Use of a Bacteriophage Cocktail in Combination with Modified Atmosphere Packaging to Control *Escherichia coli* O157:H7 Contamination on Fresh-Cut Green Leafy Vegetables

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Dedication

I dedicate this dissertation to the policemen and their families all over the world, especially those who were killed in action, and to their children who could not continue their education due to the difficulties arisen from the loss of their police dad or mom.

Biographical Sketch

Olcay Boyacioglu was born in 1978 in Izmir, Turkey. He received a Bachelor of Science Degree in Biology with a concentration in Basic and Industrial Microbiology in 1998 from Ege University, Izmir, Turkey. He also received a Master of Science Degree in Biology from the University of North Carolina at Greensboro, NC in 2002. While attending North Carolina Agricultural and Technical State University, he became a member of the Gamma Sigma Delta, honor society of agriculture, and was named a Wadaran L. Kennedy 4.0 Scholar for 2 times. Olcay is a candidate for the Doctor of Philosophy degree in Energy and Environmental Systems.

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List of Symbols and Abbreviations

A : Air package

A/E : Attaching and Effacing

ANOVA : Analysis of Variance

APC : Aerobic Plate Count

ARS : Agricultural Research Services

CA : Controlled Atmosphere, Active MAP

CDC : Centers for Disease Control and Prevention

CFU : Colony Forming Unit

DAEC : Diffusely Adherent *E. coli*

DNA : Deoxyribonucleic Acid

E. coli : *Escherichia coli*

EBI : Exponential Biotherapies Inc.

EAEC : Enteroaggregative *E. coli*

ECP-100 : EcoShield™ phage cocktail

EHEC : *Escherichia coli* O157:H7, Enterohemorrhagic *E. coli*

EIEC : Enteroinvasive *E. coli*

EMFSL : Environmental Microbial and Food Safety Laboratory

EPA : Environmental Protection Agency

EPEC : Enteropathogenic *E. coli*

ETEC : Enterotoxigenic *E. coli*

FCN : Food Contact Notification

FDA : Food and Drug Administration

G : Modified Gas (5% O_2, 35% CO_2, 60% N_2) package

GLL : Green Leaf Lettuce

GRAS : Generally Recognized as Safe

HC : Hemorrhagic Colitis

HP : Healthy People Initiative

HUS : Hemolytic Uremic Syndrome

kGy : KiloGray

LA-PAA : Lactic Acid plus Peroxyacetic Acid

LBA : Lysogeny Broth Agar

LEE : Locus for Enterocyte Effacement

LMP-102 : ListShield™ phage cocktail

LT : Heat-Labile Toxin

LWI : Lysis from Within

LWO : Lysis from Without

MA : Modified Atmosphere

MAC : MacConkey Agar

MACN : MacConkey Agar supplemented with 25 µg/ml Nalidixic Acid

MAP : Modified Atmosphere Packaging, Passive MAP

MDa : MegaDalton

MOI : Multiplicity of Infection

MVP : Moderate Vacuum Packaging

Nal : Nalidixic acid

Nal^S : Nalidixic acid sensitive, Sensitive to Nalidixic acid

Nal^R	: Nalidixic acid resistant, Resistant to Nalidixic acid
NP	: No Phage treatment
P	: Phage treatment
PBS	: Phosphate Buffered Saline
PCR	: Polymerase Chain Reaction
PFU	: Plaque Forming Unit
ppm	: Parts per million
PW	: Peptone water
PWA	: Pacific West Area
RCM	: Reinforced Clostridial Medium
RL	: Romaine Lettuce
RTE	: Ready to Eat
SLT	: Shiga-like Toxin
SMAC	: Sorbitol MacConkey Agar
Spi	: Spinach
ST	: Heat-Stable Toxin
STEC	: Shiga Toxin Producing *E. coli*
Stx	: Shiga toxin
TC	: *trans*-cinnamaldehyde
Tir	: Translocated Intimin Receptor
TSA	: Tryptic Soy Agar
TSAN	: Tryptic Soy Agar supplemented with 25 µg/ml Nalidixic Acid
TSB	: Tryptic Soy Broth

TSBN : Tryptic Soy Broth supplemented with 50 µg/ml Nalidixic Acid

TTSS : Type III Secretion System

USDA : United States Department of Agriculture

USFDA : United States Food and Drug Administration

UV : Ultra Violet

VT : Verocytotoxin

WHO : World Health Organization

Abstract

The 2006 multi-state *Escherichia coli* O157:H7 (EHEC) outbreak linked to bagged spinach raised concerns about the safety of fresh produce. One novel approach to control foodborne pathogens is lytic bacteriophages, which does not affect the produce microflora. The objectives of this study were; 1) to test and verify the efficacy of a phage cocktail against EHEC in a laboratory medium, 2) to determine the effectiveness of the phage cocktail on fresh-cut leafy greens, and 3) to test and compare the effectiveness of the phage cocktail against EHEC on fresh-cut green leafy vegetables stored under air and modified air (low O_2/high CO_2) conditions. The efficacy of the phage cocktail was determined against a nalidixic acid resistant (Nal^R) EHEC strain in Tryptic Soy Broth (TSB). Next, pieces (~2x2 cm^2) of leafy greens inoculated with 4.50 log EHEC Nal^R/cm^2 and air-dried were sprayed with 6.50 log phage cocktail/cm^2. Samples were stored at 4 or 10°C for 15 days under atmospheric or modified (5% O_2/35% CO_2/60% N_2) air. The recovered EHECs were enumerated on MacConkey agar supplemented with 25 µg/ml Nal. Phage treatment resulted in 6 log reduction ($P<0.05$) of EHEC Nal^R in TSB. At 4°C under air, the phage cocktail significantly ($P<0.05$) lowered the EHEC Nal^R counts in one day by 1.19, 3.21, and 3.25 log units on spinach, green leaf, and romaine lettuce, respectively. When stored under modified atmosphere, the reductions in EHEC Nal^R concentrations were 2.18, 3.50, and 3.13 logs. At 10°C, EHEC reductions under air were 1.99, 3.90, and 3.99 logs, and under modified air were 3.08, 3.89, and 4.34 logs on spinach, green leaf, and romaine lettuce, respectively.

The results of this study suggest that bacteriophages may be used successfully for controlling/reducing pathogenic bacterial presence and/or growth on fresh produce stored under modified atmosphere packaging.

CHAPTER 1

Introduction

Foodborne related outbreaks are a great burden on the economy. The cost of a foodborne illness per patient ranges from $26 (no medical treatment) to $6.2 million (death due to HUS) (Frenzen, Drake, & Angulo, 2005). The cost of EHEC infections to the produce industry and healthcare system is approximately $405 million (Frenzen *et al.*, 2005). Two recent studies conducted in Australia and The Netherlands calculated that the yearly cost of EHEC infections are $2.8 and $12.7 million, respectively (McPherson, Kirk, Raupach, Combs, & Butler, 2011; Tariq, Haagsma, & Havelaar, 2011). Development of produce tracking systems from farm to fork will allow smaller produce callbacks in size without paralyzing the entire produce industry. Also the development of novel sanitizing methods to control pathogen contamination on meat, poultry, fruits, and vegetables will benefit the entire food industry by reducing their costs on recalls, healthcare expenses, and possible lawsuits faced after an outbreak.

