of foodborne pathogens. Consequently, in farm to fork journey, hygiene practices should be strictly followed to prevent contamination with foodborne pathogens and foodborne illnesses (CDC, 2020b).

1.3 Climate Change

In recent years, climate change has become a global concern, and it poses a potential threat all over the world. According to the report published by Intergovernmental Panel on Climate Change (IPCC) in 2018, the ambient temperature exceeded the temperatures before Industrial Revolution by 1 °C in 2017 due to increased human activities (IPCC, 2018). If the rate of temperature increase continues at these levels, it is estimated that between 2030 and 2052, the ambient temperature will increase by 1.5 °C with respect to temperatures before Industrial Revolution. With this increase, it is expected that the challenges associated with health, water supply, food security and food safety will increase. In addition, the projected rise in temperature as a result of climate change is expected to affect the ability of survival, dissemination and persistence of bacteria, viruses and parasites in the environment. Consequently, the contamination of water and food could be affected by the changes in climate and it is predicted that the frequency of foodborne diseases might increase (WHO, 2019).

Tirado et al. (2010) reported that multiple climatic conditions might have an effect on food safety, which are temperature fluctuations, changes in precipitation patterns, cloudiness, extreme weather conditions, such as droughts, heavy rains and floods, and warming in oceans. Overflow or leaks from sewage and wastes from households, industrial regions and agricultural lands due to extreme weather events might result in the contamination of soil, water, food and fresh crops in the field with pathogens and chemicals. Besides affecting food safety directly, climate change could also impact food safety by indirect factors, such as crop production, animal and plant

health, feed health, aquaculture, processing, food handling and behavior at the household level.

Besides rainfall patterns, ocean warming and increased acidification related to climate change might affect not only chemical properties of water but also water microflora (Tirado et al., 2010). As a result, the pathogen survivability in water might be increased due to changes in the natural microflora of water.

Alterations in climate might affect the host, agent and environment or, in short, the epidemiologic triangle (Figure 1.5). The growth rate and survival of foodborne pathogens and contamination sources could be affected by climatic conditions. Most foodborne pathogenic bacteria favor temperatures above room temperature. Thus, an increase in temperature might enhance the microbial growth and proliferation in food and food-related stuffs in their journey from farm to fork. However, each region might not be affected by climate change at the same level. Geography, infrastructure, epidemiology of a disease, human behavior at the household and retail levels might affect whether each region and people are affected in the same way by the impacts of climate change. In fact, the changes in sources and pathways of contamination and projected prolonged persistence of pathogens in the environment might lead to the emergence of new zoonotic and bacterial vectors since the increased chances of interaction between microorganisms from different genus (CDC, 2012).

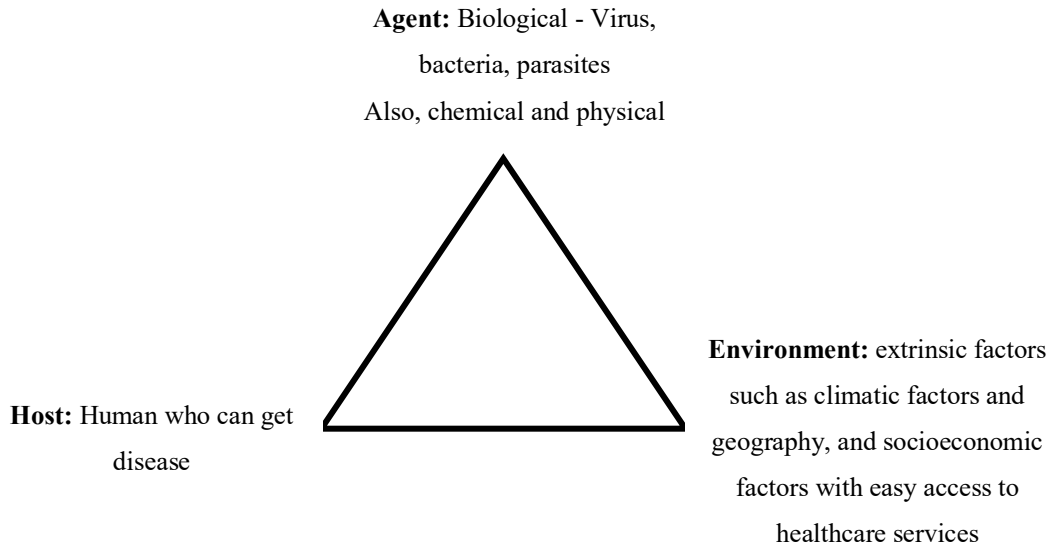


Figure 1.5 Epidemiological triangle and interactions among host, agent and environment

1.3.1 Climatic Factors

Foodborne pathogenic bacteria are dependent on environmental conditions. Therefore, it is estimated that climate change might have a strong influence over the survival and spread of foodborne pathogens. Temperature, seasonality effects, precipitation, drought and wind are the best-known effects that are resulted from climate change on foodborne pathogens. The following sections will be explaining the expected changes in each factor and the effects of them on the survivability and persistence of pathogens.

1.3.1.1 Temperature

Mean ambient temperature is predicted to rise in the coming centuries, and the magnitude of this increase will be dependent on the human activities which affect the emission levels. The emission practices adopted by people are expected to have a great impact over the elevated temperatures occurring due to climate change. If

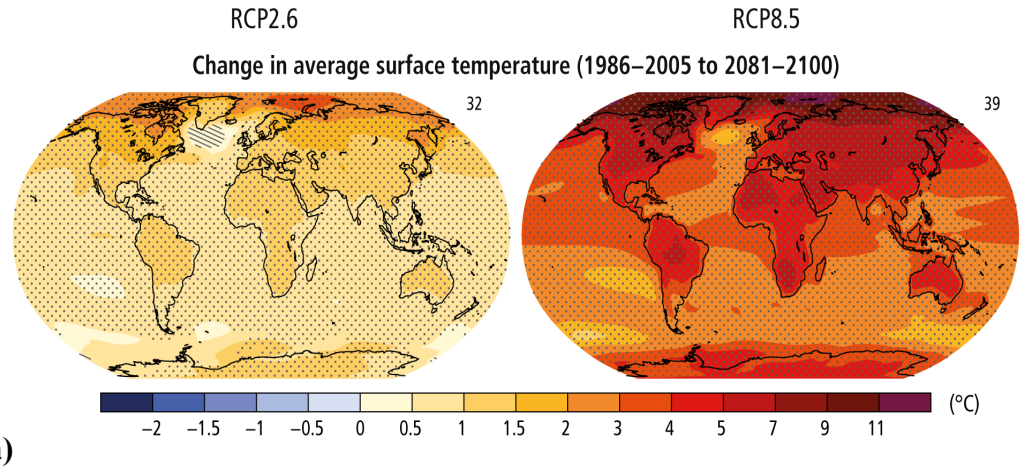
there is no major natural disaster, such as volcanic eruption, an increase by 0.3 to 0.7 °C is predicted in the global mean surface temperatures from 2016 to 2035 compared to time period 1986 and 2005 (IPCC, 2013).

The fifth Assessment Report published by Intergovernmental Panel on Climate Change (IPCC) in 2014 suggests four different representative concentration pathways (RCPs) to model the temperature elevation predictions over the greenhouse gas emissions and concentrations to put future climate scenarios forward (IPCC, 2014). These RCP models use the radiative forcing levels, which are RCP 2.6, 4.5, 6 and 8.5 for different mitigation scenarios. With these scenarios, it is projected that increase in the global mean surface temperature will increase between 0.4 and 2.6 °C in the mid-21st century and between 0.3 and 4.8 °C by the late 21st century. RCP 2.6 represents the strongest mitigation scenario, and if the radiative forcing levels are kept below 2.6 W/m^2, it is expected that the temperature rise will be in the range between 0.4 to 1.6 °C in the years between 2046 and 2065, keeping global warming below 2 °C. RCP 4.5 and 6 are intermediate scenarios with respect to RCP 2.6 and 8.5. RCP 8.5 is the scenario with the highest greenhouse gas emissions, which occurs at a level of 8.5 W/m^2. In a situation where the highest emissions occur, it is predicted that the global mean surface temperature will increase between 1.4 and 2.6 °C in the years between 2046 and 2065. The detailed predictions for RCP models that are adapted from IPCC Fifth Assessment Report (2014) are given in Table 1.6. In addition, Figure 1.6 summarizes the increase in the global mean surface temperatures over the years according to different mitigation scenarios.

Table 1.6 The estimated elevations in the global mean surface temperature (°C) with respect to different mitigation scenarios in the mid and late 21st century with respect to the period between 1986 and 2005 (Table was adapted from IPCC report in 2014)

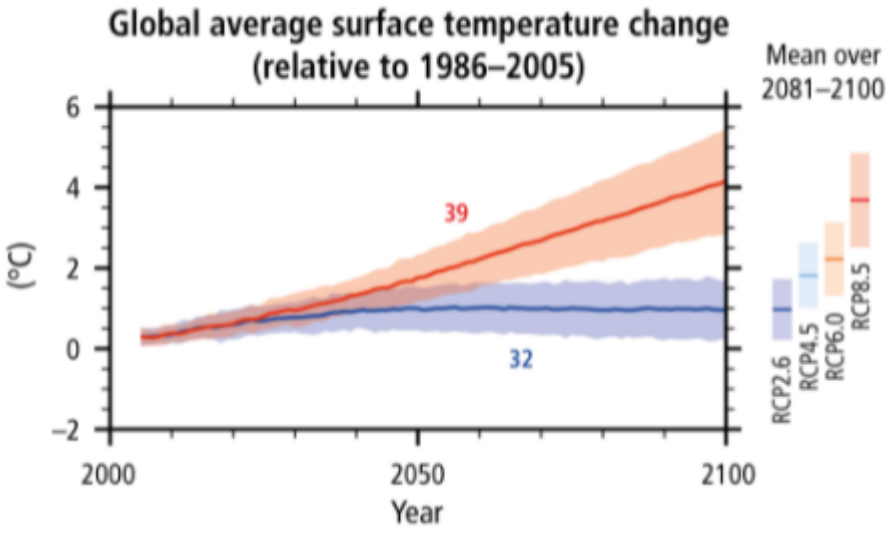
Mitigation Scenario*	2046-2065		2081-2100	
	Mean	Range	Mean	Range
RCP2.6	1.0	0.4 to 1.6	1.0	0.3 to 1.7
RCP4.5	1.4	0.9 to 2.0	1.8	1.1 to 2.6
RCP6.0	1.3	0.8 to 1.8	2.2	1.4 to 3.1
RCP8.5	2.0	1.4 to 2.6	3.7	2.6 to 4.8

*RCP: Representative Concentration Pathways, according to greenhouse gas emissions (W/m^2)



(a)

Continued on next page



(b)

Figure 1.6 Estimations of changes in global mean surface temperatures developed according to average of model studies available. (a) shows the changes in global mean surface temperature for the years between 2081 and 2100 (late 21st century) with respect to temperatures measured between 1986 and 2005. The left panel shows the temperature elevations according to the strongest mitigation scenario (RCP 2.6), and the right panel shows according to scenario where the highest emissions occur (RCP 8.5). The number of models used to calculate the means is 39 and 32 for RCP 8.5 and RCP 2.6, respectively. (b) shows the changes in global mean surface temperature per year for the years between 2006 and 2100 with respect to temperatures measured between 1986 and 2005. Solid lines represent the mean value obtained from multiple-model estimations, while shadings represent the 5 to 95 % range across the distribution. Blue and red represent RCP 2.6 and 8.5, respectively. The number of models used to calculate the means is 39 and 32 for RCP 8.5 and RCP 2.6, respectively. The mean and distributions for 2081 and 2100 for all RCP scenarios are in the right of the panel (IPCC, 2014).

These increases in temperature show that the number of extreme hot events might increase in land areas. Since the favored growing temperature for most foodborne pathogens is 37 °C, the increase in temperature might result in the enhanced growth rate of microorganisms leading to increased survival and persistence in the

environment. However, studies reported that the lower temperatures might allow some foodborne bacteria, namely *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes* and *Y. enterocolitica,* with increased survival in soil samples (García et al., 2010; Strawn et al., 2013). At lower temperatures, the metabolism of microflora in soil might slow down, which could lead to increased survival of foodborne bacteria (Fremaux et al., 2008).

Seasonality and weather conditions can affect the spread and prevalence of foodborne pathogens. The seasonality effects on STEC prevalence and shedding have been studied extensively. Depending on many other factors apart from temperature, in most of the studies, it is found that the prevalence and shedding of STEC have increased in warmer times of the year. However, studies analyzing the effects of seasonality on the contamination of cattle carcasses and hides produced inconsistent results, some reporting positive correlation (Barkocy-Gallagher et al., 2003; Rivera-Betancourt et al., 2004), while some did not show any seasonal effect (Brichta-Harhay et al., 2008). According to Liu et al. (2013), experienced higher temperatures could make livestock more vulnerable to animal diseases making them asymptomatic carriers of human enteric pathogens. In the end, the shedding rates for foodborne pathogens might increase without notice.

There are many studies reporting lower survival rates that have been observed for foodborne pathogens, *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes* and Shiga toxin producing *E. coli* in feces and manure-amended soils at elevated temperatures (Arrus et al., 2006; Fremaux et al., 2008; Mannion et al., 2007; Moriarty et al., 2011; Oliveira et al., 2012). However, it is also worth mentioning that the genetic differences between serotypes for the same species could lead to different survival levels. At 5 °C, the survival of *E. coli* O157:H7 was superior to those of *E. coli* O11:H- and O26:H11 in animal feces in a 4-week period (Fukushima et al., 1999). In addition, while decreased temperatures might have protective effects, fluctuations in temperature as well as frequent freeze-thaw events could have negative effects on the survival of *E. coli* and *Salmonella* spp. in manure-amended soils (Semenov et al., 2007). Moreover, at higher temperatures, nutrients in the soil get depleted at a higher rate due to elevated biological soil activity, which leads to

frequent usage of manure to fertilize the crops in the field (Franz, Semenov, & Van Bruggen, 2008). Therefore, the chances of using inappropriately handled manure might rise, which then increases the potential contamination of the crop.

Kim et al. (2015) have studied the relationship between foodborne diseases and temperature and relative humidity for eight different foodborne pathogens from 2003 to 2012 in South Korea. It was shown that the pathogenic *E. coli* related foodborne diseases are strongly linked to temperature and relative humidity, and it was also revealed that the strongest relationship with these climatic conditions had been observed for pathogenic *E. coli* among eight foodborne pathogens which are *Vibrio parahaemolyticus, Campylobacter jejuni, Salmonella* spp., *Bacillus cereus, Staphylococcus aureus, Clostridium perfingens* and Norovirus. They have found that higher foodborne incidences were observed in months when higher temperatures were experienced. The strongest relationship with temperature was found for pathogenic *E. coli* among eight foodborne pathogens.

The surface water temperatures are also expected to elevate (IPCC, 2013). While a minor increase in temperature might enhance microbial growth in water especially in nutrient-rich sources, higher temperatures may show inhibitory effects for some pathogens (Hofstra, 2011). It has been shown that higher survivability was observed for STEC and generic *E. coli* at 4 to 8 °C compared to 21 to 25 °C.

Due to climate change, it is also predicted to have increases in sea surface temperatures (IPCC, 2013; United States Environmental Protection Agency (EPA), 2021). It is expected that ocean surface temperatures (the top 100 m) might get warm in a range of 0.6 to 2.0 °C (IPCC, 2013). Since the presence of *Vibrio* spp. is associated with higher temperatures, an increase in sea surface temperatures is getting more concerning since it could affect the number of outbreaks related to *V. parahaemolyticus* and *V. cholerae* (Gil et al., 2004).

Consequently, the temperature increase attributed to changes in climate will have different impacts over the foodborne pathogens in terms of their survivability, growth and persistence in the environment. It is expected to have decreased survival of foodborne pathogens in soil, manure and waters with increased temperatures in

mid-latitude regions (Hellberg & Chu, 2016). However, the different geographic areas may experience things a bit differently.

1.3.1.2 Precipitation

By the end of the 21st century, it is expected to have increased heavy precipitation events with higher intensity and occurrence rates in the wet tropical regions and mid-latitude wet lands (23°26'22" and 66°33'39" north, and 23°26'22" and 66°33'39" south; for example, the USA and most of Europe) (IPCC, 2013). As seen in Figure 1.7, in mid-latitude regions, the precipitation levels are projected to increase by the late 21st century. The spread of *E. coli* O157:H7, *Salmonella* spp. and *Campylobacter* spp. is affected by the relative humidity (Dorner et al., 2006).

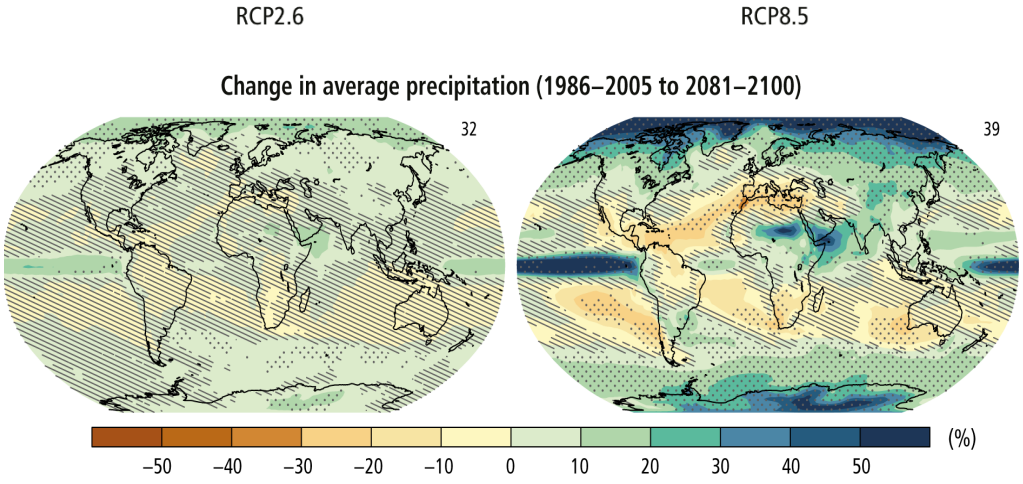


Figure 1.7 Estimations of changes in mean precipitation developed according to average of model studies available. The figure shows the changes in global mean surface mean precipitation for the years between 2081 and 2100 (late 21st century) with respect to precipitation levels measured between 1986 and 2005. The left panel shows the temperature elevations according to the strongest mitigation scenario (RCP 2.6), and the right panel shows according to scenario where the highest emissions occur (RCP 8.5). The number of models used to calculate the means is 39 and 32 for RCP 8.5 and RCP 2.6, respectively (IPCC, 2014).

Kim et al. (2015) have studied the relationship between foodborne diseases and temperature and relative humidity for eight different foodborne pathogens from 2003 to 2012 in South Korea. They have found that higher foodborne incidences were observed in months when higher relative humidity levels were experienced. The strongest relationship with precipitation was found for pathogenic *E. coli* among eight foodborne pathogens, which are *E. coli, Vibrio parahaemolyticus, Campylobacter jejuni, Salmonella* spp., *Bacillus cereus, Staphylococcus aureus, Clostridium perfingens* and Norovirus.

Contaminated water sources might be one of the pathways for the dispersion of pathogens to the environment and food since they can harbor pathogens for longer times (Liu et al., 2013). Extreme weather conditions, such as heavy rains and flooding, could lead to leaks and dissemination of pathogens to the agricultural lands, and this phenomenon is expected to increase with changing weather conditions. In addition, along with the extreme weather conditions, sewage overflows could happen due to sewer overwhelm (Hofstra, 2011). An increase in the fecal indicator organisms; namely fecal coliforms and generic *E. coli*; has been observed after rainfall events, which shows the probable overflows of sewages.

Cooley et al. (2007) reported that the levels of both generic *E. coli* and *E. coli* O157:H7 have increased in the agricultural fields in the months when the flooding events take place. This is most likely due to runoffs, leakages and drainages caused by heavy rains and floods. Likely, Rechenburg and Kistemann (2009) also revealed that leakages from sewage overflows lead to a high presence of fecal indicator organisms in a watershed in Germany. In addition, the contaminated runoffs from agricultural fields could be the reason why pathogens are detected after heavy rainfalls. There are studies reporting the presence of *E. coli* O157:H7 and other fecal indicator microorganisms in the agricultural runoff, which is filtered water from agricultural fields because of irrigation and rain. Possible dangers related to contaminated agricultural runoffs are cross-contamination of soil and subsequently the drinking water sources. However, in most of the times, the pathogen related burdens caused by heavy rains are associated with insufficient infrastructures (Miraglia et al., 2009).

The water content of the soil is an important factor for the survival of pathogens such as *E. coli, Salmonella* spp. and *L. monocytogenes.* With heavy rains, it is considered that the water availability of soil will increase due to climate change and could increase the survivability of foodborne pathogens. In addition, it is worth pointing out that *E. coli* and *Salmonella* could have higher resistance towards dry conditions. Chandler and Craven (1980) once showed that after residing in dry soil for 14 days, *E. coli* and *Salmonella* were capable of regrowth after application of sterile water to the soil.

Ge et al. (2012) have revealed that the internalization rate of *Salmonella* has increased in lettuce during heavy rain events since the pathogen concentration has increased in the soil. Moreover, the higher internalization rate of *Salmonella* was observed in lettuce under drought conditions. Therefore, the higher internalization rates in fruits and vegetables are associated with extreme weather events, in this case heavy rains and drought, which are expected to increase in the upcoming years. Furthermore, as it has been shown previously, splash events due to heavy rains are also an important contamination pathway. It is expected that the splashing of manure or soil to the leaves of crops in the field could enhance the bacterial attachment (Cevallos-Cevallos, Danyluk, Gu, Vallad, & Van Bruggen, 2012; Franz & Van Bruggen, 2008).

1.3.1.3 Drought

Although increased mean precipitation rates are expected in some regions of the world, it is expected to have decreases in the precipitation levels in most of the mid-latitude and subtropical dry lands (for example, North Africa, interior parts of North America and Eurasia) (IPCC, 2013). Dramatic decreases in the rainfall levels could end up with drought, and this could eventually change the chemical properties of soil and water systems. Since these could affect the pH and available nutrients, that can have altered effects on the growth and dispersal of foodborne pathogens. Biodiversity or the natural flora in the environment could be affected by drought events (Kohler et al., 2010). In fact, losses in biodiversity are expected, which might

lead to the growth and increased survival of pathogenic microorganisms in the environment since there are no longer competing organisms around. Van Elsas et al. (2011) showed that better survival of *E. coli* O157:H7 was obtained in soils with less biodiversity.

Yet, for their growth, microorganisms are dependent on the presence of water. In the absence of water, the salinity of water and soil systems increases (Froelich et al., 2012; J. Ma et al., 2012). It is found that the increases in the soil salinity affect *E. coli* O157:H7 survival in a negative way (J. Ma et al., 2012). However, the differences in the genome could result in different survival rates. Gutiérrez-Rodríguez et al. (2011) showed that the survival of generic *E. coli* and *E. coli* O157:H7 has differed in soils with low water content, and *E. coli* O157:H7 showed a lower survival compared to generic *E. coli*.

1.3.1.4 Wind and Dust

In some regions of the world, it is expected to have increased cyclones, in other words hurricanes and typhoons. In the next 50 years, it is estimated that the wind speed would increase by 0.6-12 % along with the increase in the global average sea surface temperature (IPCC, 2014). The spread of pathogens could be enhanced by tropical cyclones. The analysis of air samples collected before, during and after hurricanes revealed that the microbial content has dramatically changed after the hurricane in the Caribbean Sea and bacterial cells have been detected in the aerosolized air particles (DeLeon-Rodriguez et al., 2013). Thus, it is possible that the occurrence of aerosolization and spread of microorganisms could increase along with the increased cyclones. In the end, microorganisms could be transported by extreme wind events to the water sources and agricultural fields, where they can contaminate the plants and crops in the field.

Furthermore, desertification is also a concerning outcome of climate change. Dust activity could be enhanced by desertification. With cyclones, it is possible for the soil to be lifted and carried away long distances (Griffin, 2007). Besides soil;

bacteria, fungi and viruses could be transported and contaminate the water sources and agricultural fields.

The main expected changes in each climatic factor due to climate change was summarized in Figure 1.8.