The Healthy People (HP) Initiative of the US Department of Health and Human Services aims to reduce the infection incidences of four major foodborne pathogens including *Escherichia coli* O157:H7. Outbreaks linked to the consumption of tainted green leafy vegetables are considered a major health risk and one of the aims of HP 2020 is to reduce the outbreaks resulting from the consumption of the leafy greens (Healthy People 2020).

The chlorine solutions are the most widely used agents by the fresh produce industry to keep the indigenous bacteria and potential pathogen counts lower, (Takeuchi & Frank, 2001b). However, these hypochlorite solutions are only able to keep the wash water clean to prevent cross contamination of pathogenic bacteria between batches of produce (Behrsing, Winkler, Franz, & Premier, 2000). Also the oxidative effect of the free chlorine in hypochlorite solutions

is inactivated by the organic substances released from the damaged or cut surfaces of the produce (Marriott, 1999). As a result, their effectiveness against pathogens is tremendously reduced. Therefore, novel and more effective sanitation agents and methods are urgently needed to help keep fresh produce pathogen free.

One alternative method is the use of bacteriophages (phages) as natural antibacterial agents in food to eliminate pathogens. In the last decade, scientists in the West have been rediscovering and rebuilding their hopes up in phages to control the antibiotic resistant strains of bacteria. Phages are viruses that specifically interact with and infect their respective host bacterial cells. They are extremely diverse and abundant; thus, present the most promising natural weapon against the bacteria. In nature, phages are responsible for killing almost half of the bacterial population generated daily (Suttle, 2005). Considering the short generation time of the bacteria, phages represent the most effective natural tool to keep their presence under control. Currently there are 3 phage products in the market that received FDA regulatory approvals for use in food items.

Today fresh produce travel long distances, and maintaining the produce quality becomes a challenging issue. To satisfy the need to keep the fresh produce fresher longer, modified atmosphere packaging (MAP) offers indispensable advantage. During the transportation of produce the storage temperature and environmental gas concentration are controlled to reduce the physiological deterioration and prolong shelf life.

How effectively phages can perform their natural responsibilities under low oxygen/high carbon dioxide conditions is unknown in the literature. The objectives of this study are; 1) to test and verify the efficacy of a phage cocktail against *E. coli* O157:H7 in a laboratory medium, 2) to determine the effectiveness of the phage cocktail on fresh-cut leafy greens under normal

atmospheric air condition, and 3) to test and compare the effectiveness of the phage cocktail against *E. coli* O157:H7 on fresh-cut green leafy vegetables packaged under atmospheric air and modified atmosphere (low O_2/high CO_2) conditions.

CHAPTER 2

Literature Review

2.1. *Escherichia coli* as a Foodborne Pathogen

Escherichia coli is a rod-shaped (1.1x1.5 and 2.0x6.0 µm), Gram-negative, facultative anaerobic bacterium. *E. coli* was first isolated in the feces of newborn infants by German microbiologist Theodor Escherich in 1885, and described as commensal of the large intestine. He identified this bacterium as *Bacterium coli* commune (Kaper, 2005). Later, it was reclassified as *Escherichia coli,* being named after him. In 1935, *E. coli* was identified as a causative agent of diarrhea among infants (Stoppelman & Plaats, 1953). It is the most renowned member of Enterobacteriaceae family. Enteric bacteria are composed of facultative anaerobic Gram-negative rods including species of *Salmonella*, *Shigella*, and *Yersinia*. *Escherichia*, *Enterobacter*, and *Klebsiella* are also other known species belonged to the same family. *E. coli* flourishes in the large intestines of warm-blooded animals and comprises about 1% of the microbial flora in human large intestine. *E. coli* is used as a marker organism to detect fecal contamination on food and water sources. Although anaerobic *Bacteroides* spp. outnumbers the *E. coli* population in the bowel by 20:1, *E. coli* is widely used as a marker species for the determination of fecal contamination due to the culturing difficulties of anaerobic bacteria.

Before the identification of virulence factors, diarrheagenic *E. coli* strains were differentiated by serotyping. Over 700 antigenic serotypes of *E. coli* are recognized based on their somatic O, flagellar H, and capsular K antigens (Paton & Paton, 1998b). Serotype O157:H7 (O refers to somatic antigen; H refers to flagellar antigen) is uniquely responsible for causing hemolytic uremic syndrome (HUS) in many outbreaks.

2.1.1. Pathogenic *E. coli* O157:H7. *E. coli* O157:H7 is traditionally found in the gastrointestinal tracts of warm-blooded ruminant farm animals such as cattle, sheep, and goats (Erickson & Doyle, 2007). *E. coli* O157:H7 is usually introduced into the environment from these host animals. About 1-25% of the cattle is estimated to carry *E. coli* O157:H7 (Elder, Keen, Siragusa, Barkocy-Gallagher, Koohmaraie, & Laegreid, 2000). Cattle can produce 20-50 kg of feces a day. Carrier cattle may shed between 10^1 and 10^7 CFU of *E. coli* O157:H7 per gram of feces (Besser, Richards, Rice, & Hancock, 2001). Cattle shedding more than 10^4 CFU/g of *E. coli* O157:H7 are called "super-shedders," and they must be detected and removed from the herd for the wellbeing of the other animals and the environment (Matthews, Low, Gally, Pearce, Mellor, Heesterbeek *et al.*, 2006).

Most *E. coli* O157:H7 strains cannot ferment sorbitol within 24 hours in culture and do not contain β-glucuronidase enzyme (Holt, Krieg, Sneath, Staley, & Williams, 1994; Strockbine, Wells, Bopp, & Barrett, 1998). Although there are some cases of the presence of sorbitol-fermenting, β-glucuronidase-producing *E. coli* O157:H7, these are mainly in Europe (Gunzer, Bohm, Russmann, Bitzan, Aleksic, & Karch, 1992). The optimal growth temperature of *E. coli* O157:H7 is 37°C, however it can survive between 7-46°C. Optimum pH for *E. coli* O157:H7 is 6.0-7.0, but it can tolerate acidic pH as low as 3.7-4.4 (Weagant, Bryant, & Bark, 1994). Most *E. coli* O157:H7 strains ferment glucose and some ferment lactose, producing acid and gas, and are typically oxidase-negative, indole-positive, and urease negative, and produce peritrichous flagella when motile (Holt *et al.*, 1994). The unique characteristics of *E. coli* O157:H7, such as acid and cold tolerance, make monitoring this foodborne pathogen critical within the food industry.

2.1.2. Types of pathogenic *E. coli.* Diarrheagenic strains of pathogenic *E. coli* are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* usually requires that the isolates first be identified as *E. coli* before testing for virulence markers. Diarrhea-causing pathogenic *E. coli* categories are enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), enteroinvasive (EIEC), and diffusely adherent (DAEC).

Enterotoxigenic *E. coli* (ETEC). Members of this category can synthesize at least one of two enterotoxin groups: heat-stable toxins (ST) and heat-labile toxins (LT). ETECs cause diarrhea in humans through the actions of these two groups of toxins (Nataro & Kaper, 1998).

Enteropathogenic *E. coli* (EPEC). These bacteria are recognized to cause attaching-and-effacing (A/E) histopathology. EPECs recognize, attach, and disrupt the microvilli structures via the activities of eae and intimin genes to attach tightly to the epithelial cells in the intestine (Nataro & Kaper, 1998).

Enteroaggregative *E. coli* (EAEC). Bacteria in this category bind to epithelial cells as densely packed aggregates. These bacteria carry a plasmid to synthesize fimbria, which causes them to aggregate. Aggregation enhances toxicity and their ability to cause inflammation (Nataro & Kaper, 1998).

Enteroinvasive *E. coli* (EIEC). Most of the members in this category are generally non-motile. These bacteria carry an invasion plasmid that contains type III secretion system (TTSS), which secretes their own antigen to attach intestinal cell surface (Nataro & Kaper, 1998).

Diffusely Adherent *E. coli* (DAEC). Members of this category are known for having a surface fimbria that helps them diffuse into the intestinal cells without being completely

internalized. This way they can physically protect themselves from external agents (Nataro & Kaper, 1998).

Enterohemorrhagic *E. coli* (EHEC). Members of this category are known to cause hemorrhagic colitis (HC), which is bloody diarrhea with no fever, and hemolytic uremic syndrome (HUS). EHECs are a subset of Shiga toxin-producing *E. coli* (STEC), and they are characterized by expressing Shiga toxin (Stx), forming A/E lesions on epithelial cells, and possessing an approximately 60-MDa plasmid (Nataro & Kaper, 1998).

Some of the HC symptoms start as severe abdominal cramps with watery diarrhea, which typically becomes bloody within 24 h (O'Brien, Lively, Chen, Rothman, & Formal, 1983; Riley, 1987; Riley, Remis, Helgerson, McGee, Wells, Davis *et al.*, 1983). The diarrhea usually lasts 1 to 8 days. Approximately 8% of patients with *E. coli* O157:H7 and 5% of patients with HC develop a severe complication known as HUS (Slutsker, Ries, Maloney, Wells, Greene, & Griffin, 1998). Symptoms of HUS include anemia (characterized by fatigue, weakness, and light-headedness) caused by the destruction of red blood cells (hemolytic anemia), a low platelet count (thrombocytopenia), and sudden kidney failure. Some people with HUS may also develop complications of nerve or brain damage, leading to seizures or strokes. These complications typically develop in the second week of illness and may be preceded by increasing fever. HUS is more likely to occur in children under the age of 5 and in the elderly. Even without HUS and its complications, HC may cause death in older people (Blackall & Marques, 2004). HUS is associated with Shiga toxin producing enteric bacteria, such as *E. coli* O157:H7. The production of the Shiga toxin is the key for many of the pathological features of EHEC infection (Lathem, Bergsbaken, & Welch, 2004; Paton & Paton, 1998b).

Pathogenic bacteria are estimated to cause over 4 million foodborne illnesses and approximately 36,000 hospitalizations in the US yearly (Mead, Slutsker, Dietz, McCaig, Bresee, Shapiro *et al.*, 1999). *E. coli* O157:H7 has emerged in the past two decades as a significant foodborne pathogen. It is particularly important in the food industry because of its low infectious dose. Most *E. coli* O157:H7 infections occur through consumption of contaminated food and water. Ground beef, milk, apple juice, produce, and foods that have been stored, cooked, or handled improperly are potential transmission sources of *E. coli* O157:H7. The incidence of this pathogen in many food sources and its low infectious dose are causes of major concern among food processors and regulatory agencies.

Acid resistance of EHEC is another important factor that allows it to survive digestive stresses through the stomach and colonize the intestinal tract (Diez-Gonzalez & Karaibrahimoglu, 2004). After surviving the harsh environment of the stomach, the pathogen must adhere to the intestinal epithelial cells. Colon and perhaps the distal small intestine are the principle sites for EHEC colonization in humans.

Nothing is known about the colonizing antigens of EHEC but fimbria are presumed to be involved. The bacteria do not invade mucosal cells as readily as *Shigella*, but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. Shiga toxin producing *E. coli* (STEC) strains are one of the most important enteropathogens. There are more than 100 serogroups of *E. coli* that are known to produce Shiga toxins (Nataro & Kaper, 1998). *E. coli* O157:H7 is the most infamous serotype with Shiga toxin producing ability. Shiga toxin is also called as verocytotoxin (VT) and Shiga-like toxin (SLT) (Kaper, 1998). Shiga toxin producing *E. coli* O157 are derived from one clone and carry highly

similar genotypes (Law, 2000). A large amount of *E. coli* O157 isolates produce Shiga toxin 2 (Stx2) only. Shiga toxin 1 (Stx1) and Stx2 producers are occasionally found but exclusively Stx1 producing *E. coli* O157:H7 are rare (Griffin & Tauxe, 1991).

EHEC are considered to be moderately invasive. Unlike other diarrheagenic *E. coli* isolates, EHEC and EPEC isolates share the ability to induce formation of an attaching and effacing (A/E) lesion on target epithelial cells. A/E lesions are characterized by localized destruction of microvilli and the formation of polymerized actin, which helps bacteria to adhere to microvilli.

Beneath the adherent bacteria, is accumulation of cytoskeletal components, resulting in the formation of pedestals (Abe, Tatsuno, Tobe, Okutani, & Sasakawa, 2002). EHEC strains that display the A/E phenotype have a pathogenicity island homologue called Locus for Enterocyte Effacement (LEE) similar to that of EPEC (Elliott, Wainwright, McDaniel, Jarvis, Deng, Lai *et al.*, 1998). The LEE homolog contains a copy of *eaeA*, Tir (Translocated intimin receptor) homolog (Frankel & Phillips, 2008), as well as the Type III secretion system (DeVinney, Puente, Gauthier, Goosney, & Finlay, 2001).

The first gene found to be associated with A/E activity was *eae* gene and encodes the intimate bacterial adhesion known as intimin. Intimin binds its specific receptor, Tir, on intestinal epithelial cells. Tir is actually synthesized by both EHEC and EPEC, and translocated into the host intestinal epithelial cell membrane. EPEC Tir is Tyrosine phosphorylated once in the host membrane and forms a pedestal and acts as intimin receptor to bind EPEC. EHEC Tir also forms a pedestal structure and binds intimin without being Tyrosine phosphorylated, suggesting phosphorylation is not an absolutely necessary step for EHEC binding to host cells (DeVinney, Stein, Reinscheid, Abe, Ruschkowski, & Finlay, 1999). In addition, *eaeA*-negative

mutants lost the capacity to adhere intimately to the colonic epithelium of piglets (Abe *et al.*, 2002).