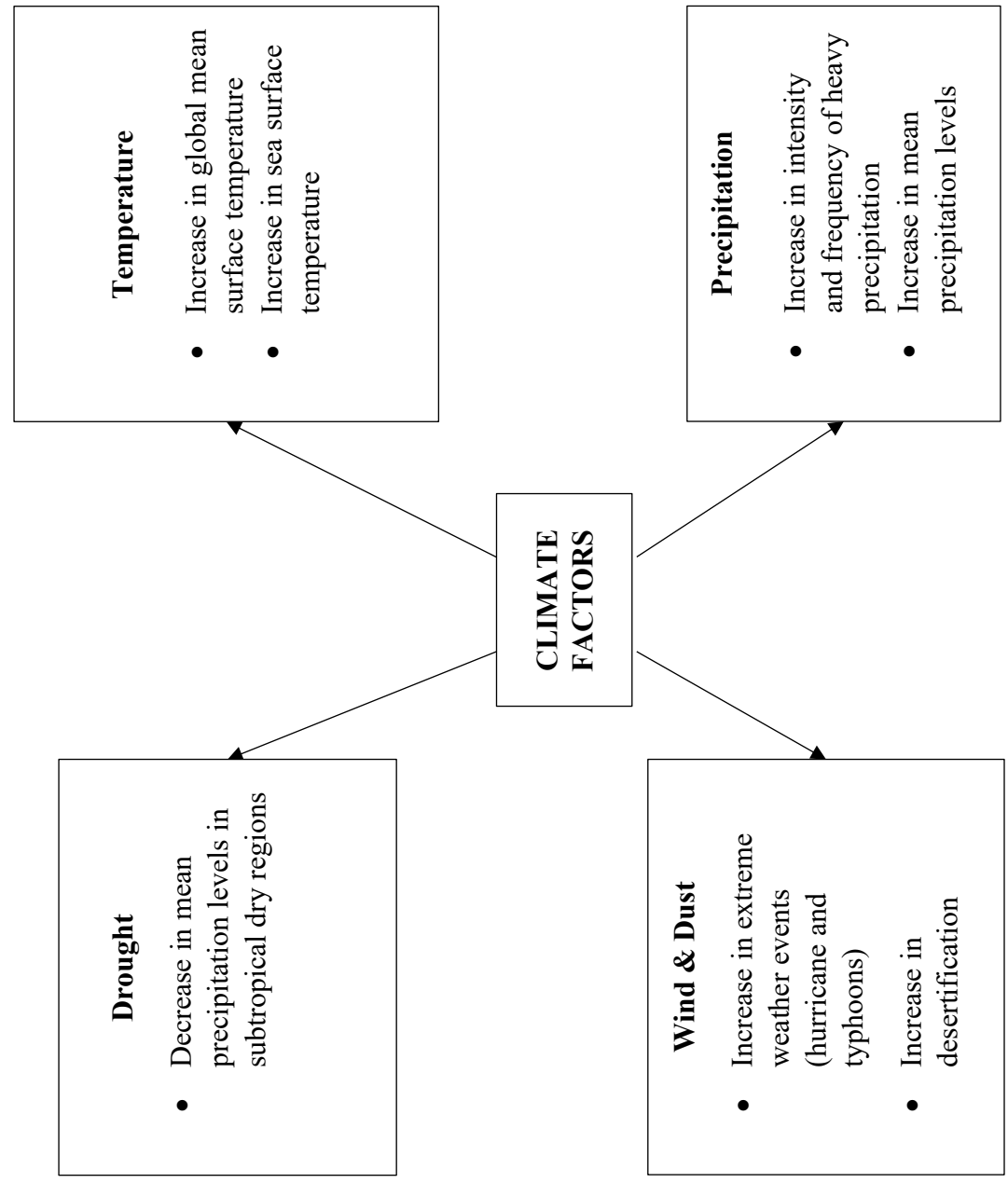


Figure 1.8 Expected changes in each climatic factor due to climate change

1.4 Biofilm Structures and Detection Methods

Leaves are not recognized as the natural host for the bacterial foodborne pathogens, such as *E. coli* and *Salmonella* spp. Therefore, bacterial pathogens should either internalize inside the plant tissue or form biofilm on the leaf surface to increase their odds of survival on harsh leaf tissue. Biofilms are defined as structures that exhibit a phenotype that changes with growth rate and genetic expression as a result of reversible attachment of bacterial populations to a surface and entrapped in the extracellular polymeric matrix (EPS) that bacteria produce. Since biofilms bring microorganisms of different classes such as viruses and bacteria together, it provides a suitable environment where horizontal gene transfer can take place. Therefore, the microorganisms in the biofilm structure get highly resistant to sanitation practices and pose a great risk especially for freshly consumed vegetables. Nearly 90 % of the biofilm structure on dry mass is composed of its matrix, i.e., the EPS, while the rest contains the microorganisms (Flemming & Wingender, 2010). Biofilm matrix protects the microorganisms entrapped against desiccation, oxidizing, biocides, antibiotics, UV radiations and most importantly host immune defenses. Therefore, biofilm formation is a great protection mechanism for bacteria on the leaf surface, which is harsh environment for bacteria presenting UV radiation and water scarcity (Melotto et al., 2008). In addition, the EPS content in biofilms may vary substantially among biofilms depending on microorganisms present, shear forces experienced, temperature and nutrient availability (Flemming & Wingender, 2010).

There are many biofilm detection methods. Congo-red agar assay, tissue culture plate assay, tube assay, electron microscopy and confocal scanning microscopy are used to detect biofilms phenotypically. However, with the help of Scanning Electron Microscopy (SEM) and other microscopic methods, the shape, size and location of the biofilms could be detected. Also, it is possible to analyze the biofilm formation steps, bacterial interactions and EPS production (Vuotto & Donelli, 2014). Therefore, using electron microscopy could help the detection of where the biofilm structures are localized on leaf tissue. Natural openings on the plant tissue, such as stomata, nectarthodes, lenticels and hydathodes, are perfect entering routes for the

enteric pathogens into plant tissue. With their high number on the leaf, stomata are the preferred routes for bacterial internalization (Melotto et al., 2008). Therefore, a SEM visualization could also supply information on whether the bacterial communities are located near stomata or not.

1.5 Objectives of the Study

In recent years, there has been an increase in the consumption of fresh vegetables due to changing dietary preferences. However, freshly consumed vegetables have become one of the common sources for foodborne outbreaks since they are not processed or minimally processed prior to consumption. Fresh produce may get contaminated at any stage from their farm to fork journey. Many factors, such as irrigation water, seeds, soil, manure, and wildlife, could lead to the contamination of freshly consumed vegetables in the field if they are inappropriately handled or contaminated due to sewage run-offs. Among many foodborne bacteria, pathogenic *Escherichia coli* is one of the agents causing devastating outbreaks, such as fenugreek sprout-related outbreak that occurred in Germany in 2011 and affected more than 3800 people with 50 deaths.

Climate change and its effects all over the world are undeniable with the increase of greenhouse gases in the atmosphere. Many climatic factors, such as temperature, changes in precipitation pattern, drought and wind and dust, are known to have effects on the bacterial survival, prevalence, and persistence in the environment. Therefore, climate change could have impacts on food safety.

There are many studies that analyzed the interaction between foodborne pathogens and fresh produce reporting the persistence of pathogenic *E. coli* in soil, seeds, irrigation water and on fresh produce itself. However, there is no research related to analysis on the effect of temperature rise due to climate change to the best of our knowledge. Therefore, this study investigates the survival of *E. coli* pathogroups on cress leaves that are grown at different temperatures to mimic the temperature rise designed according to different mitigation scenarios proposed by Intergovernmental Panel on Climate Change (IPCC) in 2014. To accomplish this, Shiga toxin producing

E. coli serovars (EHEC O157:H7 and EAEC O104:H4) that have caused many fresh produce related outbreaks and an additional EPEC strain (EPEC O26), mostly contaminating the irrigation water in underdeveloped countries were used to contaminate the cress seeds and leaves at different stages of cress growth to mimic how contamination time and route affect the pathogen load on fresh produce. At the end of each growing period of cress plants, the leaves were analyzed for the ability of *E. coli* strains used to attach and biofilm-forming capabilities on leaves. Also, Scanning Electron Microscope visuals were analyzed to locate the used bacterial strains on the leaves.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Isolates

The bacterial isolates used in this study are Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, Enteroaggregative *E. coli* (EAEC) serotype O104:H4 and Enteropathogenic *E. coli* (EPEC) serotype O26 with METU IDs MET-K1-30, MET-A1-80 and MET-A1-90, respectively. They represent three pathogroups of *E. coli*. *E. coli* O104:H4 and O26 were kindly provided by Public Health Agency of Turkey. *E. coli* O26 was isolated from water samples, and *E. coli* O104:H4 is a representative of outbreak strain that was isolated from leafy green related outbreak in Germany in 2011. *E. coli* O157:H7 was kindly provided by Ankara University Food Engineering Department, and it was isolated from a food source. All strains used in this study were described in Table 2.1.

Table 2.1 *Escherichia coli* strains used in this study

ID Codes	Pathogroup	Serotype	Source	Antimicrobial Resistance Profile*
MET-K1-30	EHEC	O157:H7	Food - Ankara University	Susceptible
MET-A1-80	EAEC	O104:H4	Public Health Agency of Turkey	TeNaSxtSAmp
MET-A1-90	EPEC	O26	Water - Public Health Agency of Turkey	Susceptible

* Te: Tetracycline, Na: Nalidixic acid, Sxt: Trimethoprim-Sulfamethoxazole S: Streptomycin, Amp: Ampicillin

2.1.2 Plant material

Due to its easy growth in a short period of time within 30 days, garden cress (*Lepidium sativum* Linn.; Zenitt Tohumculuk, Balıkesir, Turkey) was selected as model organism to represent freshly consumed vegetables in this study. The same batch of garden cress seeds were used for all experimental set ups to prevent any differences that could be resulted from seeds. The seeds were supplied as fungicide-treated form to prevent the growth of molds during storage.

2.1.3 Chemicals Used in This Study

The chemicals and their suppliers used in this study were listed in Table B.3 in Appendix B.

2.1.4 Preparation of Growth Mediums, Solutions and Chemicals

The preparation of growth mediums, solutions and chemicals were given in Appendix C.

2.2 Methods

2.2.1 Model Selection for Different Climate Change Scenarios

To determine the climate change scenarios, the Intergovernmental Panel on Climate Change (IPCC) report that was published in 2014 was taken into consideration (IPCC, 2014). In the report, four different mitigation scenarios, which are representative concentration pathways (RCPs), were introduced according to greenhouse gas emission levels to the atmosphere to predict the temperature rise situations in the future. It was proposed that the strongest mitigation scenario, which is RCP2.6, was expected to keep the global mean surface temperature between 0.3 and 1.7 ºC by the late 21st century. Moreover, the strongest mitigation scenario (RCP2.6) is expected to keep global warming below 2 ºC. On the other hand, if the

emission levels occur at the highest estimated values (RCP8.5), the projected increase in the global mean surface temperature is between 2.6 and 4.8 ºC for the same time interval. The detailed estimated values for the temperature rise situations at different RCPs for two time-intervals (2046-2065 and 2081-2100) were given in Table 1.6. In the light of these, 3 different climate change scenarios were determined to analyze the impact of an increase in global surface temperature on the bacterial attachment to the cress leaves. The optimum growing temperature for cress plant was determined as 15 ºC (Rana & Kamboj, 2018), while +2, +4 and +6 ºC rise in optimum growing temperature was selected as three climate change scenarios to mimic the temperature rise situations according to proposed RCPs. To have a better understanding how temperature rise affects bacterial attachment on leaf surface, temperatures 15, 15+2, 15+4 and 15+6 ºC have been simulated in climate chamber under controlled conditions.

2.2.2 Preparation of Inoculum for Inoculation Scenarios

Enterohemorrhagic *Escherichia coli* serotype O157:H7, Enteroaggregative *E. coli* serotype O104:H4 and Enteropathogenic *E. coli* serotype O26 strains were stored in glycerol stock at -80 ºC in Food Safety Laboratory Isolate Bank at Middle East Technical University Food Engineering Department. To prepare pre-cultures, the isolates were separately streaked onto Brain Heart Infusion (BHI) agar from glycerol stock and incubated overnight (about 18 h) at 37 ºC. Fresh colonies from BHI agar were taken and they were suspended in 100 ml BHI broth and incubated with shaking at 37 ºC for 8 h to obtain the cells in their early stationary growth phase. Then, to harvest bacterial cells, the incubated BHI broths were centrifuged at 3000 × g for 15 min at 4 ºC (Hartmann et al., 2017). The supernatant was removed, and the harvested bacterial cells were then re-suspended in 0.85 % saline (NaCl) solution. The bacterial concentration was determined according to optical density at 600 nm. The bacterial concentration to inoculate the plants were 10^8 MPN/ml. Therefore, the optical density of the inoculum was adjusted to a final concentration of 0.1 at OD_{600}, which corresponds to 10^8 MPN/ml.

2.2.3 Growth Curve Analysis

2.2.3.1 Growth Curves According to Optical Densities

To assess the in vitro growing of the bacterial isolates that were used in this study, their growth curves were obtained at climate change scenarios studied. The pre-cultures were grown in 20 ml BHI as described in Section 2.2.2. However, the harvested bacteria after centrifugation were suspended in 10 mM $MgCl_2$ solution by vigorous shaking and optical density was recorded in $MgCl_2$. Then, this suspension was used to inoculate the 20 ml BHI broth to OD_{600} of 0.001, which corresponds to 10^6 CFU/ml. 200 µl of the inoculum was subsequently transferred to 96 well plates with four technical replicates. Then, the plate was incubated at climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) and 37 ºC, which is the optimum growth temperature for *E. coli*, in BHI broth for 24 hours with shaking prior to measurement. The measurements were taken with 5-minute intervals for 24 h in ELISA reader (ThermoFisher MultiSkan). Uninoculated BHI broth was used as blank. For the calculation, the average of four replicates was taken, and the OD values of blank was subtracted from the average. Then, the data were fitted to logistic equation which describes the bacterial growth to obtain the doubling time and growth rate of the bacterial strains used in this study, and data were visualized. For this purpose, the Growthcurver package of R was used (Sprouffske & Wagner, 2016).

2.2.3.2 Growth Curves According to Colony Counts

The number of cells for the growth curve analysis was only determined for the growth at 37 ºC. The pre-cultures were grown in 20 ml BHI as described in Section 2.2.2. However, the harvested bacteria after centrifugation were suspended in 10 mM $MgCl_2$ solution by vigorous shaking and optical density was recorded in $MgCl_2$. Then, the 20 ml BHI broth was inoculated with the suspension to an OD_{600} level of 0.00001 (10^{-5}), which corresponds to 10^4 CFU/ml, in a two-step procedure. Inoculated BHI broths were subsequently left for incubation at 37 ºC for a 24-hour period with shaking at 150 rpm. The samples were collected at 0, 2, 4, 6, 8, 10, 22

and 24 h time points. At each sampling point, the samples were serially diluted by 10-fold in 180 µl of 10 mM $MgCl_2$ in 96 well plates. Afterwards, the 10 µl of the dilutions were dropped on selective medium for *E. coli*, which is brilliant-green phenol-red lactose sucrose (BPLS) agar. After an overnight incubation of the plates at 37 ºC, the colony forming units (CFU) were counted. As control, uninoculated BHI broth was used, and no growth were observed in the control samples. For the counting part, four technical replicates were used for each bacterial strain.

2.2.4 Plate Assay for Biofilm Formation

Prior to analyses, the change in biofilm forming abilities of *E. coli* O157:H7, O104:H4 and O26 on polystyrene 96-well plates have been assessed for 24, 48, 72 and 96 h at minimum and maximum climate change scenarios (15 and 15+6 ºC). To prepare the pre-cultures for the biofilm assays, *E. coli* O157:H7, O104:H4 and O26 were firstly streaked to Luria-Bertani (LB) agar and then incubated at 37 ºC for 18 h. Later on, the single colony from LB agar was transferred to and incubated in 5 ml LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl – 1000 ml) overnight at 37 ºC with continuous shaking. The active cultures were diluted to optical density of 0.2 at 550 nm, and 30 µl of this suspension were transferred to 96 well plates containing 100 µl LB in each well. The plates were incubated at 15 and 15+6 ºC for 24, 48, 72 and 96 h under static conditions. After each incubation time, wells were washed with 0.85 % NaCl 3 times to remove the planktonic, i.e., unattached cells, and they were dried under room temperature. Then, the wells were fixed with 130 µl of 95 % methanol for 15 minutes. Afterwards, the methanol was discarded, and 130 µl of 0.1 % crystal violet was added to wells, and the plates were kept under room temperature for 30 minutes to stain the biofilm forming cells. The plates were then washed by dipping them into sterile distilled water 2 times to remove the stain that was not adhered to the biofilm structure. A mixture of ethanol and acetone was used to dissolve the crystal violet stain that was adhered to biofilm structures. To do so, 130 µl of 80/20 (v/v) ethanol/acetone mixture was transferred to the wells. After an incubation of 45 minutes under room temperature, the optical density of crystal violet dye was measured at OD_{595} in ELISA reader (ThermoFisher MultiSkan). As control,

only LB containing wells were used. Results were reported as the mean of four technical replicates ± standard deviation by subtracting the OD values of control from those of samples. The ability to form biofilms were classified as Stepanović et al. (2000) suggested:

- Non-adherent: $OD \leq OD_c$
- Weakly adherent: $OD_c < OD \leq 2 \times OD_c$
- Moderately adherent: $2 \times OD_c < OD \leq 4 \times OD_c$
- Strongly adherent: $4 \times OD_c < OD$.

where OD_c is the cut-off OD value which is three standard deviations above the average OD values of the negative controls.

2.2.5 Cultivation of Plant Material

2.2.5.1 Chemical Surface Sterilization of Seeds

The garden cress (*Lepidium sativum* Linn.) seeds were rinsed with municipal water prior to use to drain off the fungicide on them. Afterwards, to prevent the microbial growth and competition due to microbiota of the seeds, the seeds were surface sterilized by dipping them into 12 % bleach solution (Domestos, Unilever) for 12 minutes and then they were vigorously rinsed with sterile distilled water three times. To prevent the microbial competition, all of the materials and equipment, which are peat, irrigation water, and pots, were autoclaved prior to use.

2.2.5.2 Seed Sowing

For the planting, one gram of surface sterilized cress seeds was mixed with autoclaved commercial peat to disperse the seeds homogenously to pot. Then, the 20.1 cm × 4.9 cm aluminum pots were filled with autoclaved commercial peat, and the mixture of peat and seeds were spread on the peat inside aluminum pots. Afterwards, mixture of peat and seed was covered by peat to sow all the seeds under

the peat. Subsequently, the pots were irrigated with 60 ml of sterile distilled water, and they were placed in the climate chamber until they are harvested.

2.2.5.3 Growth and Germination Conditions

Cress seeds were germinated and grown under controlled conditions in climate chamber at 65 % relative humidity (RH) with a light density of 162 µmol m^{-2} sec^{-1} with a 16-hour photoperiod for 30 days. For germination, the temperature inside the chamber was set differently for each inoculation group since the bacteria were introduced at different stages of growth. Thus, not to intervene with the bacterial attachment, germination was completed at different temperature setups for different groups. The plants used for control and plants that were inoculated with contaminated irrigation water at different stages were germinated at 20 ºC for 7 days to maximize the germination capacity, whereas the seed inoculation groups were germinated at designed climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) not to change the attachment levels of bacteria to the seeds and transfer of them to the leaf tissue at elevated temperatures. Upon completion of the germination 7 days post sowing (dps), the temperature was set as the designed climate change scenarios for control groups and plants inoculated with contaminated irrigation.

2.2.5.4 Irrigation Scheme

Three main groups were used for each *E. coli* serotype and climate change scenario in this study to analyze the effect of climate change on the bacterial attachment, which are control groups, contaminated irrigation groups and seed contamination groups. Control groups were only irrigated with sterile distilled water. Contaminated irrigation groups were inoculated at 7, 14, 21 and 28 dps by irrigation, whereas seed contamination groups were only inoculated at the time of sowing. According to the detailed inoculation scheme, the plants were irrigated with 60 ml of sterile distilled water at least twice a week except the days when contaminated irrigation takes place. The detailed irrigation scheme describing the growth and germination conditions as well as inoculation regimes were given in Table 2.2.

Table 2.2 The detailed irrigation plan for plants used as control, contaminated by spray irrigation at 7, 14, 21 and 28 days post-planting (dps) and grown using contaminated seeds. For each climate change scenario (15, 15+2, 15+4 and 15+6 ºC), all these groups were cultivated with 3 biological replicates for 3 *E. coli* (O157:H7, O104:H4 and O26) strains studied, and they were grown in climate chamber under controlled conditions.

				Contaminated Irrigation on Leaf by Spraying			
		Control	**Translocation of Bacteria from Seed to Leaf**	**7 dps**	**14 dps**	**21 dps**	**28 dps**
Germination	**0 dps**	Sowing	Sowing (with **Contaminated Seed**)	Sowing	Sowing	Sowing	Sowing
	3 & 5 dps	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation
	7 dps	Sterile Irrigation	Sterile Irrigation	**Contaminated Irrigation**	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation
Growth	**10 & 12 dps**	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation
	14 dps	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	**Contaminated Irrigation**	Sterile Irrigation	Sterile Irrigation
	17 & 19 dps	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation
	21 dps	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	**Contaminated Irrigation**	Sterile Irrigation
	24 & 26 dps	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation
	28 dps	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	Sterile Irrigation	**Contaminated Irrigation**
	30 dps	Harvest	Harvest	Harvest	Harvest	Harvest	Harvest

2.2.6 *Escherichia coli* Inoculation to Plant Material

2.2.6.1 Contaminated Irrigation on Leaf

The cress plants, germinated at 20 ºC and grown at 15, 15+2, 15+4 and 15+6 ºC at 65 % RH, were artificially contaminated with *E. coli* O157:H7, O104:H4 and O26 at different stages of growth separately by spray inoculation over their leaves to investigate the effect of increase in temperature on the bacterial attachment to the leaves. Four different irrigation groups were used for this purpose: at 7, 14, 21 and 28 days post sowing (dps) the plant materials were inoculated separately by spray irrigation for each climate change scenario and *E. coli* serotype. The only inoculation method for these groups was irrigation. Chemically surface sterilized seeds as well as autoclaved peat and aluminum pots were used to cultivate these groups, and the procedures described in Section 2.2.5.2 were followed for the cultivation. *E. coli* O157:H7, O104:H4 and O26 pre-cultures were prepared, and their optical densities were adjusted to 0.1 in 0.85 % saline solution at 600 nm as stated in Section 2.2.2. Until inoculation, the prepared suspension was kept in ice bath in all steps. At 7, 14, 21 and 28 dps, each plant in each group was spray-irrigated with 50 ml bacterial suspension at a level of 10^8 MPN/ml. As a control, the uninoculated plants, i.e., plants that were irrigated with sterile distilled water in all growth stages were used. The detailed experimental plan for contamination is available in Figure 2.1.

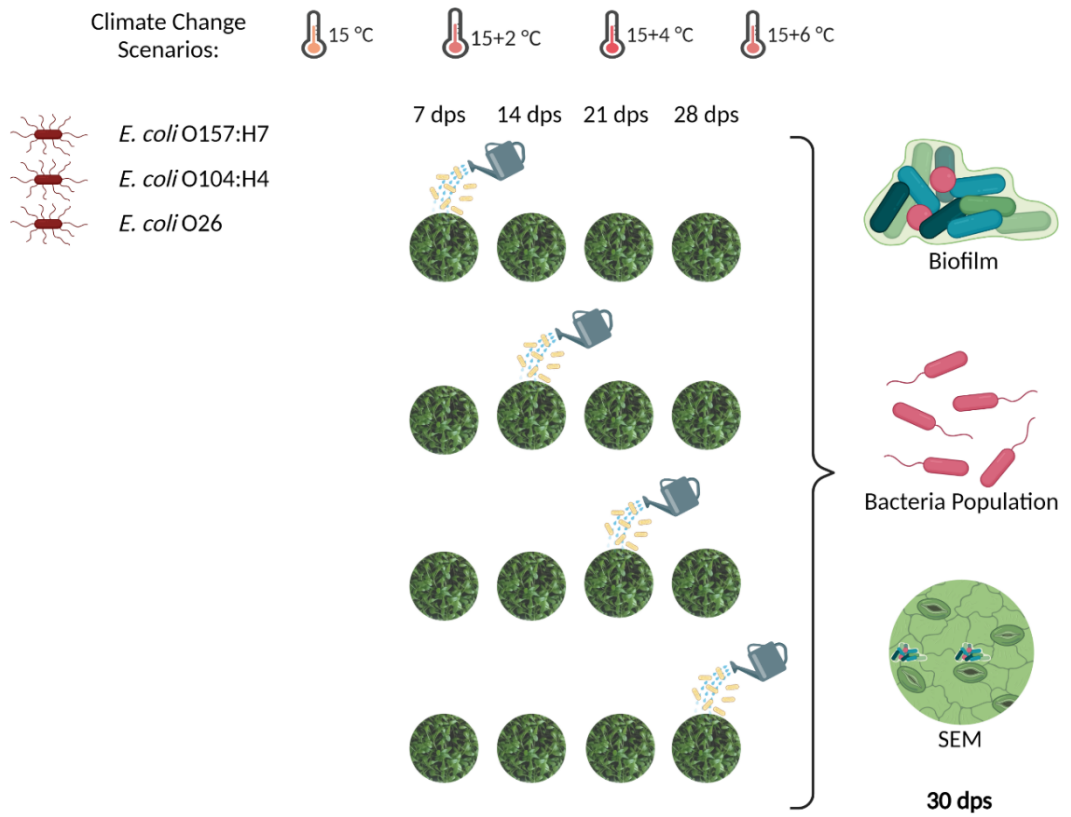


Figure 2.1 The detailed experimental plan for the Contaminated Irrigation on Cress Leaves. *dps: days post sowing. Created with BioRender.com

2.2.6.2 Translocation of Bacteria from Seed to Leaves

To study the effect of temperature on the translocation of bacteria from seeds to the phyllosphere, i.e., to the portions of plant above ground, inoculated seeds were used to cultivate cress. The bacterial suspension was prepared according to the procedures which were described in Section 2.2.2, and until usage, it was kept in ice bath. After obtaining the bacterial suspension, 1 g of surface-sterilized seeds were placed in an infuser and then inoculated by dipping them in 50 ml bacterial suspension containing 10^8 MPN/ml for 60 seconds.

The attachment level of each *E. coli* serotype to the seeds after 1 min was also determined. After 1 min of inoculation, 1 g of seed was sampled, and attachment levels were determined according to procedures mentioned in Section 2.2.7.

After that, the inoculated seeds were planted in autoclaved peat as described in Section 2.2.5.2. The only inoculation strategy for this group was inoculated seeds. During germination and growth, sterile distilled water as well as autoclaved peat and pots were used. Afterwards, they were placed in the climate chamber and germinated at designated climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) not to change the capabilities of bacterial attachment to seed and its subsequent translocation to phyllosphere through the vascular system of plant in the earlier stages of growth. As a control, the uninoculated plants, i.e., plants that were cultivated with surface sterilized seeds were used. The detailed experimental plan is available in Figure 2.2.

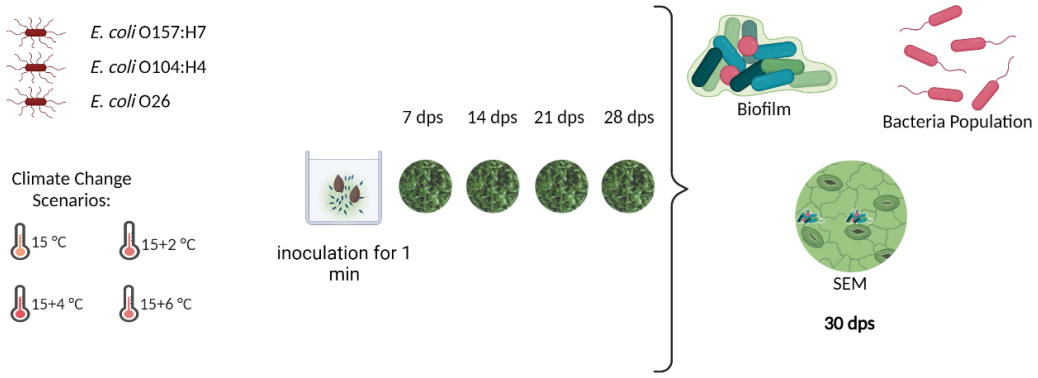


Figure 2.2 The detailed experimental plan for the Seed Inoculation analysis. *dps: days post sowing. Created with BioRender.com

2.2.7 Bacterial Attachment Assay on Leaf

After 30 dps, the cress leaves were grown enough to get harvested in their microgreen form. No wilting, any biotic damages resulting from bacterial activity, or no yellowing was observed in the leaves harvested from either contaminated or uncontaminated cress plants. For all inoculation groups, only the leaf samples were analyzed in terms of bacterial attachment. The leaf samples were cut with sterile scissors at nearly 1 cm above the peat and kept in sterile bags at 4 ºC until analyzing. The samples were analyzed at the same day when the harvest takes place.

For quantification of each group, two samples were collected:

i. for slightly attached and tightly bound cells (total corresponding *E. coli* populations) and
ii. for only tightly bound, i.e., biofilm-forming cells.

2.2.7.1 Determination of Slightly Attached and Tightly Bound *Escherichia coli* Populations

To determine the slightly attached and tightly bound *E. coli* population on the leaves, plant samples stored in the sterile bags were 10-fold diluted in buffered peptone water (BPW) without no additional treatment prior to dilution. Then, the total colonizing bacteria on the leaf was determined by 3-well most probable number (MPN) technique with minor modifications from the study of Luo et al. (2011). The 10-fold diluted leaf samples were homogenized in stomacher for 60 seconds. Then, the homogenized samples were serially diluted in BPW by 10-fold in 12 well plate up to 8 serial dilutions. The BPW amount in the wells was 2.25 ml, and to provide 10-fold dilution, 0.25 ml of homogenized sample was pipetted in the wells until the appropriate dilutions are obtained. After the serial dilutions are made, the wells were incubated at 37 ºC for 18 h. After the incubation, inoculum from turbid wells were streaked on brilliant-green phenol-red lactose sucrose (BPLS) agar for confirmation. *E. coli* can ferment lactose, and during fermentation, it produces yellow colonies on BPLS agar. Therefore, the yellow colonies formed on the BPLS agar after incubation at 37 ºC for 18 h were confirmed as *E. coli*. After the incubation, the colonies confirmed as *E. coli* were recorded, and the result for MPN/g cress was calculated. No *E. coli* growth was observed in plants used as control.

2.2.7.2 Determination of Tightly Bound *Escherichia coli* Populations

To determine the tightly bound *E. coli* cells, the harvested leaf samples were collected in a 50 ml falcon tube containing 5 ml of BPW. Then, they were vigorously washed in falcon tube by vortex for 30 seconds to remove the slightly attached *E. coli* cells from the leaves. After the removal of slightly attached cells, the procedures

for MPN method were followed as described in Section 2.2.7.1. The preparation of the pre-cultures and the quantification methods for Sections 2.2.7.1 and 2.2.7.2 were summarized in Figure 2.3.

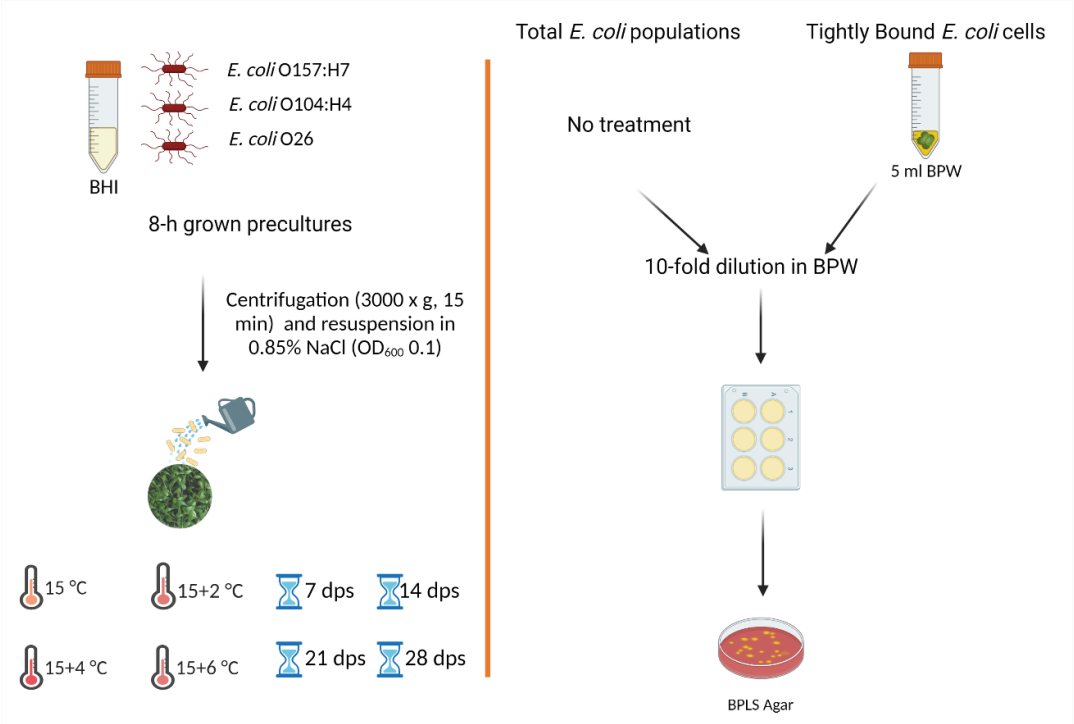


Figure 2.3 The representative visualization of the pre-culture preparation for the inoculation studies and the quantification methods for the detection of slightly attached and tightly bound *E. coli* cells and tightly bound *E. coli* cells. Created with BioRender.com

2.2.8 Scanning Electron Microscope (SEM)

After quantification of the *E. coli* cells, to visualize the structures of attached cells and biofilms and their locations on the leaf surface, the inoculated broad and curly leaf from each pot was cut with sterile scissors, and they were prepared to get visualized under Scanning Electron Microscope (SEM). For preparation, the leaves were firstly fixed in pure methanol (99.7 %) for 30 seconds (Neinhuis & Edelmann, 1996). Fixed leaves were subsequently dehydrated in serial ethanol concentrations (10, 30, 50, 70, 90, 96 %) for 10 minutes, and then plunged in pure acetone for 10

minutes (Pathan et al., 2009). The dehydrated samples were coated with gold-palladium and visualized with Field Emission SEM (Quanta 400F, Eindhoven, Holland) operating at 10-20 kV and at 5,000 – 10,000 × magnification in Middle East Technical University (METU) Central Lab and METU Department of Metallurgical and Materials Engineering (METE).

2.2.9 Statistical Analysis

All the experiments for control groups, seed inoculation and contaminated irrigation groups were planted with three biological replicates. The effects of temperature and contamination time and route on the tightly bound and slightly attached cells were determined using ANOVA followed by Tukey's test taking $p < 0.05$ statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Growth Curve Analysis

3.1.1 Growth Curve Analysis at 37 ºC

Three serotypes of *E. coli* have been used in this study to assess their attachment levels on the leaf structure. Prior to assessment, their *in-vitro* growth curves were studied to have a better understanding of their growth phases at different temperatures. The growth curves were obtained both at their optimum growth temperature (37 ºC) and at proposed climate change scenarios (15, 15+2, 15+4, 15+6 ºC). Both the cell counting, and the OD measurement were included for the optimum growth temperature, whereas only OD measurements were recorded for the proposed climate change scenarios.

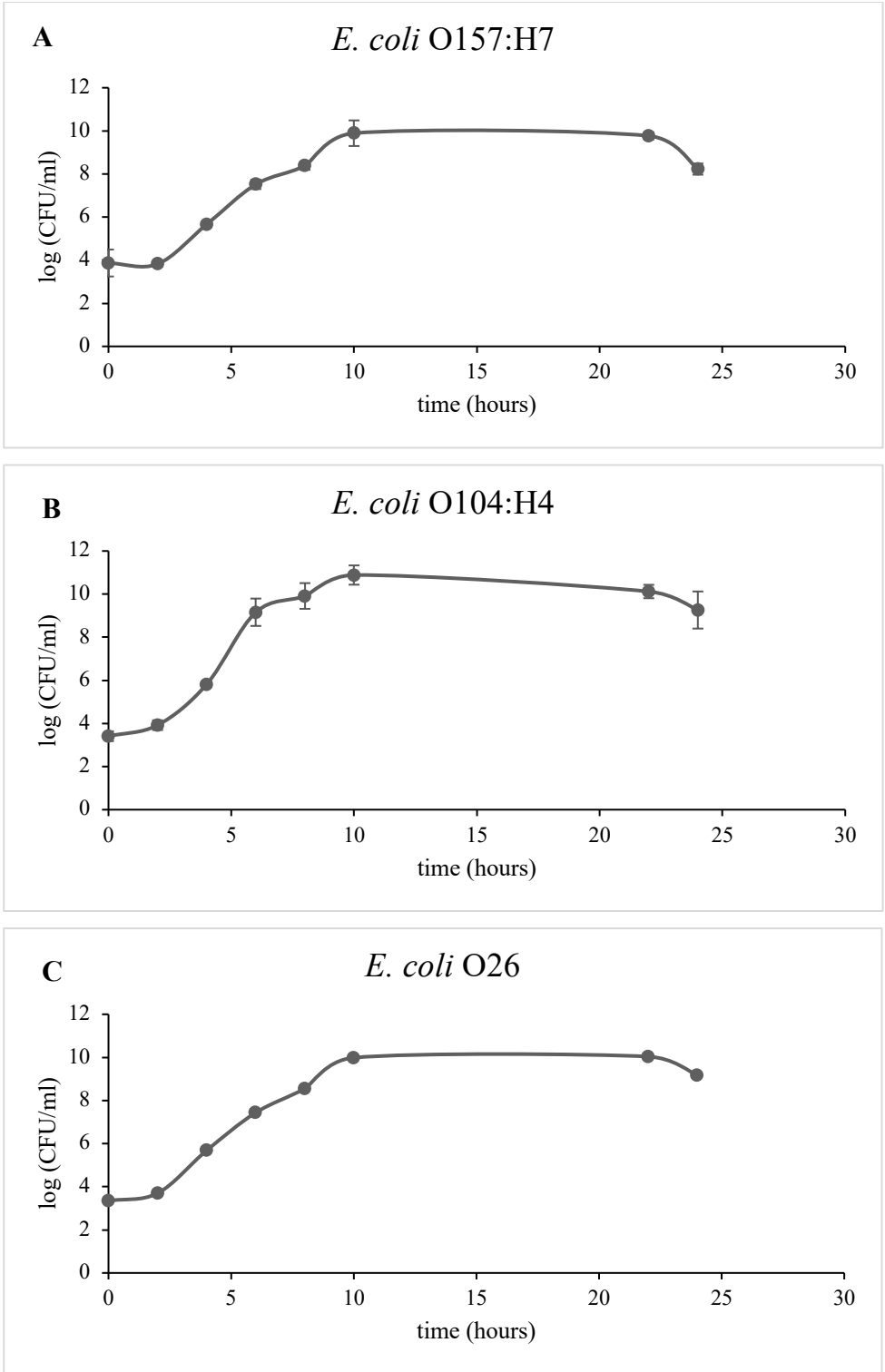


Figure 3.1 Growth curve of *E. coli* O157:H7 (A), O104:H4 (B) and O26 (C) in BHI broth for 24 hours at 37 ºC

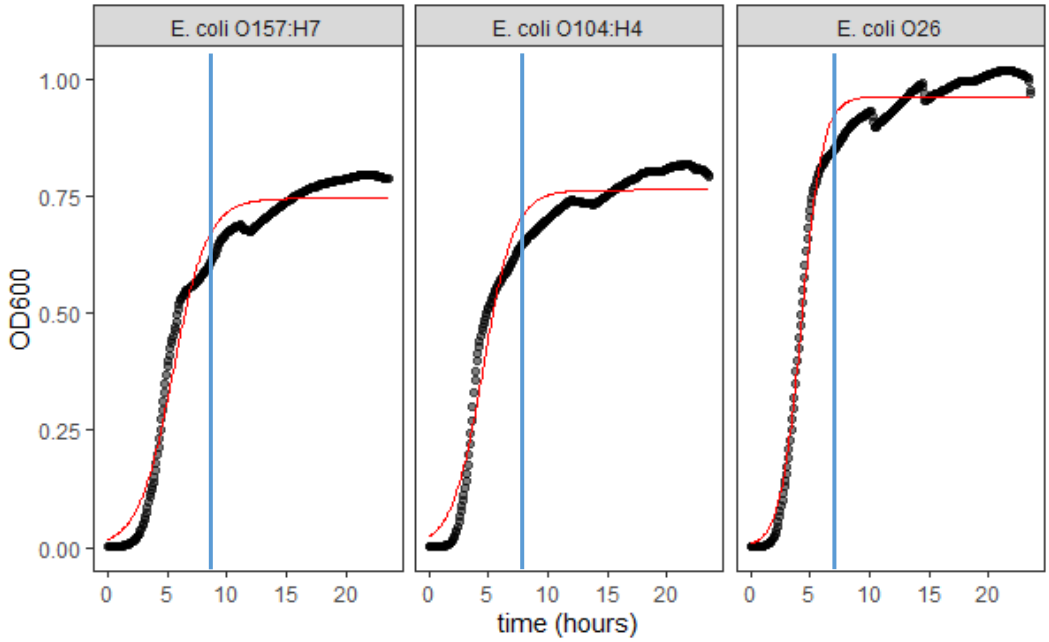


Figure 3.2 Optical density (OD) measurements of *E. coli* O157:H7, O104:H4 and O26 for 24 hours (black) and the predicted growth curves of these serotypes which are fitted to logistic equation (red) at 37 ºC. Blue line represents the incubation time selected for each bacterium to reach the early stationary phase.

In the stationary phase, the metabolic activity slows down as the nutrient in the growing medium depletes, and with this, there are changes in the morphological state of the bacteria. In addition, the bacteria get more resistant to changes in environmental conditions in the stationary phase (Bergholz et al., 2007). Therefore, the cells that were planned to be used in the inoculation assays were harvested before they go into stationary phase (Brandl & Amundson, 2008; J. Ma et al., 2012). According to Figure 3.2, *E. coli* O157:H7 and O104:H4 reach their late exponential phase nearly after a 5-hour incubation and they are at this phase until a 12-hour incubation at 37 ºC. On the other hand, *E. coli* O26 reaches its late exponential phase earlier, nearly after a 4-hour incubation. It is at late exponential phase until 11th hour of the incubation at 37 ºC. Therefore, according to these intervals, the incubation time has been determined as 8 hours for cells to reach early exponential phase.

3.1.2 Growth Curves at Climate Change Scenarios

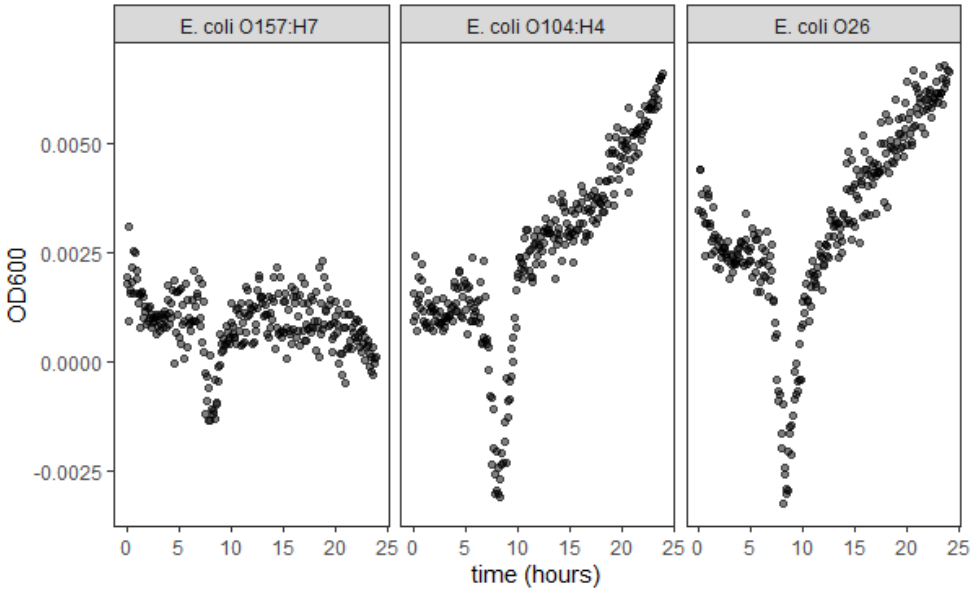


Figure 3.3 Optical density (OD) measurements of *E. coli* O157:H7, O104:H4 and O26 for 24 hours at 15 ºC.

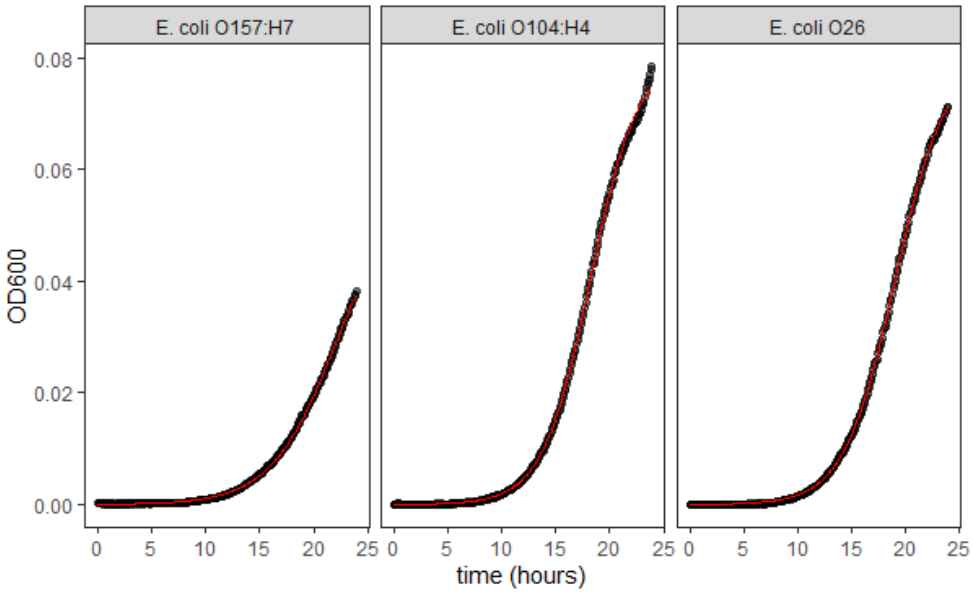


Figure 3.4 Optical density (OD) measurements of *E. coli* O157:H7, O104:H4 and O26 for 24 hours (black) and the predicted growth curves of these serotypes which are fitted to logistic equation (red) at 15+2 ºC.

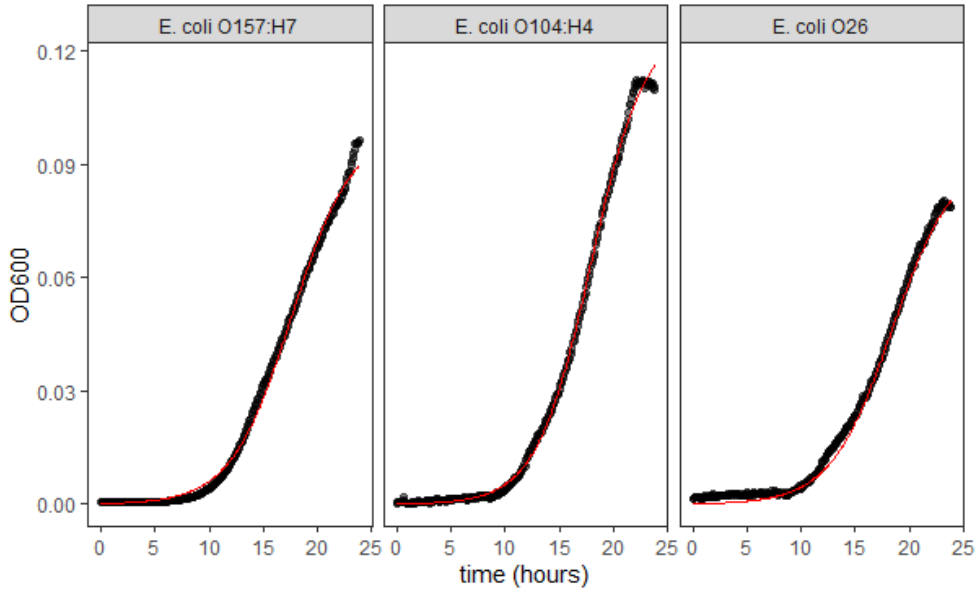


Figure 3.5 Optical density (OD) measurements of *E. coli* O157:H7, O104:H4 and O26 for 24 hours (black) and the predicted growth curves of these serotypes which are fitted to logistic equation (red) at 15+4 ºC.

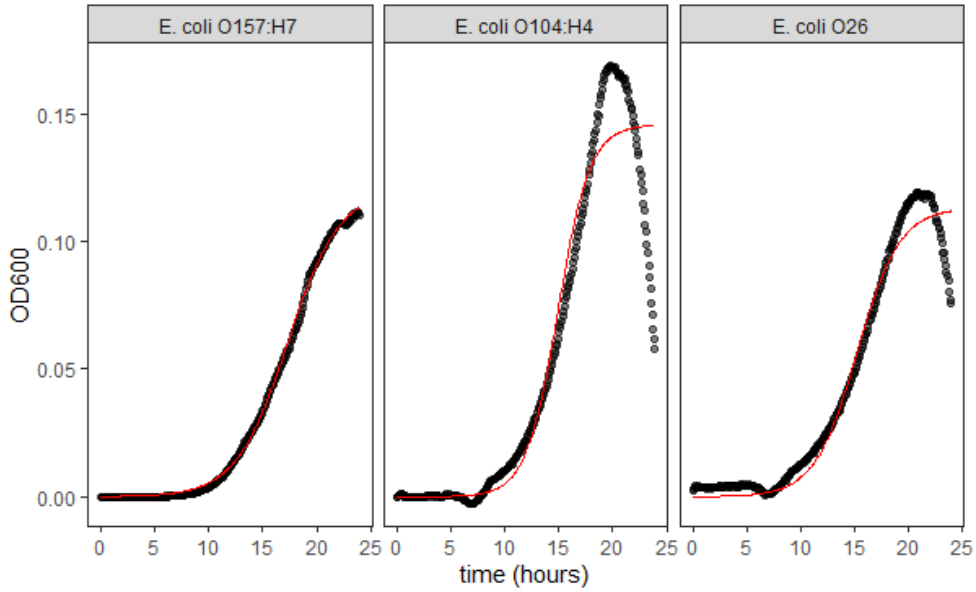


Figure 3.6 Optical density (OD) measurements of *E. coli* O157:H7, O104:H4 and O26 for 24 hours (black) and the predicted growth curves of these serotypes which are fitted to logistic equation (red) at 15+6 ºC.

As Figure 3.3 to Figure 3.6 and Table 3.1 show, *E. coli* O104:H4 and O26 showed longer lag phase at lower temperatures (15+4 and 15+6 ºC) compared to 37 ºC (Figure 3.2). Also, the growth rates of *E. coli* O104:H4 and O26 at 37 ºC was the highest, which was expected for being the optimum growth temperature. Likewise, similar issue was observed at lower temperatures. At 15+6 ºC, the growth rates were higher compared to 15 and 15+4 ºC (Table 3.1). Furthermore, *E. coli* O157:H7, O104:H4 and O26 did not grow in 24 hours at 15 ºC, but *E. coli* O104:H4 and O26 growth showed a slight increase (Figure 3.3). In fact, this was in accordance with what was reported earlier by Lee et al. (2019). They observed only a slight growth of *E. coli* O157:H7 at 15 ºC in 20 hours, and a longer lag phase at lower temperatures.

Table 3.1 The growth parameters of *E. coli* O157:H7, O104:H4 and O26 at different climate change scenarios and optimum growth temperature

		***E. coli* O157:H7**	***E. coli* O104:H4**	***E. coli* O26**
15 ºC	r (h^{-1})	NF	0.047	0.035
	t_d (h)	NF	14.748	19.804
	K	NF	34171.068	19567.441
	N_o	NF	0.003	0.004
	RSE	NF	0.0012	0.0015
15+2 ºC	r (h^{-1})	0.325	0.451	0.425
	t_d (h)	2.133	1.537	1.631
	K	0.059	0.080	0.080
	N_o	0	0	0
	RSE	0.0002	0.0005	0.0002
15+4 ºC	r (h^{-1})	0.357	0.396	0.352
	t_d (h)	1.942	1.750	1.969
	K	0.100	0.127	0.092
	N_o	0	0.000	0.000
	RSE	0.0018	0.0014	0.0009

Table 3.1 (Continued)

15+6 ºC	r (h^{-1})	0.417	0.674	0.506
	t_d (h)	1.662	1.028	1.369
	K	0.121	0.146	0.113
	N_o	0	0.000	0.000
	RSE	0.0014	0.0169	0.0068
37 ºC	r (h^{-1})	0.692	0.767	1.129
	t_d (h)	1.002	0.904	0.614
	K	0.746	0.764	0.964
	N_o	0.017	0.023	0.008
	RSE	0.0442	0.0467	0.0392

*NF: no fit to the model; r: growth rate (h^{-1}); t_d: doubling time (h); K: carrying capacity; N_o: population size in the beginning; RSE: residual standard error

3.2 Plate Assay for Biofilm Formation

Biofilms are the structures exhibiting a reversible attachment to a surface inside a matrix called extracellular polymeric matrix (EPS). Since the microorganisms inside a biofilm structure are resistant to sanitation applications and adverse conditions since the EPS matrix protects the cells against harsh environmental conditions. Thus, in terms of food safety, it is quite vital to determine to what extent the foodborne pathogens are able to form biofilms on the freshly consumed produce. However, prior to application on the leaf tissue, the biofilm formation capabilities were firstly analyzed with *in-vitro* assays to comprehend the level of biofilm forming abilities of the bacterial strains studied in this study.