2.2. Foodborne Outbreaks and Pathogens

One of the aims of Healthy People (HP) Initiative of the US Department of Health and Human Services is to reduce the infection incidences of four major foodborne pathogens (*Campylobacter* species, *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* species). The HP 2010 aimed at reducing the incidences of these 4 pathogens about 50% by year 2010 compared to their baseline levels in 1997. The objectives of HP have been updated for 2020 and now include reducing the outbreaks resulting from leafy vegetables. On average, 205 cases were reported yearly due to leafy vegetable outbreaks caused by 4 major foodborne pathogens between 2005 and 2007. The HP 2020 aims to reduce this baseline of 205 cases by about 10% by the year 2020 (Healthy People 2020).

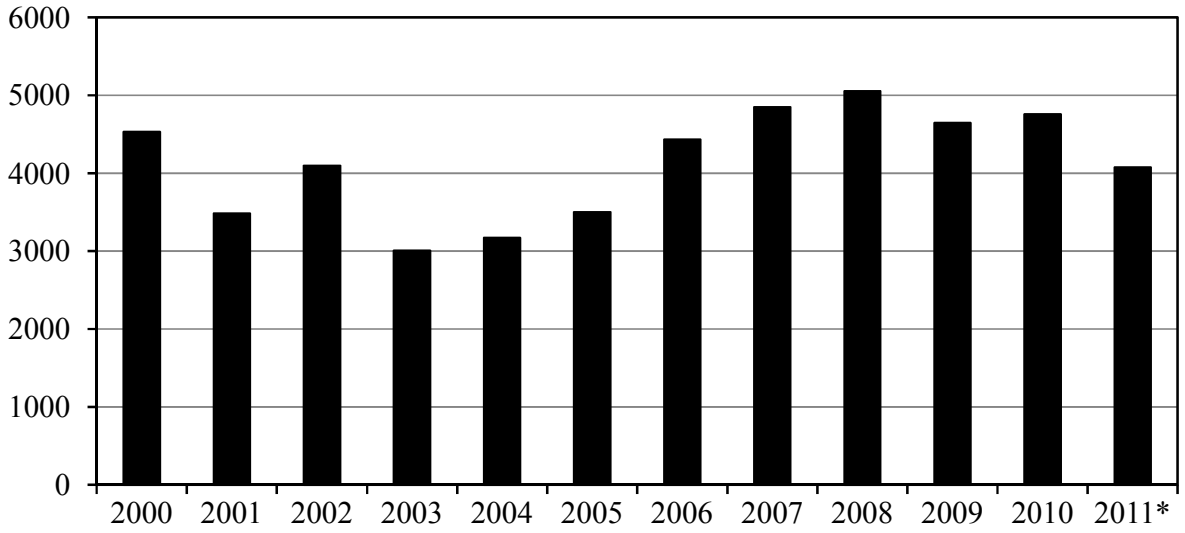
According to the review of 2008 FoodNet data, significant reductions have been achieved for the three of four major foodborne pathogens since 1997 (CDC, 2005, 2009). However, most of the improvement was observed between 1997 and 2004. Little or no progress has been made since then (Table 1). In fact, *E. coli* O157:H7 outbreaks are on the rise after 2004. FoodNet data is collected from 10 states covering about 15% of the US population on the incidence of foodborne infections. Despite the continuous improvements and research in the field, it is understood that the current applications and techniques used were unable to fulfill the 2010 goals. Especially *Salmonella* incidence level is even higher than the baseline in 1997. *E. coli* O157:H7 incidence in 2008 was higher than that of in 2004. The CDC reports published yearly also show that the numbers of Shiga toxin producing pathogenic *E. coli* (STEC) cases are on the rise since 2003 (Figure 1).

Table 1

Incidence of 4 major foodborne pathogens per 100,000 population.

Pathogen	1997 Baseline	Incidence in 2004	Incidence 2006-2008	Target 2020
Campylobacter species	24.60	12.90	12.70	8.50
E. coli O157:H7	2.10	0.90	1.20	0.60
L. monocytogenes	0.50	0.27	0.30	0.20
Salmonella species	13.70	13.00	15.20	11.40

Source: CDC 2005, 2009 and Healthy People 2020.



2011* is an approximation from the data released on 08/13/2011 based on the previous CDC reports.

Figure 1. Reported Shiga toxin producing *E. coli* (STEC) cases, 2000-2011.

2.3. Susceptibility of Fresh and Fresh-Cut Produce to Contamination

Fresh fruits and vegetables consumed after minimal or no processing are increasingly recognized as sources of foodborne disease outbreaks. In the US, produce related outbreaks have increased from 0.7% of all reported foodborne outbreaks in the 1970s to 6% in the 1990s (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). Based on the data available, 60% of the

produce related outbreaks are linked to a bacterial pathogen (Sivapalasingam *et al.*, 2004). From 1990 to 2005, the number of foodborne illnesses linked to fresh produce surpassed those linked to other foods, including poultry, beef, eggs, and seafood. In addition, the average number of illnesses per outbreak was significantly higher in fresh produce outbreaks than those caused by other foods (Solomon & Sharma, 2009). The increase in the number of outbreaks related to fresh produce might be the consequence of improving outbreak surveillance systems and/or increased consumption of fresh-cut prepackaged products.