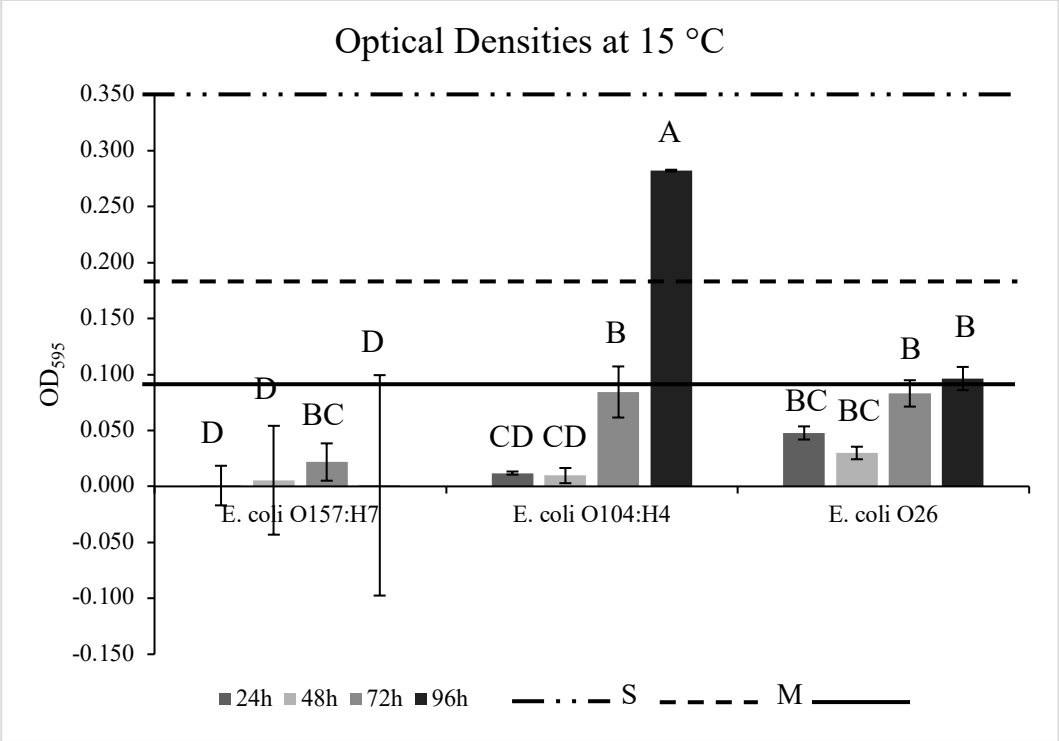


Figure 3.7 Biofilm forming abilities of *E. coli* O157:H7, O104:H4 and O26 on polystyrene 96-well plates for 24, 48, 72 and 96 h at minimum climate change scenarios (15 ºC). Results were reported as the mean of four technical replicates ± standard deviation by subtracting the OD values of control from those of samples. Horizontal lines represent the biofilm formation abilities; among weakly (W), moderately (M) and strongly (S) adherent. Statistical significance of difference in means was analyzed with ANOVA followed by Tukey test. Values that do not share the same letter show significant difference ($p < 0.05$) among the serotypes and the incubation time (24, 48, 72 and 96 h) at 15 ºC.

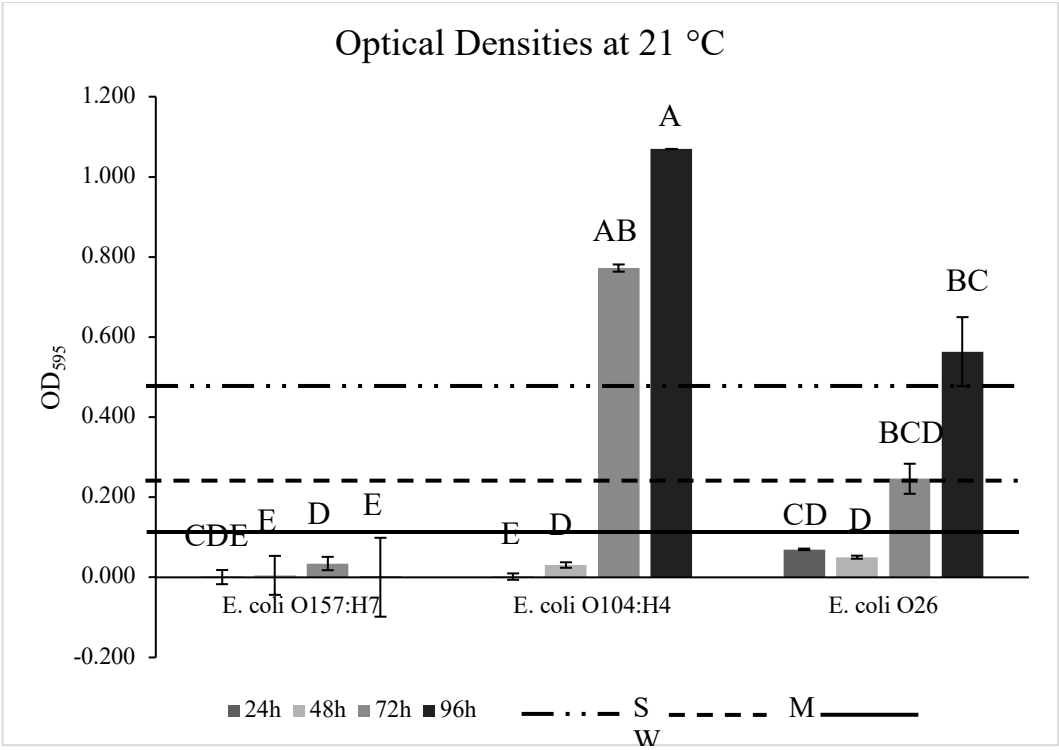


Figure 3.8 Biofilm forming abilities of *E. coli* O157:H7, O104:H4 and O26 on polystyrene 96-well plates for 24, 48, 72 and 96 h at maximum climate change scenarios (21 ºC). Results were reported as the mean of four technical replicates ± standard deviation by subtracting the OD values of control from those of samples. Horizontal lines represent the biofilm formation abilities; among weakly (W), moderately (M) and strongly (S) adherent. Statistical significance of difference in means was analyzed with ANOVA followed by Tukey test. Values that do not share the same letter show significant difference ($p < 0.05$) among the serotypes and the incubation time (24, 48, 72 and 96 h) at 21 ºC.

Figure 3.7 and Figure 3.8 demonstrate that *E. coli* serotypes showed different biofilm formation abilities on polystyrene surface. As described earlier, the biofilm formation abilities were classified according to cut-off OD_{595} values (OD_c), which is three standard deviations above the average OD values of negative controls (Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, 2000). At 15

ºC, the biofilm forming abilities of strains were classified according to cut-off values as follows (Figure 3.7),

- Non-adherent: $OD \leq 0.089$
- Weakly adherent: $0.089 < OD \leq 0.179$
- Moderately adherent: $0.179 < OD \leq 0.358$
- Strongly adherent: $0.358 < OD$.

At 21 ºC, the biofilm forming abilities of strains were classified according to cut-off values as follows (Figure 3.8),

- Non-adherent: $OD \leq 0.108$
- Weakly adherent: $0.108 < OD \leq 0.217$
- Moderately adherent: $0.217 < OD \leq 0.434$
- Strongly adherent: $0.434 < OD$.

At 15 ºC, none of the strains formed strongly adherent biofilms on polystyrene surface. It can be observed that the densities of biofilm structures are increasing as the incubation time increases for *E. coli* O104:H4 and O26 at 15 ºC. *E. coli* O104:H4 showed the highest biofilm forming capability at 96 h and it moderately adhered ($p < 0.05$). *E. coli* O157:H7 showed a slight peak at 72 h, yet it did not form any adherent structures within the incubation times provided. Similarly, *E. coli* O26 did not form biofilm at 15 ºC. It exhibited a slight increase at 96 h compared to 72 h, showing a weakly adherent pattern. However, it is not significantly different from the biofilm formation level at 72 h (Figure 3.7). At 21 ºC, *E. coli* O104:H4 and O26 formed more strong biofilms compared to 15 ºC. In addition, *E. coli* O104:H4 and O26 showed a strong adherence with prolonged incubation times. Similar to 15 ºC, *E. coli* O104:H4 had the highest biofilm biomass at 21 ºC ($p < 0.05$). Likewise, *E. coli* O157:H7 did not form biofilm at 21 ºC.

Here, *E. coli* O157:H7 did not show any biofilm forming ability and biofilm forming strains showed an increasing trend in the biofilm mass as the incubation time increases. Although it has been found that *E. coli* O157 formed weak to moderate biofilm mass on the stainless steel surface at 13 and 22 ºC in a 72 h of incubation

time, its biofilm forming ability on stainless steel has decreased as the incubation time increased (Ma et al., 2019). On the other hand, Fouladkhah et al. (2013) reported an increased biofilm mass for *E. coli* O157 and non-STEC strains on stainless steel with prolonged incubation times from day 0 to day 7 at 15 and 25 ºC. These differences might depend on different factors. The biofilm formation in STEC and other *E. coli* pathogroups is related to the surface structure and genotype of the studied strain among many other factors such as nutrient availability and temperature (Ma et al., 2019; Wang et al., 2016). Ryu et al. (2004) also revealed that STEC isolates could form strong biofilms on stainless steel at 22 ºC, but the biofilm formation abilities have decreased as the incubation time increased. However, they also reported that less biofilm structures have formed on stainless steel at lower temperatures (13 ºC), but the incubation time had a positive correlation with the biofilm densities at lower temperatures. Thus, the biofilm formation abilities are both strain and surface dependent.

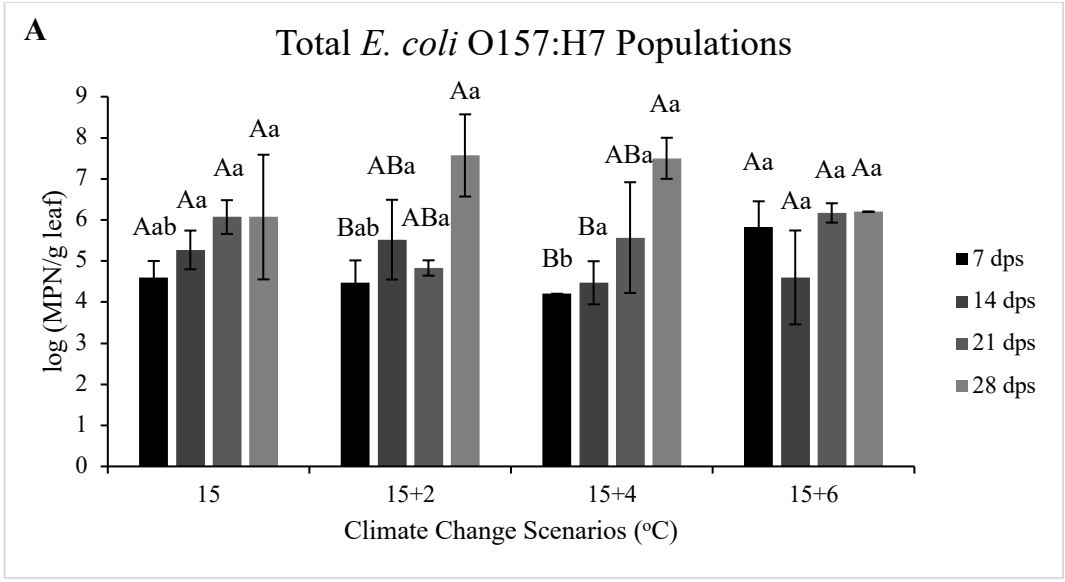
3.3 Bacterial Attachment Assay on Leaf at Different Climate Change Scenarios

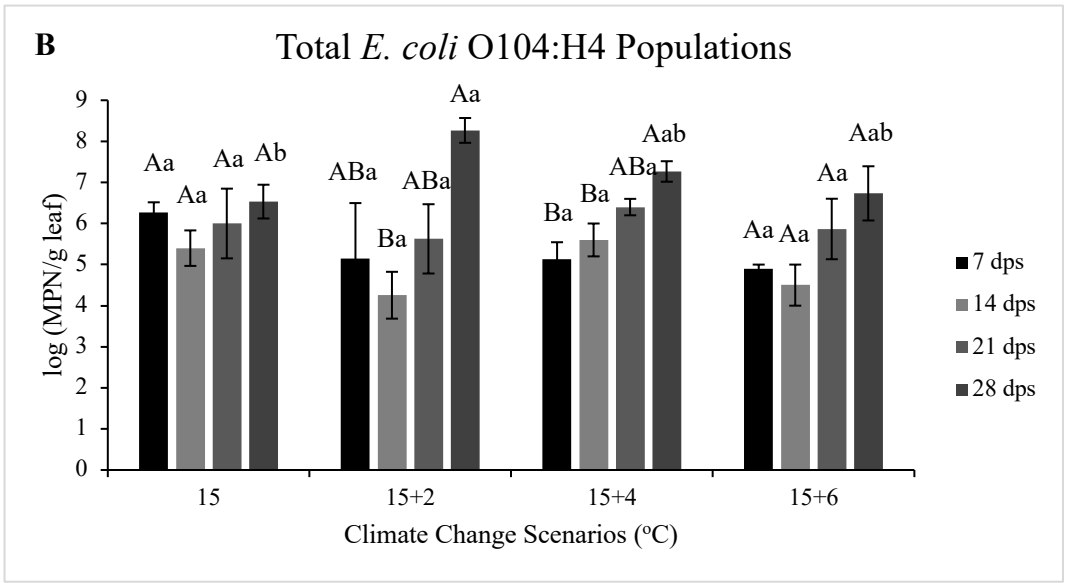
The fact that the globe is getting warmer compared to the levels recorded before the Industrial Revolution is recognized all over the world. Depending on this, the Intergovernmental Panel on Climate Change (IPCC) propose different temperature increase scenarios according to several mitigation strategies (IPCC, 2014). The IPCC report in 2014 exhibits the forecasted temperature rise scenarios according to greenhouse gas emission levels. If the strongest mitigation scenario is adopted by governments, and policymakers and the gas emission levels are reduced (RCP2.6, representative concentration pathway 2.6), the increase in the global mean surface temperature can be constrained to 0.3 ºC by the late 21st century. On the other hand, the projected rise in the global mean surface temperature is expected to be nearly 4.8 ºC by the late 21st century according to the scenario (Table 1.6). The whole ecosystem as well as the interactions between contamination sources and foodborne pathogens is subjected to get affected by the changes in climate all the world (Liu et

al., 2013). Thus, it is quite essential to determine how the behavior of foodborne pathogens on freshly consumed vegetables alters according to changes in climate.

3.3.1 Mimicking Contamination with Irrigation Water

Manure, irrigation water, soil, workers due to malpractice and even wildlife could contaminate the fresh produce in the field (Liu et al., 2013; Thao et al., 2019). However, it has been shown that the contamination through irrigation water is more common and it presents an important route for the contamination among other contamination sources in the pre-harvest stage (Van der Linden et al., 2014). In practice, many water sources including surface waters, treated wastewater and groundwater are being used to irrigate the produce in field apart from municipal water. However, the microbiological quality required for sustaining the food safety could not be achieved in various water sources (Carstens et al., 2019; Riggio et al., 2019). The transmission, survival and persistence of *E. coli* in irrigation water could be enhanced by the increasing temperatures (Liu et al., 2015). Therefore, the cress plants grown in climate chamber were spray-irrigated at their different growth stages and at different climate change scenarios by *E. coli* O157:H7, O104:H4 and O26 at an initial bacterial load of 8 log MPN/ml in irrigation water. As a result, the bacterial attachment on leaves at their latest growth stage (30 dps) was evaluated.





Continued on next page

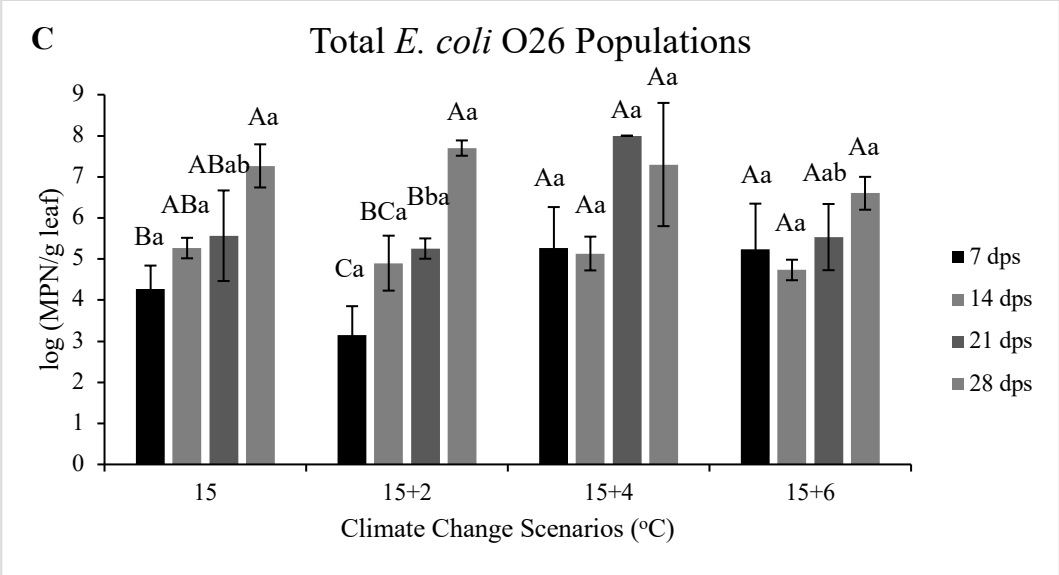
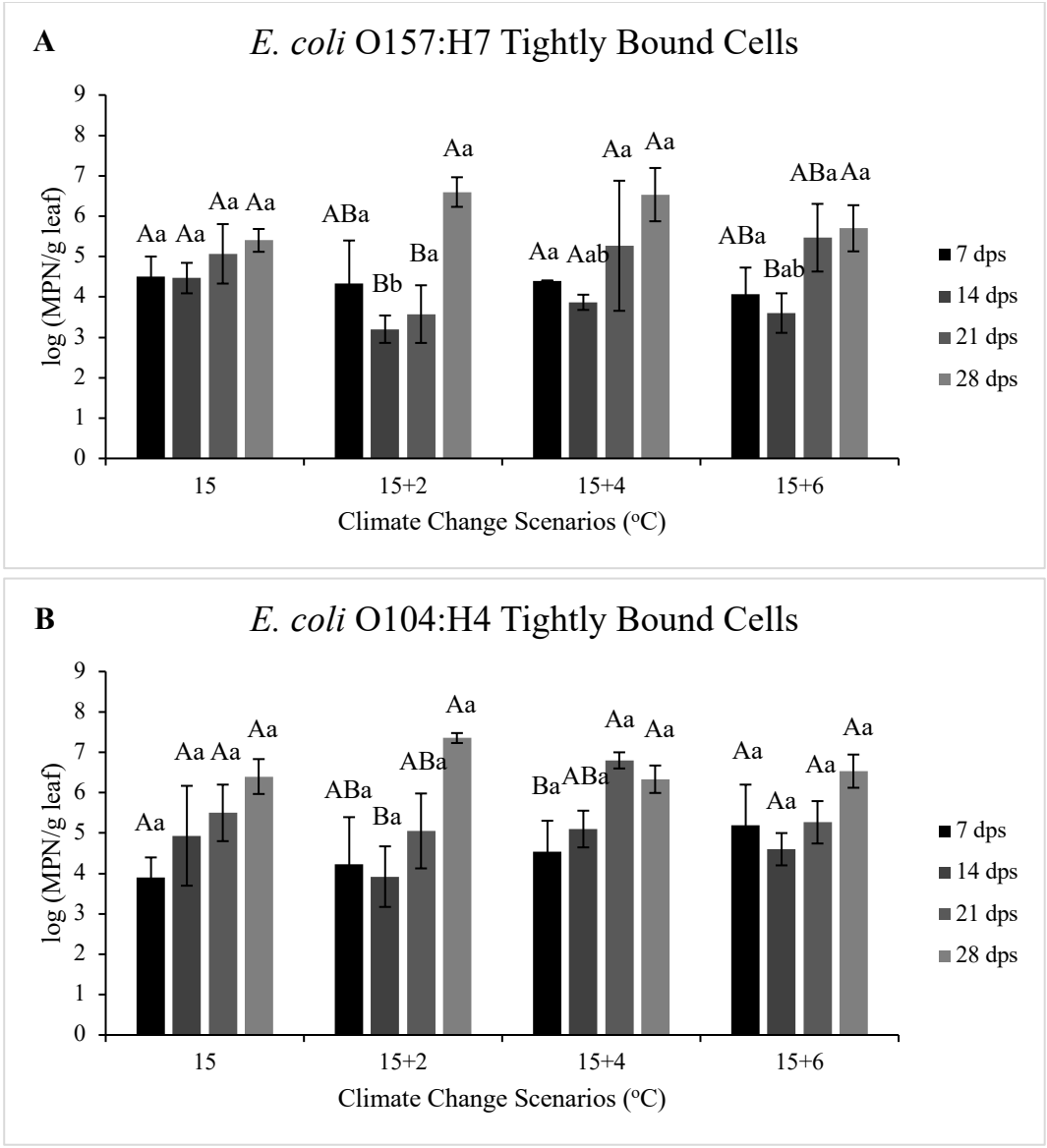


Figure 3.9 Both slightly attached and tightly bound cell levels (total populations) of *E. coli* pathogroups on cress plants. *Escherichia coli* O157:H7, O104:H4 and O26 were artificially inoculated with spray irrigation onto cress leaves at 10^8 MPN/ml level at 7, 14, 21 and 28 days post-sowing (dps). Cress plants were grown under controlled conditions in climate chamber using surface-sterilized seeds and autoclaved commercial peat at different climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) at 65 % RH. Both slightly attached and tightly bound cells (total populations) (log MPN/g leaf) were determined at 30 dps for *E. coli* O157:H7 (A), *E. coli* O104:H4 (B) and *E. coli* O26 (C). Results were reported as the mean log (MPN/g leaf) of three biological replicates ± standard deviation. Statistical significance of difference in means was analyzed with ANOVA followed by Tukey test. Values with different uppercase letters show significant difference ($p < 0.05$) among inoculation time (7, 14, 21 and 28 dps) at the same climate change scenario. Values with different lowercase letters show significant difference ($p < 0.05$) among climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) at the same inoculation time.

Artificial inoculation of cress leaves through irrigation water had resulted in significant pathogen load at the end of the growing period (30 dps) at each contamination stage (7, 14, 21 and 28 dps) and climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) (Figure 3.9). *E. coli* O157:H7 showed a slightly increase in the total attachment from 4.60 ± 0.4 log MPN/g leaf at 7 dps to 6.07 ± 1.5 log MPN/g leaf at 28 dps at 15 ºC, and from 4.20 ± 0.0 log MPN/g leaf at 7 dps to 7.50 ± 0.5 log MPN/g leaf at 28 dps at 15+2 ºC (Figure 3.9–A). Although these attachment levels are substantial and above the infectious levels (Croxen et al., 2013), the increase did not show any statistically difference. The contamination conducted at a later stage of the growth (21 and 28 dps) has resulted in more attachment levels for total *E. coli* O157:H7 at all climate change scenarios studied.

In addition, a similar pattern was observed in *E. coli* O104:H4 attachment levels. At 15+2, 15+4 and 15+6 ºC, a higher attachment level was observed in plants contaminated at later stages (5.14 ± 1.34 log MPN/g leaf at 7 dps and 8.27 ± 0.3 log MPN/g leaf at 28 dps at 15+2 ºC ($p < 0.05$); 5.13 ± 0.4 log MPN/g leaf at 7 dps and 7.27 ± 0.2 log MPN/g leaf at 28 dps at 15+4 ºC; 4.90 ± 0.1 log MPPN/g leaf at 7 dps and 6.73 ± 0.6 log MPN/g leaf at 28 dps at 15+6 ºC). However, at 15, 15+2 and 15+6 ºC, the plants inoculated at 7 dps showed higher attachment levels compared to 14 dps (Figure 3.9–B). On the other hand, plants contaminated at a later stage (21 and 28 dps) showed higher bacterial population at the harvest (30 dps) at all climate change scenarios.

For *E. coli* O26, more cell populations were observed in the plants contaminated at 21 and 28 dps. Also, there is an increase in bacterial load from 7 dps (4.27 ± 0.6 log MPN/g leaf at 15 ºC; 3.14 ± 0.7 log MPN/g leaf at 15+2 ºC) to 28 dps (7.27 ± 0.5 log MPN/g leaf at 15 ºC; 7.70 ± 0.2 log MPN/g leaf at 15+2 ºC) (Figure 3.9–C). At 15+4 ºC, more bacterial load has been assessed at 21 dps (8.00 ± 0.0 log MPN/g leaf) compared to 28 dps (7.30 ± 1.5 log MPN/g leaf). Similar to *E. coli* O157:H7 and O104:H4, *E. coli* O26 has also persisted on the leaf surface until the mature plants are formed (30 dps) with a substantial pathogen load above 3.14 ± 0.7 log MPN/g leaf at all climate change scenarios.