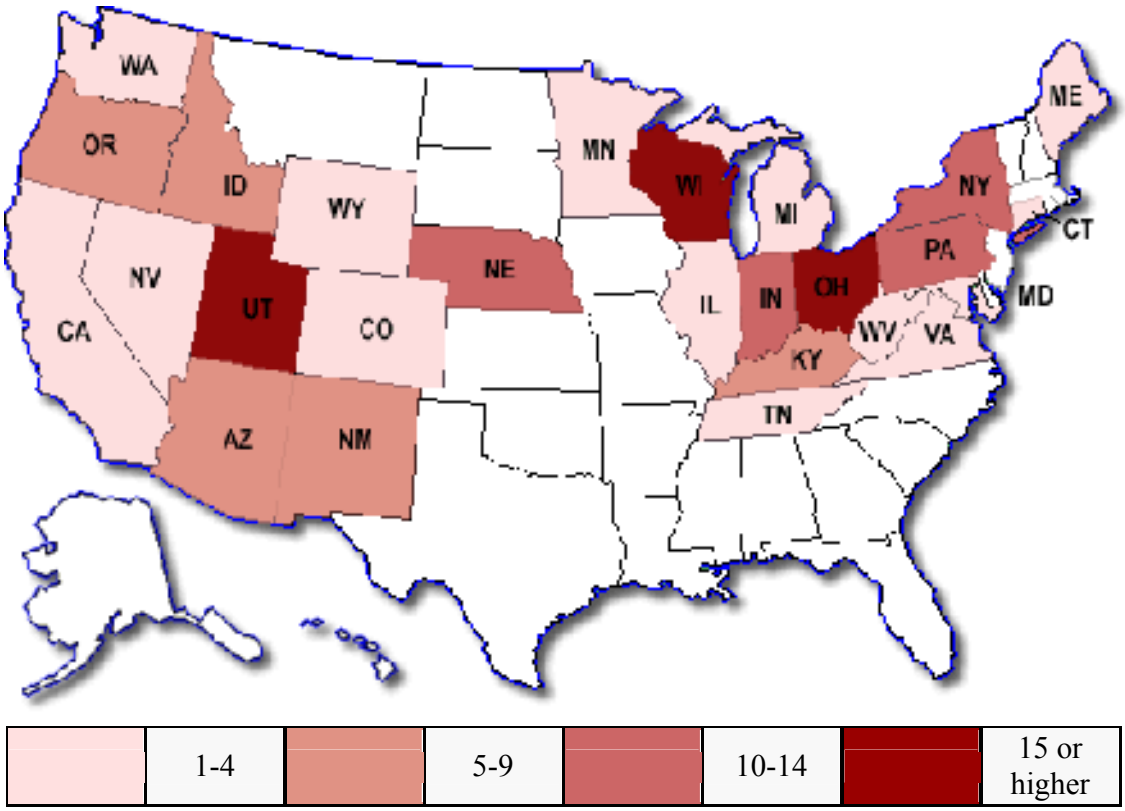
In the last decade of the 20th century, government agencies, voluntary organizations, and private companies joined their efforts to inform the American public about the healthy nutritional choices. The new MyPlate program, which replaces the Food Guide Pyramid, recommends that half of our diet should be composed of fruits and vegetables (MyPlate - USDA). The old Five-A-Day program and the current Fruits & Veggies More Matters program by the Produce for Better Health Foundation were other examples to draw public attention to importance of increasing the consumption of fruits and vegetables (Fruits & Veggies More Matters). As a result of these efforts, the *per capita* consumption of fresh produce has increased significantly in recent years (Kaufman, Handy, McLaughlin, Park, & Green, 2000). In a USDA report, it was predicted that the lettuce consumption per capita will increase by 5.1% from 2000 to 2020, with a total market growth rate of 24% by 2020 (Lin, 2004). According to the foodborne disease outbreaks surveillance data, the consumption of leafy greens increased by 17.2% during 1986-1995 compared to the previous decade and the proportion of foodborne disease outbreaks linked to the leafy green consumption also increased by 59.6% during the same period. In the following decade (1996-2005), leafy greens consumption boosted by 9.0% and the foodborne disease outbreaks arisen by 38.6% (Herman, Ayers, & Lynch, 2008). It seems that the increase in the

number of leafy green related outbreaks cannot solely be explained by the increase in leafy green consumption.

Factors such as the practices at pre- and post-harvest stages can be likelihood of contamination. Contamination at pre-harvest may occur due to the use of improperly prepared fertilizers from animal feces, irrigation water contaminated with the animal feces from a nearby animal farm, or other agents contaminating the irrigation water or the farm area (Cooley, Carychao, Crawford-Miksza, Jay, Myers, Rose *et al.*, 2007). Contamination sources at post-harvest or during processing include water used for washing, sprays, chill tanks, and ice used during shipping. Additionally, post-harvest stage contamination sources can be the contaminated surfaces and hands of infected workers with poor hygiene. Today, fresh produce travels from all over the world before it reaches the consumers in the US making oversight difficult if not impossible. The 2006 multi-state *E. coli* O157:H7 outbreak linked to bagged baby spinach was originated from a California farm (California Food Emergency Response Team, 2007). Produce grown in large amounts is distributed in the entire US in a matter of days as seen by the incidence map of 2006 spinach outbreak (Figure 2) (CDC, 2006b). A total of 254 people became sick and 3 died in two different *E. coli* O157:H7 outbreaks in 2006 which were associated with bagged baby spinach and shredded lettuce (CDC, 2006a, b). Rates of hospitalization (52-75%) and HUS (29%) were higher in these two outbreaks than previously recorded *E. coli* O157:H7 outbreaks (Mead *et al.*, 1999; Thorpe, Ritchie, & Acheson, 2002).

A Shiga toxin-producing *E. coli* (STEC) outbreak occurred in May 2011 in northern Germany, affecting thousands of people all over Europe and North America. The outbreak presented an increased incidence of HUS and bloody diarrhea, and was initially thought to be linked to the consumption of tainted raw tomatoes, cucumber, and leafy greens (Frank, Faber,

Askar, Bernard, Fruth, Gilsdorf *et al.*, 2011) . Later on, it was highly suspected that organic soybean sprouts grown in a farm in Germany were the source of the outbreak. According to the World Health Organization (WHO), 16 countries have reported significant EHEC infection cases that claimed 50 lives and over 4000 hospitalizations including more than 800 cases of HUS (WHO, 2011).



Source: (CDC, 2006b).

Figure 2. The 2006 *E. coli* O157:H7 outbreak case counts by state linked to fresh bagged baby spinach.

Recent *E. coli* O157:H7 outbreaks are more likely to be associated with the consumption of contaminated fruits and vegetables. A list of *E. coli* O157:H7 related outbreaks linked to fresh produce between years 1997-2006 is given in Table 2. *E. coli* O157:H7 and *Listeria monocytogenes* attach cut edges of produce leaves more easily than to intact leaves (Boyer,

Sumner, Williams, Pierson, Popham, & Kniel, 2007; Takeuchi, Matute, Hassan, & Frank, 2000). According to a recent study, it was found that *E. coli* O157:H7 grew faster on mechanically damaged lettuce leaves as nutrients were freely available (Brandl, 2008).

Plant surfaces are used to be known as inhabitable to enteric pathogens such as *E. coli* O157:H7 to flourish. Bacteria need to overcome harsh conditions like excessive sunlight, limited nutrients, and day/night temperature changes to survive on plant surfaces. Surprisingly, recent research shows that enteric pathogens such as *E. coli* O157:H7 and *Salmonella* spp. are able to survive and grow on plant surfaces even under these harsh conditions (Brandl, 2006; Heaton & Jones, 2008).

Table 2

E. coli O157:H7 outbreaks by produce type between years 1997-2006.

Year	**Produce type**
1997	Sprouted seeds (alfalfa)
1997	Salad
1998	Salad
1998	Fruit salad
1998	Coleslaw
1998	Sprouted seeds (clover／alfalfa)
1998	Unpasteurized apple juice
1998	Parsley
1999	Coriander (cilantro)
1999	Unpasteurized apple juice
2003	Cucumber
2003	Lettuce
2005	Lettuce
2006	Spinach
2006	Lettuce

Source: Compiled from CDC (www.cdc.gov).