Continued on next page

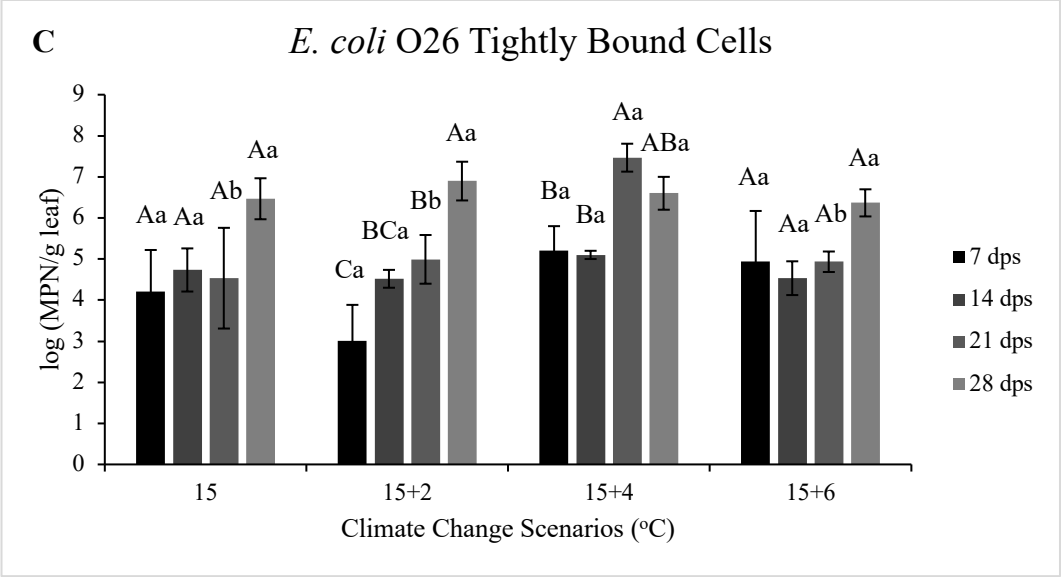


Figure 3.10 Survival of tightly bound *E. coli* pathogroups on cress leaves. *Escherichia coli* O157:H7, O104:H4 and O26 were inoculated with spray irrigation onto cress leaves at 10^8 MPN/ml level at 7, 14, 21 and 28 days post-sowing (dps). Cress plants were grown under controlled conditions in climate chamber using surface-sterilized seeds and autoclaved commercial peat at different climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) at 65 % RH. Prior to counting, leaves were washed in 5-ml BPW with vigorous shaking to remove slightly attached cells. Tightly bound cells (log MPN/g leaf) were determined at 30 dps for O157:H7 (A), O104:H4 (B) and O26 (C). Results were reported as the mean log (MPN/g leaf) of three biological replicates ± standard deviation. Statistical significance of difference in means was analyzed with ANOVA followed by Tukey test. Values with different uppercase letters show significant difference ($p < 0.05$) among inoculation time (7, 14, 21 and 28 dps) at the same climate change scenario. Values with different lowercase letters show significant difference ($p < 0.05$) among climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) at the same inoculation time.

E. coli O157:H7, O104:H4 and O26 have tightly bound to the leaf tissue with a considerable amount of bacterial load in the harvested produce (Figure 3.10). *E. coli* O157:H7 had maintained higher tightly adhered populations at 28 dps (5.40 ± 0.3 log MPN/g leaf at 15 ºC; 6.60 ± 0.4 log MPN/g leaf at 15+2 ºC; 6.53 ± 0.6 log MPN/g leaf at 15+4 ºC; 5.70 ± 0.6 log MPN/g leaf at 15+4 ºC) similar to the observation in total population levels. Analogous to the slightly attached and tightly bound cells, higher populations were observed at 7 dps (4.33 ± 1.1 log MPN/g leaf at 15+2 ºC; 4.40 ± 0.0 log MPN/g leaf at 15+4 ºC; 4.07 ± 0.6 log MPN/g leaf at 15+6 ºC) compared to 14 dps (3.20 ± 0.3 log MPN/g leaf at 15+2 ºC; 3.87 ± 0.2 log MPN/g leaf at 15+4 ºC; 3.60 ± 0.5 log MPN/g leaf at 15+6 ºC) (Figure 3.10–A). Furthermore, washing could just eliminate 0.10 to 2.32 log MPN *E. coli* O157:H7 per g leaf.

Higher tightly bound *E. coli* O104:H4 populations have been obtained at 21 dps (5.50 ± 0.7 log MPN/g leaf at 15 ºC; 5.05 ± 0.9 log MPN/g leaf at 15+2 ºC; 6.80 ± 0.2 log MPN/g leaf at 15+4 ºC; 5.27 ± 0.5 log MPN/g leaf at 15+6 ºC) and 28 dps (6.40 ± 0.4 log MPN/g leaf at 15 ºC; 7.35 ± 0.1 log MPN/g leaf at 15+2 ºC; 6.33 ± 0.3 log MPN/g leaf at 15+4 ºC; 6.53 ± 0.4 log MPN/g leaf at 15+6 ºC (Figure 3.10–B). Furthermore, only 0.13 to 2.37 log MPN of *E. coli* O104:H4 per g leaf could be eliminated by washing.

Similar to what was observed in slightly attached and tightly bound cells, tightly bound *E. coli* O26 has produced highest populations at 28 dps (6.47 ± 0.5 log MPN/g leaf at 15 ºC; 6.90 ± 0.5 log MPN/g leaf at 15+2 ºC; 6.60 ± 0.4 log MPN/g leaf at 15+4 ºC; 6.37 ± 0.3 log MPN/g leaf at 15+6 ºC) (Figure 3.10–C). Also, washing step could only reduce the bacterial populations by 0.07 to 1.03 log MPN of *E. coli* O26 per g leaf. Except the 15+2 ºC, the tightly bound populations at 7 and 14 dps are similar in other climate change scenarios.

In general, the results obtained in this study indicate that the artificial contamination through irrigation water results in considerable amount of *E. coli* populations in harvested product in each contamination scenarios (7, 14, 21, 28 dps) and climate change scenarios (15, 15+2, 15+4, 15+6 ºC). The initial load of inoculum has been maintained up to ~8.30 and 7.50 log MPN/g leaf for total corresponding *E. coli*

populations and tightly bound cells for plants inoculated at 28 dps, respectively (Figure 3.9 and Figure 3.10). In plants contaminated at a later stage (21 and 28 dps), the populations of *E. coli* O157:H7, O104:H4 and O26 were higher in general for all temperatures studied. Even though a significant amount of bacterial load has remained on leaf tissue (at least ~3.15 and 3.00 log MPN/g for total populations and tightly bound cells, respectively), the initial level of inoculum has reduced until 30 dps in plants inoculated by contaminated irrigation water at 7 and 14 dps. However, the adhered bacterial load on leaves in mature plants still poses a huge threat to both food safety and human health since *E. coli* O157:H7 is resistant to low pH environment of stomach being able to survive in high acidic mediums (Croxen et al., 2013). The ability of *E. coli* O157:H7 to internalize and colonize inside the leaf surface and its persistence on leaf have been clearly explained. Roy and Melotto (2019) have also shown that *E. coli* O157:H7 numbers on dip-inoculated store bought Butterhead lettuce leaves at 21 days post-inoculation have reduced by nearly 1 log CFU/cm^2 leaf, which was similar results to what was reported in this study. Jang and Matthews (2018) also reported a decrease in *E. coli* O157:H7 and O104:H4 populations in Arabidopsis and Romaine lettuce leaves at 5 days post-inoculation in pre-harvest conditions. Wright and Holden (2018) showed nearly 3 log CFU/g leaf decrease in *E. coli* O157:H7 strain Sakai populations on broccoli microgreens at 19 days post-inoculation.

Apart from the persistence and survival on the leaf, the present study also explored the impact of leaf age on bacterial load by artificially inoculating the plants at their different growth stages. It is well-known that the essential nutrients are being secreted during germination to promote the plant growth. Thus, younger leaf tissues are rich in nutrients that might enhance the plant growth as well as boost the growth of microorganisms (Brandl & Amundson, 2008; Ottoson et al., 2011). In addition, *E. coli* O157:H7 strain Sakai has been shown to be using plant species specific metabolic pathways during colonization of lettuce (Wright & Holden, 2018). The present study reported that both the populations of slightly attached and tightly bound cells in leaves contaminated at 7 and 14 dps are similar to each other. Particularly, a slightly more *E. coli* O104:H4 populations have attached to cress leaves

contaminated at 7 dps although any statistically significant difference was observed (Figure 3.9-B). All in all, the leaf exudates which are excreted to support the growth in the course of germination could have a positive effect on the attachment to the leaves contaminated at earlier stages, which then results in similar attachment levels for the cress plants contaminated at 7 and 14 dps.

Jang and Matthews (2018) have revealed that *E. coli* O104:H4 populations were higher than *E. coli* O157:H7 at 1 and 5 days post-inoculation on Arabidopsis leaves. The reason for this has been uncovered by the plant defense response analysis and it has been shown that the plant defense system of Arabidopsis has been induced more by the activities of *E. coli* O157:H7 compared to that of *E. coli* O104:H4. Furthermore, *E. coli* O104:H4 has secreted more extracellular compounds on plant, which was similar to what was obtained in the biofilm biomass determination on polystyrene surface in this study (Figure 3.7 and Figure 3.8). Due to this, *E. coli* O104:H4 was more protected against the plant defense mechanism. In addition, the attachment to leaf is affected by the variations in genomes of different *E. coli* strains. The surface structures of a pathogen have an impact on the first attachment and further colonization on leaf. It is already known that for the first attachment and further colonization the baby spinach leaves, *E. coli* O157:H7 used the combination of pilus, flagella, type three secretion system (T3SS) and curli (Saldaña et al., 2011). After the attachment, the important tools for the survival on the leaf are quorum sensing mechanism and biofilm-forming ability. Saldaña et al. (2011) showed that the mutations occurred in the curli production and quorum sensing mechanism related genes ended up in a significant amount of decrease in the ability to attach to leaf surface. The biofilm matrix could be changed by many factors such as genome of the strain, ambient temperature and nutrient available in the medium by altering the main component, i.e., extracellular polymeric substances in biofilms (Flemming & Wingender, 2010). Thus, it is more likely to have different attachment levels among different *E. coli* pathogroups. For example, it was reported that *E. coli* O157:H7 and O104:H4 showed different adherence abilities since *E. coli* O157:H7 synthesizes the intimin protein important for attachment to intestines, whereas *E. coli* O104:H4 could not synthesize it (Bielaszewska et al., 2011). However, in this study,

attachment levels of *E. coli* O157:H7, O104:H4 and O26 did not show any differences on cress leaves. According to Figure 3.7 and Figure 3.8, *E. coli* O104:H4 has the highest *in-vitro* biofilm forming ability among other *E. coli* serotypes and additionally, biofilm biomass by *E. coli* serotypes has increased with the prolonged incubation time and higher temperature. However, different from the *in-vitro* assays, populations of tightly bound *E. coli* O104:H4 cells on cress leaves did not show statistically difference compared to *E. coli* O157:H7 and O26 (Figure 3.10). Since the inoculated bacteria inhabits the leaf surface for a long time (30 dps), *E. coli* O157:H7, O104:H4 and O26 might have accomplished a similar attachment on the cress leaves. Thus, unlike polystyrene surface, a similar fitness has been observed by the *E. coli* serotypes on the cress leaves. Moreover, the analysis between the total *E. coli* populations (Figure 3.9) and tightly bound cells (Figure 3.10) revealed that inoculated cells onto leaves had remained until harvest on the leaf tissue at similar levels and did not differ significantly. Washing the leaves did not cause a significant reduction in the bacterial load even in the plants contaminated at a later stage (28 dps). Thus, it can be concluded that the inoculated cells have tightly bound to the leaf tissue at a considerable level, which strongly recommends that *E. coli* serotypes colonized and strongly attached to leaf tissue even in a short period of time. Likewise, Wright & Holden (2018) also revealed washing with water did not cause in reduction in colonized *E. coli* O157:H7 strain Sakai populations on various types of microgreens. Consequently, it is quite vital to take necessary precautions to prevent contamination in the production sites since it could be quite challenging to eliminate colonized foodborne pathogens and a significant bacterial load may remain until harvest (30 dps).

The outcomes from the study of Liu et al. (2015) conducted in open fields showed that ambient temperature 3 days before harvest is one of the most important factors for the *E. coli* contamination and survival on leafy greens. Therefore, it could be concluded that variations in temperature due to seasonality and climate change might affect the survival of *E. coli* on fresh produce. This present study on the mimicking climate change scenarios revealed that temperature rise designed according to different mitigation scenarios did not have a statistically significant difference on

survival of *E. coli* O157:H7, O104:H4 and O26 cells on leaf in general. For all climate change scenarios studied, a considerable amount of bacterial load has been detected at harvest (30 dps), yet no particular pattern for survival of *E. coli* serotypes at different climate change scenarios was detected. More slightly attached and tightly bound cells of *E. coli* O157:H7, O104:H4 and O26 were detected on leaves contaminated at 28 dps at 15+2 ºC and 15+4 ºC than 15 ºC. However, a less population of slightly attached and tightly bound cells of *E. coli* serotypes were obtained at 15+6 ºC. According to former studies assessing the survival of *E. coli* on fresh produce have shown that under elevated ambient temperature, the attachment of *E. coli* on the leaf surface as well as irrigation water was higher (Castro-Ibáñez, Gil, Tudela, & Allende, 2015; Decol et al., 2017). According to this, more pathogen load on leaf is expected as the ambient temperature rises. However, in the present study, this trend in bacterial load was not observed in plants contaminated at the same growth stage. Nevertheless, it would be a good point to remark that temperature rise might alter the irrigation schedule, which might have an effect on produce safety in the field since the possibilities of contaminated irrigation water usage might increase (Decol et al., 2017).

3.3.2 Mimicking Contamination with Inoculated Seeds

Seed contamination is among one of the reasons why fresh produce could get contaminated in the field (Martinez et al., 2014). It has been shown that *E. coli* can persist on seeds up to two years and with germination, *E. coli* is able to translocate and internalize towards the phyllosphere of the fresh produce through the vascular tissues; xylem and phloem (Turner et al., 2019; Van der Linden et al., 2013; van Overbeek et al., 2020). A common problem in the sprout production is that enteric pathogens are very hard to disinfect after contamination. Hence, due to a probable contamination, seed contamination could result in devastating results for the human health (Riggio et al., 2019). The leafy green related *E. coli* outbreak occurred in 2011 in the Central Europe is one of the most well-known foodborne outbreaks and more than 3800 people were reported to be affected during this outbreak with 50 deaths. Although the traceback analysis did not detect the outbreak strain (*E. coli* O104:H4),

the suspected vehicle was claimed to be fenugreek seeds (Robert Koch Institute, 2011). Increase in the temperature might positively affect the pathogen presence in environment and persistence of foodborne pathogens in environment, especially irrigation water, could be improved by temperature rise (Decol et al., 2017). Thus, the present study evaluated the behavior of *E. coli* serotypes on cress seeds and how translocation of these pathogens to edible portions is changing with increased ambient temperatures. In this regard, the cress seeds were artificially contaminated by dipping seeds into suspension containing 8 log MPN/ ml pathogen of interest for 1 min. *E. coli* serotypes have adhered to seeds at a great level after 1 min of inoculation (from 6.2 log MPN/g seed to 8 log MPN/g seed) (Figure 3.11). The seed surface characteristics and bacterial cell surface hydrophobicity might influence the attachment to seeds as reported by Cui et al. (2017) and it has been revealed that the lowest attachment was obtained *E. coli* O104:H4 among *Salmonella enterica* and *E. coli* O157:H7 serotypes on alfalfa, lettuce, tomato and fenugreek seeds. Nevertheless, the present study showed that *E. coli* O104:H4 and O157:H7 adhered to cress seeds at a similar level except for 15^{o}C, lower cell populations were observed for *E. coli* O26 compared to *E. coli* O104:H4 and O157:H7 (Figure 3.11).

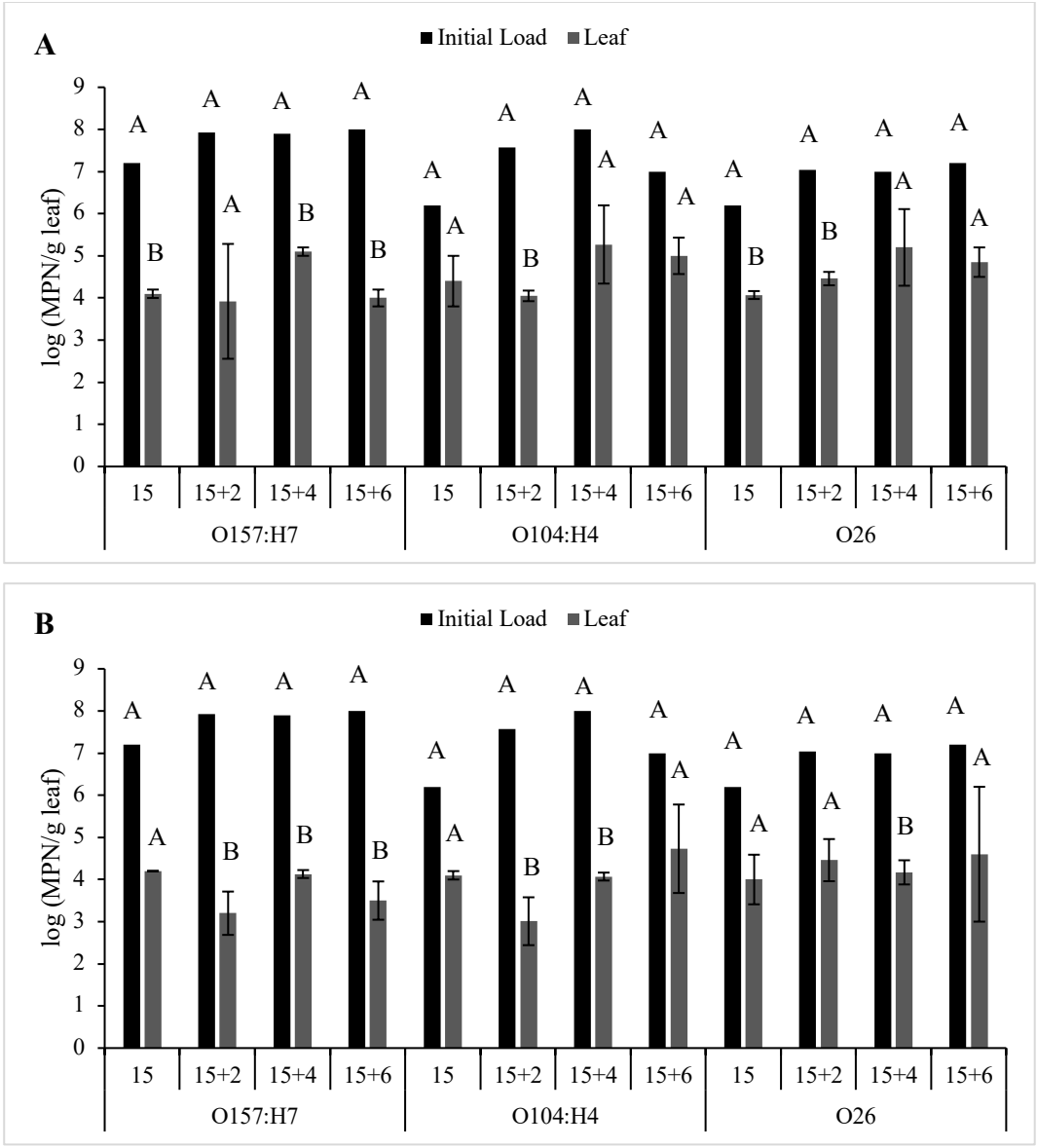


Figure 3.11 Translocation of *E. coli* serotypes from seeds to cress leaves. Surface-sterilized cress seeds were inoculated with *Escherichia coli* O157:H7, O104:H4 and O26 for 1 min at 10^8 MPN/ml level. Initial loads attached on seeds at the sowing day (log MPN/g seed) were also determined. Cress seeds were sown using autoclaved commercial peat and grown under controlled conditions in climate chamber with sterile irrigation at different climate change scenarios (15, 15+2, 15+4 and 15+6 ºC) at 65 % RH. Microbiological load (log MPN/g leaf) on cress leaves were determined at 30 days post-sowing (dps) for *E. coli* O157:H7, O104:H4 and O26. (A) reports slightly attached and tightly bound cells on cress leaves. (B) shows tightly bound cells on cress leaves in which leaves were washed in 5-ml BPW by vigorously

shaking to remove slightly attached cells prior to counting. Results were reported as the mean log (MPN/g leaf) of three biological replicates ± standard deviation. Statistical significance of difference in the means was analyzed with ANOVA followed by Tukey test. Values with different letters show significant difference ($p < 0.05$) between initial load on seeds and microbiological load on seeds at 30 dps at the same climate change scenario.

At all climate change scenarios, *E. coli* serotypes were translocated from inoculated seeds towards leaf tissue, and they were survived on leaf until harvest (30 dps). A substantial amount of initially inoculated cells was transferred to the leaf portion and the slightly attached and tightly bound cell populations were between 3.92 ± 1.4 log and 5.27 ± 0.9 log MPN/g leaf (Figure 3.11–A). Moreover, washing step did not eliminate all the attached bacteria and tightly bound cell populations on cress leaves were detected as from 3.01 ± 0.6 and 4.73 ± 1.0 log MPN/g leaf after washing (Figure 3.11–B). This means that nearly all the translocated bacteria towards leaf have tightly bound and they cannot be eliminated effectively by washing. Similar to contamination through irrigation water cases, the resulting bacterial levels were above the infectious dose of *E. coli* O157:H7, that is therefore critical for the food safety and human health. Xiao et al. (2014) earlier revealed that attachments of *E. coli* O157:H7 and O104:H4 on radish microgreens grown using contaminated seeds (4 log CFU/g) were similar. In addition, they reported that all the initial bacterial load was attained on the leaves, which were harvested 5 days after sowing. Radish seeds contaminated with a lower initial load of bacteria has resulted in microgreens with more pathogen load than the inoculum. Hence, *E. coli* O157:H7 and O104:H4 might multiply while translocating towards leaf tissue (Xiao et al., 2014). The most decrease in the bacterial load with respect to initial load of seeds has occurred at 15+2 ºC even though there is no significant difference among the climate change scenarios for *E. coli* serotypes. Gómez-Aldapa et al. (2013) revealed that 10 days after inoculation, different *E. coli* pathogroup populations on mung bean sprouts have increased from 2 log CFU/g to 5 and 7 log CFU/g at 20 and 30 ºC, respectively. However, since sprouting requires more humid environments unlike microgreen or

leafy green cultivations, humidity could have positively affected the microbial growth on mung bean sprouts. Therefore, it could be concluded that the difference in cultivation techniques could change the microbial behavior on germinating seeds, and temperature rise could lead to increase in the *E. coli* populations in humid environments. All in all, this present study concludes that seed contamination with *E. coli* could lead to translocation of pathogens to edible portions from seeds and substantial attachment on leaf tissue.

3.4 Biofilm Structure Visualization on Leaf by Scanning Electron Microscope

Leaf surface presents a hostile environment for the survival of enteric pathogens. That's why the leaf surface is not a primary host for human enteric pathogens. However, they are considered to be alternative hosts (Dublan et al., 2014). Pathogens use biofilm structures to protect themselves from UV radiation, nutrient limitations, and desiccation on leaf surface. Therefore, the human enteric pathogens could use two ways to achieve survival against unfavorite environment, which are either forming a biofilm matrix or internalizing through the inside of the plant. However, bacterial pathogens cannot internalize through the plant tissue since they do not have the enzymes required for the degradation of cuticle and plant cell wall, so natural openings on the leaf are used to further internalization (Melotto et al., 2008). For the persistence of foodborne pathogens on leave, the biofilm forming ability is also important. It has been demonstrated that *E. coli* O157:H7 can internalize into the plant tissue (Roy & Melotto, 2019). Thus, human enteric pathogens that reside near stomata or other natural openings found on the leaf structure, such as hydathodes, lenticels, and nectarthodes, could further colonize and internalize into the plant material where these pathogens can reach nutrient-rich sites so that they can avoid restricted nutrient resources and harsh conditions on leaf. Among the natural openings present on leaf structure, stomata are one of the most preferred routes for the bacterial entry to the inside of the plant since stomata outnumber the other natural openings. To analyze where the *E. coli* serotypes used in this study localize on the leaf tissue, the leaf surfaces were analyzed with SEM. The broad and well-grown

leaves were collected at harvest day (30 dps) from cress plants contaminated through irrigation water at 7, 14, 21 and 28 dps and cress plants grown using contaminated seeds. In control samples, no bacterial communities near stomata or on the leaf crevices were detected (Figure 3.12).