Animal feces are widely used in agriculture and proper composting is critical to kill possible pathogens in the manure. Live pathogens in partially composted manure can infect the growing plants. Contaminated ovine manure with 10^6 CFU/g of *E. coli* O157:H7 was not free of pathogen after 21 months when not aerated (Kudva, Blanch, & Hovde, 1998). *E. coli* O157:H7 was also shown to survive more than 40 days depending on the initial concentration and composting temperature (Wang, Zhao, & Doyle, 1996). Another pathogen, *Salmonella* spp., was found to survive longer than *E. coli* O157:H7 in bovine manure under the same conditions (Himathongkham, Bahari, Riemann, & Cliver, 1999). Enteric pathogens can also survive in sterile water for long periods (Wang & Doyle, 1998). Addition of organic matter similar to animal manure greatly increased the survival of *E. coli* O157:H7 in water (Hutchison, Walters, Moore, & Avery, 2005). It is also found that the pathogen load in water sources close to animal fields increases after heavy rains (Cooley *et al.*, 2007). Use of improperly composted animal manure and irrigation with contaminated water may result in introduction of live pathogens in agricultural fields.

Since large scale food-borne outbreaks started to draw attention, a tendency has been noticed that certain pathogens are more linked to certain food items. Green leafy vegetables such as lettuce and spinach have been linked to 29 *E. coli* O157:H7 outbreaks occurred between 1990 and 2005 (Solomon & Sharma, 2009).

Plant surfaces are mostly covered with waxy cuticle. Aerial plant surfaces are thought to be inhabitable by enteric pathogens as there is limited nutrient availability, large temperature swing between day and night, and high UV exposure from sunlight. Often cracks and damages in the cuticle that expose the epidermal cells are the sites for bacterial colonization. Plant surfaces are also colonized by natural foliar microflora, which competes for nutrient (Lindow & Brandl,

2003). When introduced to plant surfaces, pathogens must be able to first attach and proliferate to be transmitted to the produce. *E. coli* was shown to strongly attach to lettuce and green pepper after a short time (Beuchat, 1999; Han, Sherman, Linton, Nielsen, & Nelson, 2000). Washing and agitation were not enough to remove the attached *E. coli* cells. Damaged plant tissues are more susceptible to pathogen colonization than intact tissues (Seo & Frank, 1999). Fresh-cut produce are more susceptible to bacterial attachment and colonization than whole plants.

In the period from 1990 to 2005, the lettuce was the number one produce example associated with several outbreaks (Solomon & Sharma, 2009). The reasons for this problem might be listed as follows: 1) Lettuce is grown directly on the ground; 2) Its leaves are easily damaged creating easy access points for any pathogen; and 3) Lettuce is consumed raw as main salad ingredient. However, survey studies on lettuce samples from farm and screened grocery stores did not yield any pathogens to prove these hypotheses (Delaquis, Bach, & Dinu, 2007).

E. coli contamination on cut lettuce even at low levels (10^2 CFU/g) was still present after 15 days of incubation at 4°C (Beuchat, 1999). Chlorine wash (200 ppm) was not effective any more than washing with water alone. Similar result was collected after washing with cold and warm water including 100 ppm chlorine (Delaquis, Stewart, Cazaux, & Toivonen, 2002). *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were shown to attach more to the cut surfaces of lettuce compared to the intact surfaces (Takeuchi & Frank, 2000). Similar to the known phytopathogens, *E. coli* attaches to the stomatas on intact lettuce plants (Seo & Frank, 1999).

In the field, plants spray–irrigated with water contaminated with 10^7 CFU/ml of *E. coli* O157:H7 were tested positive up to 20 days, and chlorine treatment (200 ppm) of these plants did not inactivate the organism completely (Solomon, Yaron, & Matthews, 2002). Another study showed that lettuce plants grown in soil fertilized with contaminated manure were tested positive

for *E. coli* 77 days after seeding (Islam, Doyle, Phatak, Millner, & Jiang, 2004). According to a recent study, *E. coli* O157:H7 cells used filamentous type III secretion system to attach to green leafy vegetables such as spinach, lettuce, and arugula when leaves were dipped in 10^6 CFU/ml of *E. coli* O157:H7 solutions at 37°C for extended periods of times (Shaw, Berger, Feys, Knutton, Pallen, & Frankel, 2008).

Intestinal pathogens attached on produce surfaces have been found to colonize and survive inside the plant tissues through a process called internalization. Internalized pathogens pose a greater threat as they can continue their presence through the food delivery system undetected. Currently no sanitation method used in the produce industry has been proven to be fully effective against internalized pathogens. Internalization may occur through the cuts and bruises on the surface, plant roots, and infiltration and cross contamination during post-harvest processing. Rapid cooling of the harvested produce from the farm may promote internalization. When a warm fruit is immersed in a cooler liquid, it creates a positive temperature difference and the gases in internal spaces of fruit contract to draw some amount of cooling water inside the fruit through the pores and openings on the surface (Buchanan, Edelson, Miller, & Sapers, 1999). If the cooling water is contaminated, it is possible that bacteria will be driven into the produce. Warm mangoes (46°C) immersed in 22°C cool water contaminated with *S.* Enteritidis were found to have internalized bacteria on the cut stem end. However the opposite blossom end was less likely to have bacteria (Penteado, Eblen, & Miller, 2004). Type of the produce is important for internalization. Oranges immersed in *E. coli* O157:H7 and *Salmonella* containing liquids had internalization rates of 2.5% and 3.0%, respectively (Eblen, Walderhaug, Edelson-Mammel, Chirtel, De Jesus, Merker *et al.*, 2004).

Several studies focused on *E. coli* O157:H7 internalization in spinach and lettuce plants through root uptake as *E. coli* O157:H7 outbreaks are often linked to these produce. *E. coli* O157:H7 cells were recovered from internal tissues of lettuce 5 days after the plants were treated with contaminated irrigation water or manure slurry with 10^7 *E. coli* O157:H7 CFU/ml (Solomon *et al.*, 2002). As the concentration of *E. coli* O157:H7 on the lettuce plants increased to 10^9 CFU/ml, the number of internalized *E. coli* O157:H7 also increased to 10^2 CFU/g in tests conducted 5 days after the exposure (Solomon & Matthews, 2005). *E. coli* O157:H7 count did not increase in samples taken after 5 days showing that the cells are not dividing inside the lettuce tissue.

In a study, the effect of the presence of other bacteria was tested on internalization and growth of *E. coli* O157:H7 inside of 6-week old spinach plants. Bacteria were vacuum infiltrated to the roots of spinach. The presence of *Pseudomonas syringae* did not result in significant increase in the counts of *E. coli* O157:H7 compared to *E. coli* O157:H7 alone treatment 1 week after the exposure, 4.9 and 4.6 log CFU/g spinach respectively (Hora, Warriner, Shelp, & Griffiths, 2005). The researchers also tested for the effect of nematode presence in the soil contaminated with *E. coli* O157:H7 (10^7 CFU/g). The roots of all 24 plants were positive for *E. coli* O157:H7, whereas the leaves were all negative for the same bacteria. In another study, *E. coli* O157:H7 counts were determined in hydroponically grown cress, lettuce, radish, and spinach plants 9 and 49 days after exposure to 10^2 CFU/ml of *E. coli* O157:H7 (Jablasone, Warriner, & Griffiths, 2005). Internalized *E. coli* O157:H7 was positive in lettuce, radish, and spinach plants after nine days of growth, but not in cress (Jablasone *et al.*, 2005). *E. coli* O157:H7 counts in spinach dropped from 2.5 log CFU/g at nine days to undetectable levels (<1 log CFU/g) at 49 days. Internalization of *Salmonella* was also investigated under the same

conditions. Internalized *Salmonella* was present (1 to 1.6 log CFU/g) in lettuce and radish plants after nine days (Jablasone *et al.*, 2005). Nevertheless, all samples were negative for internalized *Salmonella* after 49 days. *E. coli* O157:H7 and *Salmonella* are not able to survive long periods inside the plant tissues probably due to the physiological and nutritional stress inside the plants or the plant defense mechanisms are effective in killing enteric human pathogens. Internalization of the pathogens inside the plant tissue happens at a higher rate when the plants are grown in contaminated soil than in hydroponic media (Franz, Visser, Van Diepeningen, Klerks, Termorshuizen, & van Bruggen, 2007; Jablasone *et al.*, 2005). This may be due to the root damage in the plants when they are grown in soil. Eight-day old lettuce seedlings were placed in *E. coli* O157:H7 or *Salmonella*-inoculated hydroponic media (10^7 CFU/ml) or soil (7-8 log CFU/g) and grown for 18 days. No *E. coli* O157:H7 were recovered from the roots or leaves of surface-sterilized lettuce plants grown hydroponically (Franz *et al.*, 2007). Under hydroponic conditions, *Salmonella* was found to be internalized in 4/10 lettuce root samples and 2/10 leaf samples. When the lettuce seedlings were grown in contaminated soil, internalization of *E. coli* O157:H7 and *Salmonella* was observed in all lettuce samples at levels of 3.95 log CFU/g and 2.57 log CFU/g, respectively (Franz *et al.*, 2007). These results were contradicted by Sharma *et al.* (2009a), who were not able to recover internalized *E. coli* O157:H7 from mature spinach grown in hydroponic medium and inoculated with 10^7 CFU/ml *E. coli* O157:H7 (Sharma, Ingram, Patel, Millner, Wang, Hull *et al.*, 2009a). The researchers were also not able to detect internalization in spinach exposed to *E. coli* O157:H7 at 10^3 or 10^7 CFU/g in pasteurized soil.

These results demonstrate that internalization of pathogenic bacteria is feasible through washing process that reduces produce core temperature and root uptake given there is a high concentration of the bacteria available in close proximity. Root uptake of bacteria requires the

bacteria to travel long distance to the leaf in order to be ingested by the consumers, which does not seem to be likely as understood by the current research. Spray washing of the green leafy vegetables with water contaminated with the pathogens or floods in the growing field after heavy rains that carry manure from a nearby animal raising facility are more probable ways of contamination.

After the initial attachment of the pathogen on produce surface, bacteria may come together and form a structure called biofilm (Annous, Solomon, Cooke, & Burke, 2005). Biofilms are structures formed by bacteria adherent to each other and covered in an extracellular matrix (Costerton, Stewart, & Greenberg, 1999). Between 30-80% of the total bacteria that exist on a plant surface are proposed to be embedded in biofilms (Lindow & Brandl, 2003). It is known that natural plant flora forms biofilms (Annous *et al.*, 2005; Danhorn & Fuqua, 2007); but a similar biofilm formation was simply not expected from enteric bacteria. Lately the topic of biofilm formation by foodborne pathogens has been a growing research area. Pathogens like *E. coli*, *Salmonella*, *Shigella*, and *Campylobacter* were all found to form biofilms on plant surfaces such as parsley, tomatoes, and melons (Annous *et al.*, 2005).

Bacteria form biofilms to resist harsh conditions on plant surfaces such as large temperature changes, unavailability of water, and high UV exposure. Biofilms on plant surfaces resemble biofilms on food processing surfaces. Both harbor high number of bacteria that are difficult to remove or inactivate as they are protected in extracellular matrix (Chmielewski & Frank, 2003). Biofilm embedded cells can withstand harsh conditions of processing steps such as disinfectants; and therefore, they could reach the consumers.

Postharvest plant lesions also promote attachment and growth of pathogens. Cut or damaged lettuce heads release latex rich in organic matter including sugar. Latex was shown to

support the rapid growth of *E. coli* O157:H7 on cut lettuce surface. When the amount of surface damage was increased, *E. coli* O157:H7 growth was increased in parallel (Brandl, 2008).

2.3.1. Use of sanitizers to disinfect fresh produce. Bacterial flora on healthy spinach leaves, phyllobacteria, is about 5 log CFU/cm^2 (Warner, Rothwell, & Keevil, 2008). These bacteria associated with plant leaves do not localize uniformly on the leaf surface. They were shown to attach and colonize at selected areas such as stomata, the base of trichomes, epidermal cell wall junctions, grooves along the veins, depression regions in the cuticle, and beneath the cuticle. More bacteria are present on the bottom leaf surface compared to the top side, which might be due to the distribution of stomata and trichomes (Beattie & Lindow, 1999). Further attachment of phyllobacteria on cut surface of fresh produce items makes it more difficult to remove or inactivate these bacteria. Similar to the natural phyllobacteria, a human pathogen *Salmonella* Thompson was found to attach stomatas of spinach leaves (Warner *et al.*, 2008).

In the literature, various compounds, methods, and physical conditions have been tested for their efficacies to reduce the live microorganism count on fresh produce. Some of these are hot or cold water, sodium hypochlorite, chlorine dioxide, organic acids, acidified water, ozone, ozonated water, UV radiation, and irradiation.

2.3.1.1. Chlorine solutions. Chlorine solutions up to 200 ppm are by far the most widely used method by the fresh produce industry (Takeuchi & Frank, 2001b). However, hypochlorite solutions are not effective in inactivating *E. coli* O157:H7 internalized in cut edges or stomata of lettuce leaves (Takeuchi et al., 2000). Studies have shown that foodborne pathogens attach well to cut surfaces on fresh produce, where the nutrients are readily available (Boyer et al., 2007; Takeuchi & Frank, 2000). Cut lettuce leaves were heavily colonized by *E. coli* O157:H7 and *L. monocytogenes* compared to whole leaf. However, surface intactness was not important for

Salmonella Typhimurium, which attached well on both cut and whole leaves (Takeuchi & Frank, 2000). *E. coli* O157:H7 is able to penetrate cut surfaces of lettuce more efficiently at 4°C compared to 7, 25, and 37°C (Takeuchi & Frank, 2000). The more *E. coli* O157:H7 cells penetrate into the cut surfaces or stomatas, the better protected they are from the harmful effect of free chlorine in the produce wash solutions. In a study, it was found that 200 mg/ml chlorine solution did not eliminate *E. coli* O157:H7 cells attached to cut surfaces completely, washing reduced the pathogen concentration by only 1 log CFU/g (Takeuchi & Frank, 2001a, b).