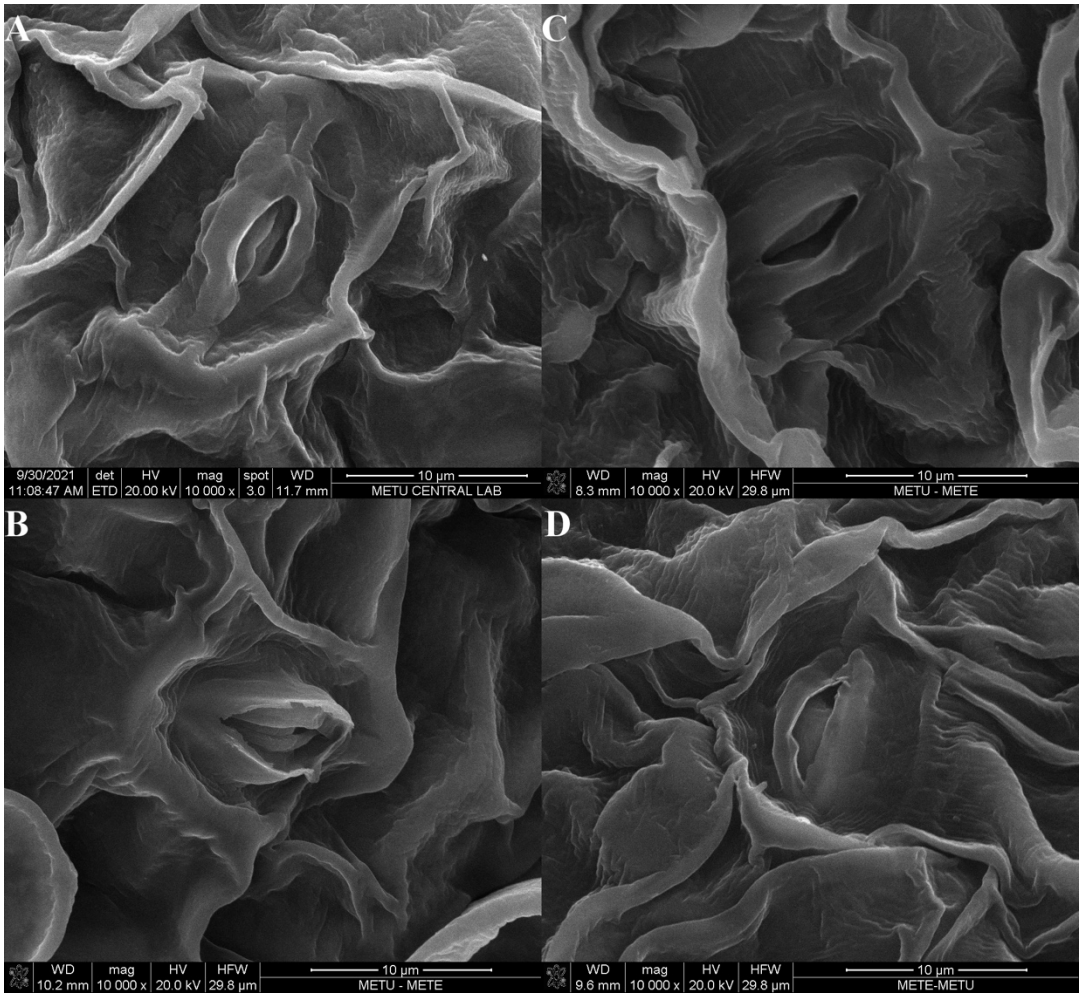


Figure 3.12 The control samples only irrigated with sterile distilled water and grown with surface-sterilized seeds and autoclaved commercial peat. The samples were grown at 15 ºC (A), 15+2 ºC (B), 15+4 ºC (C), 15+6 ºC (D). Scale bars: 10 µm.

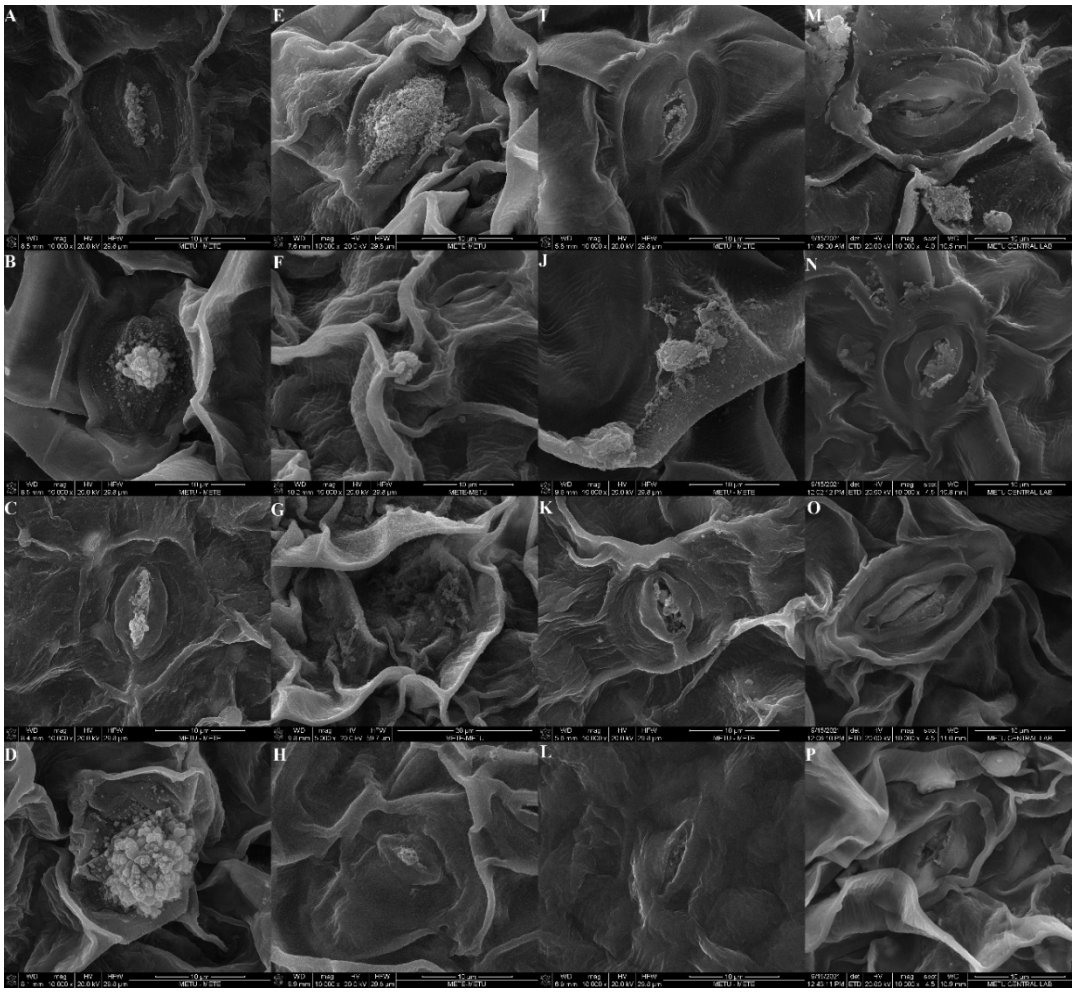


Figure 3.13 Attachment of *E. coli* O157:H7 serotype to cress leaves at different climate change scenarios (A-D: 15 ºC; E-H: 15+2 ºC; I-L: 15+4 ºC; M-P: 15+6 ºC). Leaf samples were irrigated with O157:H7 bacterial suspension at 7, 14, 21 and 28 dps by spraying (A, E, I, M: leaves contaminated at 7 dps; B, F, J, N: leaves contaminated at 14 dps; C, G, K, O: leaves contaminated at 21 dps; D, H, L, P: leaves contaminated at 28 dps). Scale bars: 10 µm except for G=30 µm.

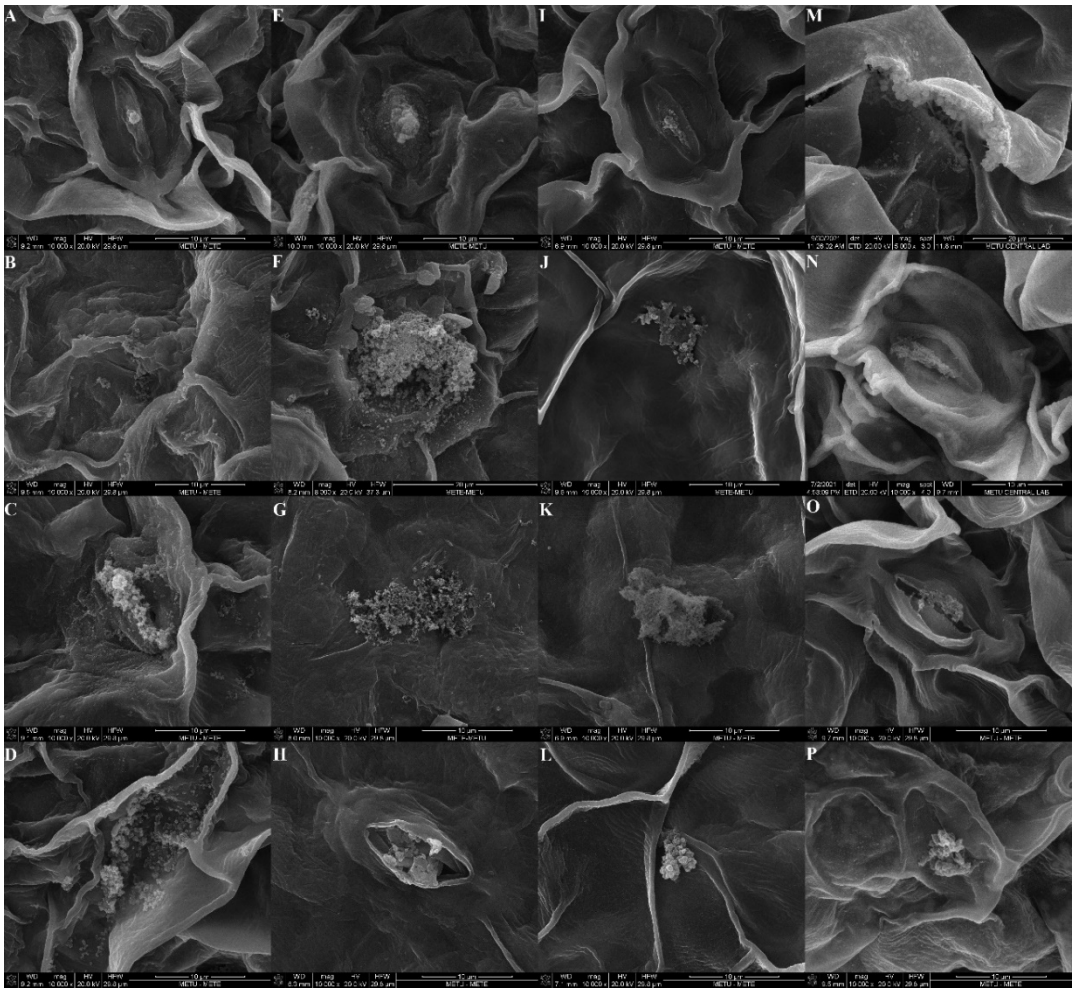


Figure 3.14 Attachment of *E. coli* O104:H4 serotype to cress leaves at different climate change scenarios (A-D: 15 ºC; E-H: 15+2 ºC; I-L: 15+4 ºC; M-P: 15+6 ºC). Leaf samples were irrigated with O104:H4 bacterial suspension at 7, 14, 21 and 28 dps by spraying (A, E, I, M: leaves contaminated at 7 dps; B, F, J, N: leaves contaminated at 14 dps; C, G, K, O: leaves contaminated at 21 dps; D, H, L, P: leaves contaminated at 28 dps). Scale bars: 10 µm except for M, F=20 µm.

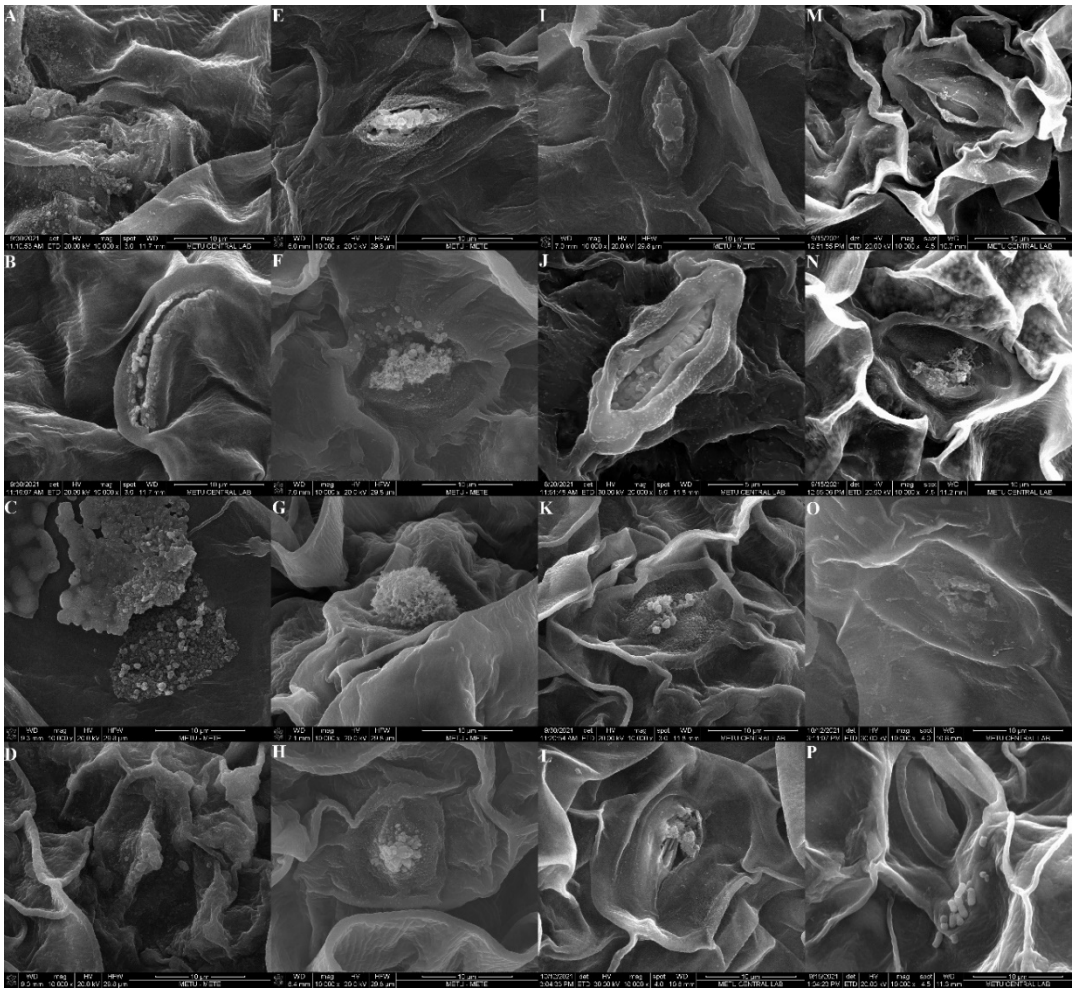


Figure 3.15 Attachment of *E. coli* O26 serotype to cress leaves at different climate change scenarios (A-D: 15 ºC; E-H: 15+2 ºC; I-L: 15+4 ºC; M-P: 15+6 ºC). Leaf samples were irrigated with O26 bacterial suspension at 7, 14, 21 and 28 dps by spraying (A, E, I, M: leaves contaminated at 7 dps; B, F, J, N: leaves contaminated at 14 dps; C, G, K, O: leaves contaminated at 21 dps; D, H, L, P: leaves contaminated at 28 dps). Scale bars: 10 µm except for J=5 µm.

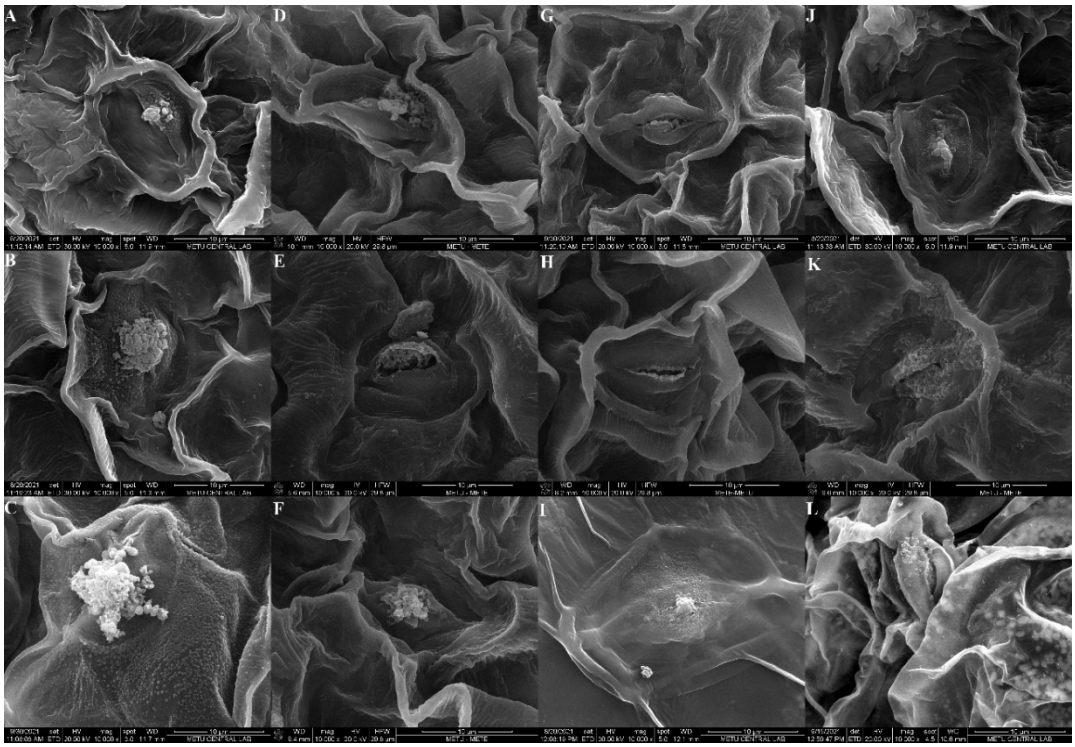


Figure 3.16 Attachment of *E. coli* O157:H7, O104:H4 and O26 serotypes to cress leaves at different climate change scenarios (A-C: 15 ºC; D-F: 15+2 ºC; G-I: 15+4 ºC; J-L: 15+6 ºC). Plants were grown using seeds contaminated with O157:H7, O104:H4 and O26 (A, D, G, J: leaves contaminated with *E. coli* O157:H7; B, E, H, K: leaves contaminated with *E. coli* O104:H4; C, F, I, L: leaves contaminated with *E. coli* O26). Scale bars: 10 µm.

E. coli serotypes used in this study attached to cress as the bacterial attachment assays show and as well as, they were visualized under the SEM (Figure 3.13, Figure 3.14, Figure 3.15 and Figure 3.16). As compatible with the earlier evidence, bacterial aggregates were visualized on either stomata or leaf crevices on leaf structure. Yet, no differences have been detected in the architecture of the aggregation patterns with respect to both inoculation time and climate change scenarios studied. The rod-shaped single cells were identified on leaf inoculated with *E. coli* O26 at 28 dps at 15+6 ºC (Figure 3.15–P). Detection of single cells under SEM was not common. Even in the plants inoculated at 28 dps, the aggregated structures on leaf were detected though their harvesting time (30 dps) was three days ahead their inoculation

(28 dps). This result is in accordance with the results reported for tightly bound cells recorded at 28 dps (5-7 log MPN/g leaf) for all *E. coli* serotypes and climate change scenarios (Figure 3.10). In conclusion, all the *E. coli* serotypes did manage to attach and form biofilm matrices to protect themselves regardless of their inoculation time on the leaf.

CHAPTER 4

CONCLUSIONS

The current study provides a comprehensive understanding for the survival and persistence of different *E. coli* pathogroups on cress leaves at different climate change scenarios. The fresh produces are the materials which are consumed in their raw or minimally processed forms in the salads or ready-to-eat foods. A probable contamination of fresh produce in the field could result in a considerable amount of pathogen load on the edible portions. The results of the present study revealed that increase in ambient temperature which was designed according to mitigation scenarios for climate change proposed by Intergovernmental Panel on Climate Change (IPCC) did not lead to any significant increase in the survival of pathogens on the leaf tissue. The *E. coli* serotypes were introduced at different routes (seed and leaves) and at different periods during growth (7, 14, 21 and 28 dps). The survival of *E. coli* O157:H7, O104:H4 and O26 did not differ significantly with respect to temperature, inoculation time and inoculation route; however, the pathogen load detected on the cress leaves was reported to be above the infection doses of *E. coli* O157:H7 and O104:H4. It was also demonstrated that *E. coli* O157:H7, O104:H4 and O26 have transferred from contaminated seeds to the leaf portions within a 30-day growth period.

One of the striking results of the present study was that washing did not cause a considerable reduction in the pathogen load on cress leaves, regardless of the inoculation route was seed, or the plant was inoculated a very early stage (7 dps). This means that nearly all the pathogenic bacteria inoculated on the plants have tightly bounded to the leaf tissue so that a simple washing practice could not eliminate the bacterial load on the leaves. Additionally, the SEM visuals validate the presence of residing cell aggregates near and on the stomata. Thus, it is quite vital to follow and implement Good Agricultural Practices to prevent the contamination of the fresh produce in the field. Furthermore, it is also important to reinforce the Good

Hygiene Practices, Good Manufacturing Practices and Good Storage Practices to be able to supply safe food from farm to fork continuum. Here would be decent to underline the significance of "One Health" approach. Contamination of fresh produce in the field is dependent on so many factors from animals to water sources used for irrigation. Hence, having a collaboration between the scientists from other fields would present more solid solutions to produce safer food. Thus, "One Health" concept should be adopted to predict and analyze (i) the behavior of foodborne pathogens in the environment, (ii) how they are spread to water sources, manure, seeds, or other contamination sources, and (iii) how the climatic factors, concerning not only temperature rise, but also changes in precipitation pattern, drought, and changes in concentration of greenhouse gases in atmosphere, affect the bacterial load on the cress leaves to present comprehensive solutions.

To conclude, the present study investigates and compares the survival of *E. coli* O157:H7, O104:H4 and O26 under the same controlled conditions and shows that protective measures should be taken to prevent a probable contamination in the field. The understanding of *E. coli* O104:H4 survival on fresh produce is also essential since it may shed light on potential threats that can be resulted from similar hybrid pathogroup bacteria in future.

Last but not least, the present study did evaluate the survival of *E. coli* under controlled conditions. The seeds, growing mediums, water and pots were all sterilized prior to use to be able to present the survival capability of solely *E. coli* pathogroups on fresh produce. However, this is not the case in real life. The soil or the plants have their own microbiome, and the microbiome might present a more challenging and competitive environment for the *E. coli* survival. Furthermore, open field makes everything even harder. The possibilities of where a contamination could come from are tremendous in an open field with extreme weather conditions or with a bird itself. In addition, temperature rise is not the one possible outcome of climate change. Humidity, cloudiness, changes in the precipitation patterns or any other climatic factors could affect the bacterial survival on fresh produce. Therefore, the analysis of these factors could present a more comprehensive understanding towards relationship between climate change and pathogen survival on fresh produce.

REFERENCES

Afset, J. E., Bevanger, L., Romundstad, P., & Bergh, K. (2004). Association of atypical enteropathogenic *Escherichia coli* (EPEC) with prolonged diarrhoea. *Journal of Medical Microbiology*, *53*(11), 1137–1144. https://doi.org/10.1099/jmm.0.45719-0

Ahmed, W., Gardner, T., & Toze, S. (2011). Microbiological quality of roof-harvested rainwater and health risks: A review. *Journal of Environmental Quality*, *40*(1), 13–21. https://doi.org/10.2134/jeq2010.0345

Allende, A., Castro-Ibáñez, I., Lindqvist, R., Gil, M. I., Uyttendaele, M., & Jacxsens, L. (2017). Quantitative contamination assessment of *Escherichia coli* in baby spinach primary production in Spain: Effects of weather conditions and agricultural practices. *International Journal of Food Microbiology*, *257*, 238–246. https://doi.org/10.1016/j.ijfoodmicro.2017.06.027

Allende, A., & Monaghan, J. (2015). Irrigation water quality for leafy crops: A perspective of risks and potential solutions. *International Journal of Environmental Research and Public Health*, *12*(7), 7457–7477. https://doi.org/10.3390/ijerph120707457

Alum, A., Enriquez, C., & Gerba, C. P. (2011). Impact of drip ırrigation method, soil, and virus type on tomato and cucumber contamination. *Food and Environmental Virology*, *3*(2), 78–85. https://doi.org/10.1007/s12560-011-9060-y

Amoah, P., Drechsel, P., & Abaidoo, R. C. (2005). Irrigated urban vegetable production in Ghana: Sources of pathogen contamination and health risk elimination. *Irrigation and Drainage*, *54*(SUPPL. 1), 49–61. https://doi.org/10.1002/ird.185

Arrus, K. M., Holley, R. A., Ominski, K. H., Tenuta, M., & Blank, G. (2006). Influence of temperature on *Salmonella* survival in hog manure slurry and seasonal temperature profiles in farm manure storage reservoirs. *Livestock Science*, *102*(3), 226–236. https://doi.org/10.1016/j.livsci.2006.03.021

Barkocy-Gallagher, G. A., Arthur, T. M., Rivera-Betancourt, M., Nou, X., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2003). Seasonal prevalence of Shiga Toxin-Producing *Escherichia coli*, Including O157:H7 and Non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *Journal of Food Protection*, *66*(11), 1978–1986. https://doi.org/10.4315/0362-028X-66.11.1978

Behiry, I. K., Abada, E. A., Ahmed, E. A., & Labeeb, R. S. (2011). Enteropathogenic *Escherichia coli* associated with diarrhea in children in Cairo, Egypt. *TheScientificWorldJournal*, *11*, 2613–2619. https://doi.org/10.1100/2011/485381

Berends, I. M. G. A., Graat, E. A. M., Swart, W. A. J. M., Weber, M. F., van de Giessen, A. W., Lam, T. J. G. M., Heuvelink, A. E., & van Weering, H. J. (2008). Prevalence of VTEC O157 in dairy and veal herds and risk factors for veal herds. *Preventive Veterinary Medicine*, *87*(3–4), 301–310. https://doi.org/10.1016/j.prevetmed.2008.05.004

Bergholz, T. M., Wick, L. M., Qi, W., Riordan, J. T., Ouellette, L. M., & Whittam, T. S. (2007). Global transcriptional response of *Escherichia coli* O157:H7 to growth transitions in glucose minimal medium. *BMC Microbiology*, *7*, 1–27. https://doi.org/10.1186/1471-2180-7-97

Bieber, D., Ramer, S. W., Wu, C. Y., Murray, W. J., Tobe, T., Fernandez, R., & Schoolnik, G. K. (1998). Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli. Science*, *280*(5372), 2114–2118. https://doi.org/10.1126/science.280.5372.2114

Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., Peters, G., & Karch, H. (2011). Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: A microbiological study. *The Lancet Infectious Diseases*, *11*(9), 671–676. https://doi.org/10.1016/S1473-3099(11)70165-7

Brandl, M. T., & Amundson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *Applied*

and Environmental Microbiology, *74*(8), 2298–2306. https://doi.org/10.1128/AEM.02459-07

Brichta-Harhay, D. M., Guerini, M. N., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2008). *Salmonella* and *Escherichia coli* O157:H7 contamination on hides and carcasses of cull cattle presented for slaughter in the United States: An evaluation of prevalence and bacterial loads by immunomagnetic separation and direct plating methods. *Applied and Environmental Microbiology*, *74*(20), 6289–6297. https://doi.org/10.1128/AEM.00700-08

Carstens, C. K., Salazar, J. K., & Darkoh, C. (2019). Multistate outbreaks of foodborne illness in the United States associated with fresh produce from 2010 to 2017. *Frontiers in Microbiology*, *10*(November), 1–15. https://doi.org/10.3389/fmicb.2019.02667

Castillo, A., Mercado, I., Lucia, L. M., Martínez-Ruiz, Y., Ponce De León, J., Murano, E. A., & Acuff, G. R. (2004). *Salmonella* contamination during production of cantaloupe: A binational study. *Journal of Food Protection*, *67*(4), 713–720. https://doi.org/10.4315/0362-028X-67.4.713

Castro-Ibáñez, I., Gil, M. I., Tudela, J. A., & Allende, A. (2015). Microbial safety considerations of flooding in primary production of leafy greens: A case study. *Food Research International*, *68*, 62–69. https://doi.org/10.1016/j.foodres.2014.05.065

Castro-Ibáñez, I., Gil, M. I., Tudela, J. A., Ivanek, R., & Allende, A. (2015). Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain. *Food Microbiology*, *49*, 173–181. https://doi.org/10.1016/j.fm.2015.02.004

CDC. (2002). Surveillance for Waterborne-Disease Outbreaks — United States, 1999–2000 Sherline. *Morbidity and Mortality Weekly Report*, *51*, 1994–1999.

CDC. (2012). *Principles of Epidemiology in Public Health Practice, Third Edition An Introduction to Applied Epidemiology and Biostatistics: Lesson 1: Introduction to Epidemiology*.

https://www.cdc.gov/csels/dsepd/ss1978/lesson1/index.html

CDC. (2014). *Questions and answers*. https://doi.org/10.1016/j.actpha.2017.09.025

CDC. (2018a). *Burden of Foodborne Illness: Findings*. Centers for Disease Control and Prevention. http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html

CDC. (2018b). *Multistate Outbreak of E. coli O157:H7 Infections Linked to Romaine Lettuce (Final Update)*. https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.

CDC. (2020a). *Outbreak of E. coli Infections Linked to Clover Sprouts*. https://doi.org/10.1111/ajt.15211

CDC. (2020b). *Refrigerate Food Quickly*. https://www.cdc.gov/foodsafety/newsletter/refrigerate-food-quickly.html

CDC. (2022). *One Health Basics The Foundation of One Health*. https://www.cdc.gov/onehealth/basics/index.html

CDC NORS Dashboard. (2018). *Foodborne Person to Person Waterborne Animal Contact Environmental Indeterminate / Unknown*. https://wwwn.cdc.gov/norsdashboard/

Cevallos-Cevallos, J. M., Danyluk, M. D., Gu, G., Vallad, G. E., & Van Bruggen, A. H. C. (2012). Dispersal of *Salmonella* Typhimurium by rain splash onto tomato plants. *Journal of Food Protection*, *75*(3), 472–479. https://doi.org/10.4315/0362-028X.JFP-11-399

Chandler, D. S., & Craven, J. A. (1980). Relationship of soil moisture to survival of *Escherichia coli* and *Salmonella* Typhimurium in soils. *Australian Journal of Agricultural Research*, *31*(3), 547–555. https://doi.org/10.1071/AR9800547

Chitarra, W., Decastelli, L., Garibaldi, A., & Gullino, M. L. (2014). Potential uptake of *Escherichia coli* O157:H7 and *Listeria monocytogenes* from growth substrate into leaves of salad plants and basil grown in soil irrigated with contaminated water. *International Journal of Food Microbiology*, *189*, 139–145. https://doi.org/10.1016/j.ijfoodmicro.2014.08.003

Codex Alimentarius Commission. (2013). *Code of Hygienic Practice for Fresh Fruits and Vegetables: Vol. CAC/RCP 53*. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/tr/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXC%2B53-2003%252FCXC_053e.pdf

Codex Alimentarius Commission. (2020). *Codex Alimentarius Commission General Principles of Food Hygiene CXC 1-1969*. https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXC%2B1-1969%252FCXC_001e.pdf

Cooley, M. B., Miller, W. G., & Mandrell, R. E. (2003). Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.69.8.4915-4926.2003

Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M. T., Myers, C., Rose, C., Keys, C., Farrar, J., & Mandrell, R. E. (2007). Incidence and tracking of *Escherichia coli* O157:H7 in a major produce production region in California. *PLoS ONE*, *2*(11). https://doi.org/10.1371/journal.pone.0001159

Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M., & Finlay, B. B. (2013). Recent advances in understanding enteric pathogenic *Escherichia coli. Clinical Microbiology Reviews*, *26*(4), 822–880. https://doi.org/10.1128/CMR.00022-13

Cui, Y., Walcott, R., & Chen, J. (2017). Differential attachment of *Salmonella enterica* and Enterohemorrhagic *Escherichia coli* to alfalfa, fenugreek, lettuce, and tomato seeds. *Applied and Environmental Microbiology*, *83*(7), 1–12.

Cull, C. A., Renter, D. G., Dewsbury, D. M., Noll, L. W., Shridhar, P. B., Ives, S. E., Nagaraja, T. G., & Cernicchiaro, N. (2017). Feedlot- and pen-level prevalence of enterohemorrhagic *Escherichia coli* in feces of commercial

feedlot cattle in two major U.S. cattle feeding areas. *Foodborne Pathogens and Disease*, *14*(6), 309–317. https://doi.org/10.1089/fpd.2016.2227

de Oliveira Elias, S., Noronha, T. B., & Tondo, E. C. (2018). Assessment of *Salmonella* spp. and *Escherichia coli* O157:H7 growth on lettuce exposed to isothermal and non-isothermal conditions. *Food Microbiology*, *72*, 206–213. https://doi.org/10.1016/j.fm.2017.11.016

Decol, L. T., Casarin, L. S., Hessel, C. T., Batista, A. C. F., Allende, A., & Tondo, E. C. (2017). Microbial quality of irrigation water used in leafy green production in Southern Brazil and its relationship with produce safety. *Food Microbiology*, *65*, 105–113. https://doi.org/10.1016/j.fm.2017.02.003

DeLeon-Rodriguez, N., Lathem, T. L., Rodriguez-R, L. M., Barazesh, J. M., Anderson, B. E., Beyersdorf, A. J., Ziemba, L. D., Bergin, M., Nenes, A., & Konstantinidis, K. T. (2013). Microbiome of the upper troposphere: Species composition and prevalence, effects of tropical storms, and atmospheric implications. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(7), 2575–2580. https://doi.org/10.1073/pnas.1212089110

Destoumieux-Garzón, D., Mavingui, P., Boetsch, G., Boissier, J., Darriet, F., Duboz, P., Fritsch, C., Giraudoux, P., Roux, F. Le, Morand, S., Paillard, C., Pontier, D., Sueur, C., & Voituron, Y. (2018). The one health concept: 10 years old and a long road ahead. *Frontiers in Veterinary Science*, *5*(FEB), 1–13. https://doi.org/10.3389/fvets.2018.00014

Dorner, S. M., Anderson, W. B., Slawson, R. M., Kouwen, N., & Huck, P. M. (2006). Hydrologic modeling of pathogen fate and transport. *Environmental Science and Technology*, *40*(15), 4746–4753. https://doi.org/10.1021/es060426z

Dublan, M. D. L. A., Ortiz-Marquez, J. C. F., Lett, L., & Curatti, L. (2014). Plant-adapted *Escherichia coli* show increased lettuce colonizing ability, resistance to oxidative stress and chemotactic response. *PLoS ONE*, *9*(10). https://doi.org/10.1371/journal.pone.0110416

Erickson, M. C., Webb, C. C., Davey, L. E., Payton, A. S., Flitcroft, I. D., & Doyle, M. P. (2014). Biotic and abiotic variables affecting internalization and fate of *Escherichia coli* O157:H7 isolates in leafy green roots. *Journal of Food Protection*, *77*(6), 872–879. https://doi.org/10.4315/0362-028X.JFP-13-432

European Commission (EC). (2004). *Regulation (EC) No 852/2004 of the European Parliament and of th Council on the Hygiene of foodstuffs*. https://eur-lex.europa.eu/eli/reg/2004/852/oj

European Commission (EC). (2013). *Commission Implementing Regulation (EU) No 208/2013 of 11 March 2013 on traceability requirements for sprouts and seeds intended for the production of sprouts.* http://www.legislation.gov.uk/eur/2013/208/body/adopted

FDA, U. S. F. and D. A. (2019). FSMA Final Rule on Produce Safety. *Food and Drug Administration*, *000*, 1–15.

Feng, P. (2017). Pathogenic Escherichia coli Group. In *Bad Bug Book: Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins* (2nd ed., pp. 68–81). Center for Food Safety and Applied Nutrition, Food and Drug Administration. https://doi.org/10.1016/B978-0-323-40181-4.00148-1

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, *8*(9), 623–633. https://doi.org/10.1038/nrmicro2415

Food Availability (Per Capita) Data System. Summary FindingsUSDA. (2020). *Food Availability (Per Capita) Data System. Summary Findings*. USDA's Economic Research Service United States Department of Agriculture. Economic Research Service. https://www.ers.usda.gov/data-products/food-availability-per-capita-data-system/

Forslund, A., Ensink, J. H. J., Markussen, B., Battilani, A., Psarras, G., Gola, S., Sandei, L., Fletcher, T., & Dalsgaard, A. (2012). *Escherichia coli* contamination and health aspects of soil and tomatoes (Solanum lycopersicum L.) subsurface drip irrigated with on-site treated domestic wastewater. *Water Research*, *46*(18), 5917–5934. https://doi.org/10.1016/j.watres.2012.08.011

Fouladkhah, A., Geornaras, I., & Sofos, J. N. (2013). Biofilm Formation of o157

and non-O157 Shiga Toxin-Producing *Escherichia coli* and multidrug-resistant and susceptible *Salmonella* Typhimurium and Newport and their inactivation by sanitizers. *Journal of Food Science*, *78*(6). https://doi.org/10.1111/1750-3841.12123

Franz, E., Semenov, A. V., & Van Bruggen, A. H. C. (2008). Modelling the contamination of lettuce with *Escherichia coli* O157:H7 from manure-amended soil and the effect of intervention strategies. *Journal of Applied Microbiology*, *105*(5), 1569–1584. https://doi.org/10.1111/j.1365-2672.2008.03915.x

Franz, Eelco, Semenov, A. V., Termorshuizen, A. J., De Vos, O. J., Bokhorst, J. G., & Van Bruggen, A. H. C. (2008). Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils. *Environmental Microbiology*, *10*(2), 313–327. https://doi.org/10.1111/j.1462-2920.2007.01453.x

Franz, Eelco, & Van Bruggen, A. H. C. (2008). Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. *Critical Reviews in Microbiology*, *34*(3–4), 143–161. https://doi.org/10.1080/10408410802357432

Fremaux, B., Prigent-Combaret, C., & Vernozy-Rozand, C. (2008). Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: An updated review. *Veterinary Microbiology*, *132*(1–2), 1–18. https://doi.org/10.1016/j.vetmic.2008.05.015

Froelich, B. A., Williams, T. C., Noble, R. T., & Oliver, J. D. (2012). Apparent loss of vibrio vulnificus from north carolina oysters coincides with a drought-induced increase in salinity. *Applied and Environmental Microbiology*, *78*(11), 3885–3889. https://doi.org/10.1128/AEM.07855-11

Fukushima, H., Hoshina, K., & Gomyoda, M. (1999). Long-term survival of Shiga toxin-producing *Escherichia coli* O26, O111, and O157 in bovine feces. *Applied and Environmental Microbiology*, *65*(11), 5177–5181. https://doi.org/10.1128/aem.65.11.5177-5181.1999

García, R., Bælum, J., Fredslund, L., Santorum, P., & Jacobsen, C. S. (2010). Influence of temperature and predation on survival of *Salmonella enterica* serovar Typhimurium and expression of inva in soil and manure-amended soil. *Applied and Environmental Microbiology*, *76*(15), 5025–5031. https://doi.org/10.1128/AEM.00628-10

Gautam, R., Bani-Yaghoub, M., Neill, W. H., Döpfer, D., Kaspar, C., & Ivanek, R. (2011). Modeling the effect of seasonal variation in ambient temperature on the transmission dynamics of a pathogen with a free-living stage: Example of *Escherichia coli* O157:H7 in a dairy herd. *Preventive Veterinary Medicine*, *102*(1), 10–21. https://doi.org/10.1016/j.prevetmed.2011.06.008

Ge, C., Lee, C., & Lee, J. (2012). The impact of extreme weather events on *Salmonella* internalization in lettuce and green onion. *Food Research International*, *45*(2), 1118–1122. https://doi.org/10.1016/j.foodres.2011.06.054

Gelting, R. (2007). Investigation of an *Escherichia coli* O157:H7 Outbreak Associated with Dole Pre-Packaged Spinach. *Centers for Disease Control and Prevention (CDC) National Center for Environmental Health/Environmental Health Services Branch*, 1–24.

Gil, A. I., Louis, V. R., Rivera, I. N. G., Lipp, E., Huq, A., Lanata, C. F., Taylor, D. N., Russek-Cohen, E., Choopun, N., Sack, R. B., & Colwell, R. R. (2004). Occurrence and distribution of *Vibrio cholerae* in the coastal environment of Peru. *Environmental Microbiology*, *6*(7), 699–706. https://doi.org/10.1111/j.1462-2920.2004.00601.x

Gómez-Aldapa, C. A., Rangel-Vargas, E., Bautista-De León, H., Vázquez-Barrios, M. E., Gordillo-Martínez, A. J., & Castro-Rosas, J. (2013). Behavior of enteroaggregative *Escherichia coli,* non-O157-shiga toxin-producing *E. coli,* enteroinvasive *E. coli*, enteropathogenic *E. coli* and enterotoxigenic *E. coli* strains on mung bean seeds and sprout. *International Journal of Food Microbiology*, *166*(3), 364–368. https://doi.org/10.1016/j.ijfoodmicro.2013.08.008

Green, B. W. (2015). Fertilizers in aquaculture. In *Feed and Feeding Practices in Aquaculture*. Elsevier Ltd. https://doi.org/10.1016/b978-0-08-100506-4.00002-7

Griffin, D. W. (2007). Atmospheric movement of microorganisms in clouds of desert dust and implications for human health. *Clinical Microbiology Reviews*, *20*(3), 459–477. https://doi.org/10.1128/CMR.00039-06

Gu, G., Hu, J., Cevallos-Cevallos, J. M., Richardson, S. M., Bartz, J. A., & van Bruggen, A. H. C. (2011). Internal colonization of *Salmonella enterica* serovar Typhimurium in tomato plants. *PLoS ONE*, *6*(11). https://doi.org/10.1371/journal.pone.0027340

Gutiérrez-Rodríguez, E., Gundersen, A., Sbodio, A. O., & Suslow, T. V. (2011). Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach. *Journal of Applied Microbiology*, *112*(1), 109–118. https://doi.org/10.1111/j.1365-2672.2011.05184.x

Harris, J. R., Mariano, J., Wells, J. G., Payne, B. J., Donnell, H. D., & Cohen, M. L. (1985). Person-to-person transmission in an outbreak of enteroinvasive *Escherichia coli. American Journal of Epidemiology*, *122*(2), 245–252. https://doi.org/10.1093/oxfordjournals.aje.a114095

Hartmann, R., Fricke, A., Stützel, H., Mansourian, S., Dekker, T., Wohanka, W., & Alsanius, B. (2017). Internalization of *Escherichia coli* O157:H7 gfp+ in rocket and Swiss chard baby leaves as affected by abiotic and biotic damage. *Letters in Applied Microbiology*, *65*(1), 35–41. https://doi.org/10.1111/lam.12742

Hebbelstrup Jensen, B., Olsen, K. E. P., Struve, C., Krogfelt, K. A., & Petersen, A. M. (2014). Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli. Clinical Microbiology Reviews*, *27*(3), 614–630. https://doi.org/10.1128/CMR.00112-13

Hellberg, R. S., & Chu, E. (2016). Effects of climate change on the persistence and dispersal of foodborne bacterial pathogens in the outdoor environment: A

review. *Critical Reviews in Microbiology*, *42*(4), 548–572. https://doi.org/10.3109/1040841X.2014.972335

Herman, K. M., Hall, A. J., & Gould, L. H. (2015). Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. *Epidemiology and Infection*, *143*(14), 3011–3021. https://doi.org/10.1017/S0950268815000047

Hofstra, N. (2011). Quantifying the impact of climate change on enteric waterborne pathogen concentrations in surface water. *Current Opinion in Environmental Sustainability*, *3*(6), 471–479. https://doi.org/10.1016/j.cosust.2011.10.006

Holvoet, K., Sampers, I., Seynnaeve, M., & Uyttendaele, M. (2014). Relationships among hygiene indicators and enteric pathogens in irrigation water, soil and lettuce and the impact of climatic conditions on contamination in the lettuce primary production. *International Journal of Food Microbiology*, *171*, 21–31. https://doi.org/10.1016/j.ijfoodmicro.2013.11.009

Hong, W., Wu, Y. E., Fu, X., & Chang, Z. (2012). Chaperone-dependent mechanisms for acid resistance in enteric bacteria. *Trends in Microbiology*, *20*(7), 328–335. https://doi.org/10.1016/j.tim.2012.03.001

IPCC. (2013). *Climate Change 2013: The Physical Science Basis*. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

IPCC. (2014). *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]*.

IPCC. (2018). Summary for Policymakers. In *Ipcc - Sr15* (Vol. 2, Issue October). www.environmentalgraphiti.org

Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2005). Survival of *Escherichia coli* O157:H7 in soil and on carrots and onions grown in fields treated with contaminated manure composts or irrigation water. *Food Microbiology*, *22*(1), 63–70. https://doi.org/10.1016/j.fm.2004.04.007

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2004). Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes

grown in fields treated with contaminated manure composts or irrigation water. *Applied and Environmental Microbiology*, *70*(4), 2497–2502. https://doi.org/10.1128/AEM.70.4.2497-2502.2004

Itoh, Y., Nagano, I., Kunishima, M., & Ezaki, T. (1997). Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. *Journal of Clinical Microbiology*, *35*(10), 2546–2550. https://doi.org/10.1128/jcm.35.10.2546-2550.1997

Jang, H., & Matthews, K. R. (2018). Survival and interaction of *Escherichia coli* O104:H4 on *Arabidopsis thaliana* and lettuce (*Lactuca sativa*) in comparison to *E. coli* O157:H7: Influence of plant defense response and bacterial capsular polysaccharide. *Food Research International*, *108*(March), 35–41. https://doi.org/10.1016/j.foodres.2018.03.029

Jongman, M., & Korsten, L. (2018). Irrigation water quality and microbial safety of leafy greens in different vegetable production systems: A review. *Food Reviews International*, *34*(4), 308–328. https://doi.org/10.1080/87559129.2017.1289385

Kim, Y. S., Park, K. H., Chun, H. S., Choi, C., & Bahk, G. J. (2015). Correlations between climatic conditions and foodborne disease. *Food Research International*, *68*, 24–30. https://doi.org/10.1016/j.foodres.2014.03.023

Kinnula, S., Hemminki, K., Kotilainen, H., Ruotsalainen, E., Tarkka, E., & Salmenlinna, S. (2018). *Outbreak of multiple strains of non-O157 Shiga toxin-producing and enteropathogenic Escherichia coli associated with rocket salad , Finland , autumn 2016*. 1–8.

Kohler, J., Knapp, B. A., Waldhuber, S., Caravaca, F., Roldán, A., & Insam, H. (2010). Effects of elevated CO2, water stress, and inoculation with Glomus intraradices or *Pseudomonas mendocina* on lettuce dry matter and rhizosphere microbial and functional diversity under growth chamber conditions. *Journal of Soils and Sediments*, *10*(8), 1585–1597. https://doi.org/10.1007/s11368-010-0259-6

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., &

Sela, S. (2009). Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Applied and Environmental Microbiology*, *75*(19), 6076–6086. https://doi.org/10.1128/AEM.01084-09

Kroupitski, Y., Gollop, R., Belausov, E., Pinto, R., & Sela, S. (2019). *Salmonella enterica* growth conditions influence lettuce leaf internalization. *Frontiers in Microbiology*, *10*(MAR), 1–11. https://doi.org/10.3389/fmicb.2019.00639

Lee, J. I., Kim, S. S., & Kang, D. H. (2019). Susceptibility of *Escherichia coli* O157:H7 grown at low temperatures to the krypton-chlorine excilamp. *Scientific Reports*, *9*(1), 1–9. https://doi.org/10.1038/s41598-018-37060-1

Levine, M. M., & Edelman, R. (1984). Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: Epidemiology and pathogenesis. *Epidemiologic Reviews*, *6*.

Liu, C., Hofstra, N., & Franz, E. (2013). Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by *Escherichia coli* O157 and *Salmonella* spp. *International Journal of Food Microbiology*, *163*(2–3), 119–128. https://doi.org/10.1016/j.ijfoodmicro.2013.02.026

Liu, C., Hofstra, N., & Leemans, R. (2015). Preparing suitable climate scenario data to assess impacts on local food safety. *Food Research International*, *68*, 31–40. https://doi.org/10.1016/j.foodres.2014.08.017

Lopez-Galvez, F., Allende, A., Pedrero-Salcedo, F., Alarcon, J. J., & Gil, M. I. (2014). Safety assessment of greenhouse hydroponic tomatoes irrigated with reclaimed and surface water. *International Journal of Food Microbiology*, *191*, 97–102. https://doi.org/10.1016/j.ijfoodmicro.2014.09.004

Lothigius, Å., Sjöling, Å., Svennerholm, A. M., & Bölin, I. (2010). Survival and gene expression of enterotoxigenic *Escherichia coli* during long-term incubation in sea water and freshwater. *Journal of Applied Microbiology*, *108*(4), 1441–1449. https://doi.org/10.1111/j.1365-2672.2009.04548.x

Luo, Y., Nou, X., Yang, Y., Alegre, I., Turner, E., Feng, H. A. O., Abadias, M., &

Conway, W. (2011). Determination of free chlorine concentrations needed to prevent *Escherichia coli* O157:H7 cross-contamination during fresh-cut produce wash. *Journal of Food Protection*, *74*(3), 352–358. https://doi.org/10.4315/0362-028X.JFP-10-429

Ma, J., Ibekwe, A. M., Crowley, D. E., & Yang, C. H. (2012). Persistence of *Escherichia coli* O157:H7 in major leafy green producing soils. *Environmental Science and Technology*, *46*(21), 12154–12161. https://doi.org/10.1021/es302738z

Ma, Z., Bumunang, E. W., Stanford, K., Bie, X., Niu, Y. D., & McAllister, T. A. (2019). Biofilm formation by shiga toxin-producing *Escherichia coli* on stainless steel coupons as affected by temperature and incubation time. *Microorganisms*, *7*(4). https://doi.org/10.3390/microorganisms7040095

Machado-Moreira, B., Richards, K., Brennan, F., Abram, F., & Burgess, C. M. (2019). Microbial contamination of fresh produce: What, where, and how? *Comprehensive Reviews in Food Science and Food Safety*, *18*(6), 1727–1750. https://doi.org/10.1111/1541-4337.12487

Mannion, C., Lynch, P. B., Egan, J., & Leonard, F. C. (2007). Seasonal effects on the survival characteristics of *Salmonella* Typhimurium and *Salmonella* Derby in pig slurry during storage. *Journal of Applied Microbiology*, *103*(5), 1386–1392. https://doi.org/10.1111/j.1365-2672.2007.03384.x

Manyi-Loh, C. E., Mamphweli, S. N., Meyer, E. L., Makaka, G., Simon, M., & Okoh, A. I. (2016). An overview of the control of bacterial pathogens in cattle manure. *International Journal of Environmental Research and Public Health*, *13*(9). https://doi.org/10.3390/ijerph13090843

Martinez, B. M., Fink, R. C., Diez-Gonzalez, F., & Sadowsky, M. J. (2014). Enteric pathogen-plant interactions: Molecular connections leading to colonization and growth and implications for food safety. *Microbes and Environments*, *29*(2), 123–135. https://doi.org/10.1264/jsme2.ME13139

Mellmann, A., Harmsen, D., Cummings, C. A., Zentz, E. B., Leopold, S. R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S. F.,

Henkhaus, J. K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P. M., Moore, R. L., Guenther, S., Rothberg, J. M., & Karch, H. (2011). Prospective genomic characterization of the german enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS ONE*, *6*(7), 1–9. https://doi.org/10.1371/journal.pone.0022751

Melotto, M., Underwood, W., & He, S. Y. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. *Annual Review of Phytopathology*, *46*(1), 101–122. https://doi.org/10.1146/annurev.phyto.121107.104959

Mercanoglu Taban, B., & Halkman, A. K. (2011). Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens? *Anaerobe*, *17*(6), 286–287. https://doi.org/10.1016/j.anaerobe.2011.04.004

Miraglia, M., Marvin, H. J. P., Kleter, G. A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., De Santis, B., Dekkers, S., Filippi, L., Hutjes, R. W. A., Noordam, M. Y., Pisante, M., Piva, G., Prandini, A., Toti, L., van den Born, G. J., & Vespermann, A. (2009). Climate change and food safety: An emerging issue with special focus on Europe. *Food and Chemical Toxicology*, *47*(5), 1009–1021. https://doi.org/10.1016/j.fct.2009.02.005

Moriarty, E. M., Mackenzie, M. L., Karki, N., & Sinton, L. W. (2011). Survival of *Escherichia coli, Enterococci,* and *Campylobacter* spp. in sheep feces on pastures. *Applied and Environmental Microbiology*, *77*(5), 1797–1803. https://doi.org/10.1128/AEM.01329-10

Mukherjee, M., Hu, Y., Tan, C. H., Rice, S. A., & Cao, B. (2018). Engineering a light-responsive, quorum quenching biofilm to mitigate biofouling on water purification membranes. *Science Advances*, *4*(12). https://doi.org/10.1126/sciadv.aau1459

Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli. Clinical Microbiology Reviews*, *11*(1), 142–201. https://doi.org/10.1128/cmr.11.1.142

Neely, M. N., & Friedman, D. I. (1998). Functional and genetic analysis of regulatory regions of coliphage H- 19B: Location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. *Molecular*

Microbiology, *28*(6), 1255–1267. https://doi.org/10.1046/j.1365-2958.1998.00890.x

Neinhuis, C., & Edelmann, H. G. (1996). Methanol as a rapid fixative for the investigation of plant surfaces by SEM. *Journal of Microscopy*, *184*(1), 14–16. https://doi.org/10.1046/j.1365-2818.1996.d01-110.x

Oliveira, M., Viñas, I., Usall, J., Anguera, M., & Abadias, M. (2012). Presence and survival of *Escherichia coli* O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. *International Journal of Food Microbiology*, *156*(2), 133–140. https://doi.org/10.1016/j.ijfoodmicro.2012.03.014

Orozco, L. R., Iturriaga, M. H., Tamplin, M. L., Fratamico, P. M., Call, J. E., Luchansky, J. B., & Escartin, E. F. (2008). Animal and environmental impact on the presence and distribution of *Salmonella* and *Escherichia coli* in hydroponic tomato greenhouses. *Journal of Food Protection*, *71*(4), 676–683. https://doi.org/10.4315/0362-028x-71.4.676

Ottoson, J. R., Nyberg, K., Lindqvist, R., & Albihn, A. (2011). Quantitative microbial risk assessment for *Escherichia coli* O157 on lettuce, based on survival data from controlled studies in a climate chamber. *Journal of Food Protection*, *74*(12), 2000–2007. https://doi.org/10.4315/0362-028X.JFP-10-563

Pathan, A. K., Bond, J., & Gaskin, R. E. (2009). Sample preparation for SEM of plant surfaces. *Materials Today*, *12* (Electron Microscopy Special Issue), 32–43. https://doi.org/10.1016/S1369-7021(10)70143-7

Rana, M. K., & Kamboj, N. K. (2018). Garden Cress. In M. K. Rana (Ed.), *Vegetable Crop Science* (pp. 345–351). CRC Press.

Rasko, D. A., Webster, D. R., Sahl, J. W., Bashir, A., Boisen, N., Scheutz, F., Paxinos, E. E., …, & Waldor, M. K. (2011). Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. *The New England Journal of Medicine*, *365*(8), 709–717.

Rechenburg, A., & Kistemann, T. (2009). Sewage effluent as a source of

Campylobacter sp. in a surface water catchment. *International Journal of Environmental Health Research*, *19*(4), 239–249. https://doi.org/10.1080/09603120802460376

Riggio, G. M., Wang, Q., Kniel, K. E., & Gibson, K. E. (2019). Microgreens—A review of food safety considerations along the farm to fork continuum. *International Journal of Food Microbiology*, *290*, 76–85. https://doi.org/10.1016/j.ijfoodmicro.2018.09.027

Rivera-Betancourt, M., Shackelford, S. D., Arthur, T. M., Westmoreland, K. E., Bellinger, G., Rossman, M., Reagan, J. O., & Koohmaraie, M. (2004). Prevalence of *Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* in two geographically distant commercial beef processing plants in the United States. *Journal of Food Protection*, *67*(2), 295–302. https://doi.org/10.4315/0362-028X-67.2.295

Robert Koch Institute. (2011). *Report: Final presentation and evaluation of epidemiological findings in the EHEC O104:H4 outbreak, Germany 2011*. https://www.rki.de/EN/Content/infections/epidemiology/outbreaks/EHEC_O104/ehec_O104_inhalt_en.html

Roy, D., & Melotto, M. (2019). Stomatal response and human pathogen persistence in leafy greens under preharvest and postharvest environmental conditions. *Postharvest Biology and Technology*, *148*, 76–82. https://doi.org/10.1016/j.postharvbio.2018.10.013

Ryu, J., Kim, H., & Beuchat, L. R. (2004). Attachment and biofilm formation by *Escherichia coli* O157:H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. *67*(10), 2123–2131.

Safadi, R. Al, Abu-Ali, G. S., Sloup, R. E., Rudrik, J. T., Waters, C. M., Eaton, K. A., & Manning, S. D. (2012). Correlation between in vivo biofilm formation and virulence gene expression in *Escherichia coli* O104:H4. *PLoS ONE*, *7*(7). https://doi.org/10.1371/journal.pone.0041628

Saldaña, Z., Sánchez, E., Xicohtencatl-Cortes, J., Puente, J. L., & Girón, J. A.

(2011). Surface structures involved in plant stomata and leaf colonization by Shiga-toxigenic *Escherichia coli* O157: H7. *Frontiers in Microbiology*, *2*(MAY), 1–9. https://doi.org/10.3389/fmicb.2011.00119

Sánchez, A. S., Cohim, E., & Kalid, R. A. (2015). A review on physicochemical and microbiological contamination of roof-harvested rainwater in urban areas. *Sustainability of Water Quality and Ecology*, *6*, 119–137. https://doi.org/10.1016/j.swaqe.2015.04.002

Semenov, A. V., Van Bruggen, A. H. C., Van Overbeek, L., Termorshuizen, A. J., & Semenov, A. M. (2007). Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cow manure. *FEMS Microbiology Ecology*, *60*(3), 419–428. https://doi.org/10.1111/j.1574-6941.2007.00306.x

Sharapov, U. M., Wendel, A. M., Davis, J. P., Keene, W. E., Farrar, J., Sodha, S., Hyytia-Trees, E., Leeper, M., Gerner-Smidt, P., Griffin, P. M., & Braden, C. (2016). Multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of fresh spinach: United States, 2006. *Journal of Food Protection*, *79*(12), 2024–2030. https://doi.org/10.4315/0362-028X.JFP-15-556

Shaw, R. K., Berger, C. N., Feys, B., Knutton, S., Pallen, M. J., & Frankel, G. (2008). Enterohemorrhagic *Escherichia coli* exploits EspA filaments for attachment to salad leaves. *Applied and Environmental Microbiology*, *74*(9), 2908–2914. https://doi.org/10.1128/AEM.02704-07

Simmons, G., Hope, V., Lewis, G., Whitmore, J., & Gao, W. (2001). Contamination of potable roof-collected rainwater in Auckland, New Zealand. *Water Research*, *35*(6), 1518–1524. https://doi.org/10.1016/S0043-1354(00)00420-6

Smolinski, H. S., Wang, S., Ren, L., Chen, Y., Kowalcyk, B., Thomas, E., Van Doren, J., & Ryser, E. T. (2018). Transfer and redistribution of *Salmonella* Typhimurium LT2 and *Escherichia coli* O157:H7 during pilot-scale processing of baby spinach, cilantro, and romaine lettuce. *Journal of Food*

Protection, *81*(6), 953–962. https://doi.org/10.4315/0362-028X.JFP-17-420

Sprouffske, K., & Wagner, A. (2016). Growthcurver: An R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics*, *17*(1), 17–20. https://doi.org/10.1186/s12859-016-1016-7

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *40*, 175–179.

Strawn, L. K., Fortes, E. D., Bihn, E. A., Nightingale, K. K., Gröhn, Y. T., Worobo, R. W., Wiedmann, M., & Bergholz, P. W. (2013). Landscape and meteorological factors affecting prevalence of three food-borne pathogens in fruit and vegetable farms. *Applied and Environmental Microbiology*, *79*(2), 588–600. https://doi.org/10.1128/AEM.02491-12

Taormina, P. J., Beuchat, L. R., & Slutsker, L. (1999). Infections associated with eating seed sprouts: An international concern. *Emerging Infectious Diseases*, *5*(5), 626–634. https://doi.org/10.3201/eid0505.990503

Taylor, R. G., Scanlon, B., Döll, P., Rodell, M., Van Beek, R., Wada, Y., Longuevergne, L., Leblanc, M., Famiglietti, J. S., Edmunds, M., Konikow, L., Green, T. R., Chen, J., Taniguchi, M., Bierkens, M. F. P., Macdonald, A., Fan, Y., Maxwell, R. M., Yechieli, Y., … Treidel, H. (2013). Ground water and climate change. *Nature Climate Change*, *3*(4), 322–329. https://doi.org/10.1038/nclimate1744

Thao, S., Brandl, M. T., & Carter, M. Q. (2019). Enhanced formation of shiga toxin-producing *Escherichia coli* persister variants in environments relevant to leafy greens production. *Food Microbiology*, *84*, 103241. https://doi.org/10.1016/j.fm.2019.103241

Tirado, M. C., Clarke, R., Jaykus, L. A., McQuatters-Gollop, A., & Frank, J. M. (2010). Climate change and food safety: A review. *Food Research International*, *43*(7), 1745–1765. https://doi.org/10.1016/j.foodres.2010.07.003

Turner, K., Moua, C. N., Hajmeer, M., Barnes, A., & Needham, M. (2019).

Overview of leafy greens–related food safety incidents with a California link: 1996 to 2016. *Journal of Food Protection*, *82*(3), 405–414. https://doi.org/10.4315/0362-028X.JFP-18-316

United States Environmental Protection Agency (EPA). (2011). *Graywater Discharges from Vessels*. https://www3.epa.gov/npdes/pubs/vgp_graywater.pdf

United States Environmental Protection Agency (EPA). (2021). *Climate Change Indicators: Sea Surface Temperature | US EPA*. https://www.epa.gov/climate-indicators/climate-change-indicators-sea-surface-temperature

Uyttendaele, M., Jaykus, L. A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., & Rao Jasti, P. (2015). Microbial hazards in irrigation water: standards, norms, and testing to manage use of water in fresh produce primary production. *Comprehensive Reviews in Food Science and Food Safety*, *14*(4), 336–356. https://doi.org/10.1111/1541-4337.12133

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., & Heyndrickx, M. (2013). Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butterhead lettuce seeds, and their subsequent survival and growth on the seedlings. *International Journal of Food Microbiology*, *161*(3), 214–219. https://doi.org/10.1016/j.ijfoodmicro.2012.12.015

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., & Heyndrickx, M. (2014). Evaluation of an attachment assay on lettuce leaves with temperature-and starvation-stressed *Escherichia coli* O157:H7 MB3885. *Journal of Food Protection*, *77*(4), 549–557. https://doi.org/10.4315/0362-028X.JFP-13-332

Van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *ISME Journal*, *5*(2), 173–183. https://doi.org/10.1038/ismej.2010.80

van Overbeek, L. S., der Plas, C. L. Van, & van der Zouwen, P. (2020). The role of pea (Pisum sativum) seeds in transmission of entero-aggregative *Escherichia*

coli to growing plants. *Microorganisms*, *8*(1271), 1–17. https://doi.org/10.3390/microorganisms8091271

Vuotto, C., & Donelli, G. (2014). Microbial Biofilms: Methods and Protocols. In G. Donelli (Ed.), *Springer* (Vol. 1147, pp. 73–84). Springer Science+Business Media. https://doi.org/10.1007/978-1-4939-0467-9

Wang, J., Stanford, K., McAllister, T. A., Johnson, R. P., Chen, J., Hou, H., Zhang, G., & Niu, Y. D. (2016). Biofilm formation, virulence gene profiles, and antimicrobial resistance of nine serogroups of non-O157 shiga toxin-producing *Escherichia coli*. *Foodborne Pathogens and Disease*, *13*(6), 316–324. https://doi.org/10.1089/fpd.2015.2099

Wennerås, C., Erling, V., Wennerâs, C., & Erling, V. (2004). Prevalence of Enterotoxigenic *Escherichia coli*-associated diarrhoea and carrier state in the developing world. *Journal of Health, Population and Nutrition*, *22*(4), 370–382.

WHO. (2003). Report of a Joint WHO/FAO Expert: Diet, nutrition and the prevention of chronic diseases. In *World Health Organization - Technical Report Series* (Issue 916). https://doi.org/10.1093/ajcn/60.4.644a

WHO. (2017). The Burden of Foodborne Diseases in the WHO European Region. *World Health Organization*. http://www.euro.who.int/en/health-topics/disease-prevention/food-safety/publications/2017/the-burden-of-foodborne-diseases-in-the-who-european-region-2017

WHO. (2019). *Food Safety Climate Change and the role of WHO* (Vol. 47, Issue January). https://doi.org/10.1016/j.fct.2009.02.005

Wright, K. M., & Holden, N. J. (2018). Quantification and colonisation dynamics of *Escherichia coli* O157:H7 inoculation of microgreens species and plant growth substrates. *International Journal of Food Microbiology*, *273*(March), 1–10. https://doi.org/10.1016/j.ijfoodmicro.2018.02.025

Xiao, Z., Nou, X., Luo, Y., & Wang, Q. (2014). Comparison of the growth of *Escherichia coli* O157:H7 and O104:H4 during sprouting and microgreen production from contaminated radish seeds. *Food Microbiology*, *44*, 60–63.

https://doi.org/10.1016/j.fm.2014.05.015

Zarkani, A. A., Schierstaedt, J., Becker, M., Krumwiede, J., Grimm, M., Grosch, R., Jechalke, S., & Schikora, A. (2019). *Salmonella* adapts to plants and their environment during colonization of tomatoes. *FEMS Microbiology Ecology*, *95*(11), 1–14. https://doi.org/10.1093/femsec/fiz152

APPENDICES

A. Leafy vegetables and leafy vegetable-related outbreaks in the US (2009 to 2018)

Table A.1 Leafy vegetables and leafy vegetable-related multistate outbreaks from 2009 to 2018 reported in the US

Year	Month	Etiology	Serotype or Genotype	Etiology Status	Illnesses	Hospitalizations	Deaths	Food Vehicle	IFSAC Category
2009	4	STEC	O157:H7	Confirmed	16		0	lettuce; ambrosia	Multiple
2009	9	STEC	O157:H7	Confirmed	22			romaine lettuce, unspecified	Vegetable Row Crops
2009	9	STEC	O157:H7	Confirmed	10	0	0	romaine lettuce, unspecified	Vegetable Row Crops
2010	4	STEC	O145	Confirmed	31	14	0	romaine lettuce, unspecified	Vegetable Row Crops
2011	10	STEC	O157:H7	Confirmed	60	35	0	romaine lettuce, unspecified	Vegetable Row Crops
2011	10	STEC	O157:H7	Confirmed	26	5	0	lettuce	Vegetable Row Crops
2011	12	STEC	O26	Confirmed	29	7	0	clover sprouts	Sprouts
2012	10	STEC	O157:H7	Confirmed	33	13	0	prepackaged leafy greens	Vegetable Row Crops
2012	10	STEC	O145	Confirmed	16	6	0	lettuce	Vegetable Row Crops
2012	6	STEC	O157:H7	Confirmed	52			romaine lettuce, unspecified	Vegetable Row Crops
2012	3	STEC	O157:H7	Confirmed	24			leaf lettuce	Vegetable Row Crops
2012	11	STEC	O157:NM (H-)	Confirmed	10		0	spinach, unspecified	Vegetable Row Crops
2013	4	STEC	O157:H7	Confirmed	14	9	1	prepackaged leafy greens	Vegetable Row Crops

Table A.1 (Continued)

Year	Month	Etiology	Serotype or Genotype	Etiology Status	Illnesses	Hospitalizations	Deaths	Food Vehicle	IFSAC Category
2014	4	STEC	O157:H7	Confirmed	4	1	0	spinach	Vegetable Row Crops
2014	5	STEC	O121	Confirmed	19	5	0	clover sprouts	Sprouts
2014	6	STEC	O157:H7	Confirmed	16	13	0	pre-packaged salad	Multiple
2014	11	STEC	O157:H7	Confirmed	11	2	0	salad	Vegetable Row Crops
2014	6	STEC	O111	Confirmed	16	2	0	cabbage	Vegetable Row Crops
2015	4	STEC	O145	Confirmed	7	5	0	prepackaged leafy greens	Vegetable Row Crops
2015	3	STEC	O157:H7	Confirmed	16	10	0	romaine lettuce, unspecified	Vegetable Row Crops
2015	10	STEC	O157:H7	Confirmed	5	3	0	pre-packaged salad	Multiple
2016	1	STEC	O157:NM (H-)	Confirmed	11	2	0	alfalfa sprouts	Sprouts
2016	6	STEC	O157:H7	Confirmed	11	4	0	iceberg lettuce, unspecified	Vegetable Row Crops
2017	9	STEC	O26	Confirmed	8	3	0	spinach	Vegetable Row Crops
2017	8	STEC	O157:H7	Confirmed	68	18	0	spring salad; baby leaf, unspecified;	Vegetable Row Crops

Table A.1 (Continued)

Year	Month	Etiology	Serotype or Genotype	Etiology Status	Illnesses	Hospitalizations	Deaths	Food Vehicle	IFSAC Category
2018	3	STEC; STEC	O157:H7; other	Confirmed; Confirmed	239	104	5	romaine lettuce, unspecified	Vegetable Row Crops
2018	10	STEC	O157:H7	Confirmed	25	8	0	leafy greens	Vegetable Row Crops
2018	10	STEC	O157:H7	Confirmed	62	25	0	romaine lettuce, leaf	Vegetable Row Crops
2018	10	STEC	O157:H7	Confirmed	22	10	0	iceberg lettuce, unspecified	Vegetable Row Crops

Table A.2 Leafy vegetables and leafy vegetable-related single state outbreaks from 2009 to 2018 reported in the US

Year	Month	State	Etiology	Serotype or Genotype	Etiology Status	Illnesses	Hospitalizations	Deaths	Food Vehicle	IFSAC Category
2011	5	New York	ETEC	O6:H16	Confirmed	19	0	0	tabouleh salad; spinach struedel	Multiple
2010	10	Maryland	STEC	O157:H7	Confirmed	7	4	0	apple cider, unpasteurized	Fruits
2011	7	Minnesota	STEC	O157:H7	Confirmed	6	1	0	fruit	Fruits
2011	7	Oregon	STEC	O157:H7	Confirmed	15	7	2	strawberries	Fruits
2011	10	Minnesota	STEC; *Cryptosporidium parvum*	O111:NM;	Confirmed; Confirmed	14	0	0	apple cider, unpasteurized	Fruits
2012	4	Louisiana	STEC	O145	Confirmed	5	3	1	ground beef; lettuce; sprouts	Multiple
2012	4	California	STEC	O157:H7	Confirmed	12	1	0	vegetable-based salads unspecified	Vegetable Row Crops
2012	9	Pennsylvania	STEC	O157:H7	Confirmed	9	7	0	salads	Vegetable Row Crops
2012	10	Michigan	STEC	O157:NM (H-)	Confirmed	3	0	0	apple cider, unpasteurized	Fruits

Table A.2 (Continued)

Year	Month	State	Etiology	Serotype or Genotype	Etiology Status	Illnesses	Hospitalizations	Deaths	Food Vehicle	IFSAC Category
2012	11	Massachusetts	STEC	O157:H7	Confirmed	8	4	0	leaf lettuce, unspecified	Vegetable Row Crops
2013	4	Michigan	STEC	O157:H7	Suspected	20	7	0	salad, unspecified	Seeded Vegetables
2013	6	Missouri	STEC	O157:H7	Confirmed	6	4	0	lettuce-based salads unspecified	Multiple
2013	9	Connecticut	EAEC		Confirmed	34	0	0	salad, unspecified; vegetable tray; cheese dish, other; dips;	Multiple
2013	7	Arizona	STEC	O157:H7	Confirmed	94	22	0	lettuce	Vegetable Row Crops
2013	9	Florida	STEC	O157:H7	Confirmed	7	5	0	kale	Vegetable Row Crops
2013	10	Colorado	STEC	O157:H7	Confirmed	9	1	0	sandwich, cucumber	Seeded Vegetables
2013	12	Connecticut	STEC	O157:H7	Confirmed	9	8	0	lettuce	Vegetable Row Crops
2013	7	California	STEC	O157:H7	Confirmed	5	5	0	green leaf lettuce	Vegetable Row Crops
2013	10	California	STEC	O157:H7	Confirmed	8	1	0	green beans	Seeded Vegetables
2014	4	Minnesota	EAEC	O126	Confirmed	4	0	0	romaine lettuce, unspecified	Vegetable Row Crops
2014	7	Minnesota	STEC	O157:H7	Confirmed	57	9	0	potato salad; celery, unspecified	Vegetable Row Crops

Table A.2 (Continued)

Year	Month	State	Etiology	Serotype or Genotype	Etiology Status	Illnesses	Hospitalizations	Deaths	Food Vehicle	IFSAC Category
2015	10	Minnesota	STEC	O157	Confirmed	2	1	0	apple cider, unpasteurized	Fruits
2015	10	Michigan	STEC	O45	Confirmed	2	0	0	apple cider, unpasteurized	Fruits
2015	8	Washington	STEC	O121	Confirmed	2	0	0	kale, unspecified; pineapple juice, unpasteurized	Multiple
2015	10	California	STEC	O111:NM	Confirmed	15	1	0	apple cider, unspecified	Fruits
2016	5	Missouri	STEC		Suspected	11	4	0	salad	Vegetable Row Crops
2016	5	Colorado	STEC	O157:H7	Confirmed	9	1	0	tomato (see fruit); cucumber; lettuce	Multiple
2016	9	Kansas	STEC	O157:H7	Confirmed	56	10	0	apple cider, unspecified	Fruits
2016	6	Illinois	STEC	O157:H7	Confirmed	96	19	0	cilantro	Herbs
2017	12	Wisconsin	STEC	O157	Confirmed	3	3	0	mixed green salad	Vegetable Row Crops
2018	8	Oregon	STEC	O157:H7	Confirmed	23	1	0	potato salad	Multiple
2018	8	Rhode Island	EAEC; ETEC; *E. coli,* Other; ETEC; EAEC; EAEC	O6:H16; ; O148:H28; ;	Confirmed; Confirmed; Confirmed; Suspected; Confirmed; Suspected	64	1	0	menu items w/ lettuce	Vegetable Row Crops
2018	5	South Dakota	STEC	O103	Confirmed	30	0	0	lettuce	Vegetable Row Crops

B. Chemicals Used in This Study

Table B.3 List and the suppliers of the chemicals

Chemicals	**Supplier**
Acetone	Isolab
Bacteriological Agar	CondaLab (Madrid, Spain)
Bleach	Domestos, Unilever (London, UK)
Brain Hearth Infusion (BHI) Broth	Merck (Darmstadt, Germany)
Brilliant-Green Phenol-Red Lactose Sucrose (BPLS) Agar	Merck (Darmstadt, Germany)
Buffered Peptone Water (BPW)	Merck (Darmstadt, Germany)
Crystal Violet	Sigma Aldrich (St, Louis, Missouri, USA)
Ethanol (96 %)	Isolab
Magnesium Chloride Hexahydrate ($MgCl_2 \cdot 6H_2O$)	Merck (Darmstadt, Germany)
Methanol (99.7 %)	Isolab
Sodium Chloride (NaCl)	Merck (Darmstadt, Germany)
Tryptone	Sigma Aldrich (St, Louis, Missouri, USA)
Yeast Extract	Amresco (Ohio, USA)

C. Preparation of Growth Mediums, Solutions and Chemicals

1. Buffered Peptone Water (BPW)

10 g of buffered peptone water was dissolved in 500 ml of deionized water by heating and continuous agitation. Then, it was sterilized at 121 ºC for 15 minutes.

2. Brain Hearth Infusion (BHI) Broth

3.7 g of brain hearth infusion broth was dissolved in 100 ml of deionized water by heating and continuous agitation in an Erlenmeyer flask. Then, the flask was covered by cotton plugs, and it was sterilized at 121 ºC for 15 minutes.

3. Brain Hearth Infusion (BHI) Agar

18.5 g of brain hearth infusion broth and 7.5 g of bacteriological agar were dissolved in 500 ml of deionized water by heating and continuous agitation. Then, it was sterilized at 121 ºC for 15 minutes. Afterwards, to cool it down, the agar was transferred to water bath which is at 50 ºC. After cooling to about 50 ºC, agar was poured into the petri dish plates near the Bunsen burner.

4. Brilliant-Green Phenol-Red Lactose Sucrose (BPLS) Agar

20.6 g of brilliant-green phenol-red lactose sucrose (BPLS) agar was completely dissolved in 400 ml of sterile deionized water by heating and continuous agitation. Then, the solution was brought to boiling. Afterwards, to cool it down, the agar was transferred to water bath which is at 50 ºC. After cooling to about 50 ºC, agar was poured into the petri dish plates near the Bunsen burner.

5. 0.85 % Sodium Chloride (NaCl) Solution

8.5 g of NaCl was dissolved in 1000 ml of deionized water. Then, it was sterilized at 121 ºC for 15 minutes.

6. 10 mM Magnesium Chloride (MgCl2) Solution

2.033 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$; molecular weight: 203.30 g/mole) was dissolved in 1000 ml of deionized water. Then, it was sterilized at 121 ºC for 15 minutes.

7. Luria-Bertani (LB) Broth

10 g of tryptone, 5 g of yeast extract and 5 g of NaCl were dissolved in 1000 ml of deionized water. Then, it was sterilized at 121 ºC for 15 minutes.

8. Luria-Bertani (LB) Agar

10 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 15 g of bacteriological agar were dissolved in 1000 ml of deionized water. Then, it was sterilized at 121 ºC for 15 minutes. Afterwards, to cool it down, the agar was transferred to water bath which is at 50 ºC. After cooling to about 50 ºC, agar was poured into the petri dish plates near the Bunsen burner.

9. Crystal Violet

0.1 g of crystal violet was dissolved in 80 ml of deionized water. Then, 20 ml of methanol (99.7%) was added to make the volume 100 ml. Do not autoclave the crystal violet solution. Cover the bottle with aluminum foil to protect it from the light.