Hypochlorite solutions are mainly used to keep the wash water clean to prevent cross contamination of pathogenic bacteria (Behrsing *et al.*, 2000). Nevertheless chlorine wash step is extensively used by the fresh produce industry. Non-pathogenic *E. coli* count was reduced by 2 log CFU/g when the inoculated lettuce was washed in 100 ppm hypochlorite solution (Behrsing *et al.*, 2000). *Salmonella* Baildon inoculated on diced tomatoes and shredded lettuce was reduced by less than 1 log CFU/g after washing in 200 ppm hypochlorite solution for 40 seconds (Weissinger, Chantarapanont, & Beuchat, 2000). *L. monocytogenes* populations on lettuce were reduced by only 0.7 log CFU/g and 1.7 log CFU/g after washing in 100 ppm chlorinated water and peracetic acid, respectively (Hellstrom, Kervinen, Lyly, Ahvenainen-Rantala, & Korkeala, 2006). The oxidative effect of free chlorine in hypochlorite solutions used to wash fresh produce is inactivated by the organic substances released from the damaged or cut surfaces (Marriott, 1999). Survival chance of bacteria localized on fresh produce is higher when the produce is cut and washed in chlorine solution as the cut surfaces will release organic matter, which quickly inactivates the free chlorine. The exopolysaccharide found in bacterial biofilms on produce may also have the same effect against the free chlorine in hypochlorite solutions (Solomon & Sharma, 2009). Sodium hypochlorite (200 ppm) treatment of fresh-cut lettuce at 50°C for 1 min was

found to reduce *E. coli* O157:H7, *S.* Typhimurium, and *Staphylococcus aureus* by up to 1.7 log CFU/g without increasing browning (Kondo, Murata, & Isshiki, 2006).

Oxidizing effect of chlorine can also be employed in gaseous sanitizers to control and kill foodborne pathogens. Oxidative capacity of chlorine dioxide (ClO_2) is higher than liquid hypochlorite sanitizers, which may overcome inactivation problem by the presence of residual organic matter. Chlorine dioxide gas was used to successfully kill *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* on apples, tomatoes, and onions but it was ineffective against these foodborne pathogens on peaches, cabbage, carrots, or lettuce (Sy, Murray, Harrison, & Beuchat, 2005). In another study, researchers used ClO_2 gas (total 100 mg) against *Salmonella* spp. and could not recover any pathogen on cucumbers, obtaining up to 6 log CFU/g reduction. Authors concluded that ClO_2 gas is more effective on smooth surfaces like cucumber as gas treatment was able to reduce *Salmonella* counts only by 2-4 log CFU/g on bell peppers and strawberries, respectively (Yuk, Bartz, & Schneider, 2006).

2.3.1.2. Warm or hot water wash. Warm or hot water wash treatments have been used more efficiently than cold water to reduce microbial load on fruits and vegetables. Nonetheless, due to the delicate nature of the fresh leafy greens the use of warm and hot water is limited. Iceberg lettuce treated for 5 min with 50°C acidified (pH 4.9) water had lower total bacteria and Enterobacteriaceae counts by 2.9 and 3.7 log CFU/g, respectively, compared to control samples after 13 days at 4°C (Wei, Wolf, & Hammes, 2005).

Recently, Chiquita's Fresh Express brand, a ready-to-eat bagged salad producer, has announced to start using a mixture of organic acids; lactic acid plus peroxyacetic acid (LA-PAA), (Fresh Rinse™) to enhance the efficiency of their fresh produce wash process (Fresh Express, 2011). The company invented the LA-PAA mixture (Ho, 2009), and claims it to be eco-

friendly and 7 times more effective than the traditional chlorine wash used by the industry for reducing the total aerobic bacterial count (Ho, Luzuriaga, Rodde, Tang, & Phan, 2011).

2.3.1.3. Vacuum cooling. Vacuum cooling is an industry standard method in the leafy green industry. The purpose of this method is to reduce the inner heat of the produce quickly to slow down the plant metabolism and any further bacterial growth on produce surface. The effect of vacuum cooling on bacterial attachment and infiltration was recently investigated on lettuce tissue. It was found that vacuum cooling disrupts the surface structure of lettuce tissue causing stomatas to open. *E. coli* O157:H7 that was experimentally inoculated on the lettuce was able to attach deeper and infiltrate through the stomatas and cause external damages on lettuce surface under vacuum conditions. More than 1 log CFU/g *E. coli* O157:H7 was recovered from the vacuum cooled lettuce leaves after surface washing in hypochlorite solution compared to the control leaves that were not subjected to vacuum cooling (Li, Tajkarimi, & Osburn, 2008). Especially, the pressure change while switching the conditions to normal atmosphere after the vacuum cooling process was effective to force the surface bacteria further into the lettuce tissue (Li *et al.*, 2008).

2.3.1.4. Ozone. Ozone has a rapid oxidative activity and is more powerful disinfectant than chlorine. It has been approved as GRAS and was used in food and bottled water industries for years (Marriott & Gravani, 2006). Ozone treatment (64 min) followed by pressurized (83 kPa) ozone treatment (64 min) reduced *E. coli* O157:H7 and *Salmonella* counts by 3 and 2.6 log CFU/g on strawberries and 3.8 and 3.6 log CFU/g on raspberries, respectively (Bialka & Demirci, 2007). Pathogen count on *E. coli* O157:H7 or *L. monocytogenes* inoculated lettuce was reduced by 1.1 and 0.9 log CFU/g, respectively, after 5 min treatment with 5 ppm ozonated water (Yuk, Yoo, Yoon, Moon, Marshall, & Oh, 2006). Based on these findings, gaseous ozone may

be more effective than ozonated water. However, ozone treatments require expensive treatment chambers and; thus, it may be applied to the small-scale produce farming operations. Ozone treatments are not seen feasible to be adopted by the large-scale produce industry in the near future.

Cold plasma treatments (3 min) of apples inoculated with *E. coli* O157:H7 or *Salmonella* Stanley resulted in 3.7 and 3.6 log CFU/ml reductions of the pathogen count, respectively (Niemira & Sites, 2008). Even though some of the sanitizing agents discussed here offer promising results, the consumer attitudes towards these types of marginal sanitation methods are not known, and should be investigated.

2.3.1.5. Irradiation. Irradiation is a non-thermal method utilizing radiation to kill insects, molds, bacteria, and other foodborne microorganisms. Irradiation was developed in 1940s and has been used on different food products for various purposes by a number of FDA approvals since 1963. It was approved by FDA in 1986 to be used on fresh fruits and vegetables to eliminate insects and inhibit ripening and sprouting processes. Due to the continuous occurrences of foodborne pathogen outbreaks linked to the leafy greens, a renewed interest has grown to use irradiation against foodborne pathogens on fresh produce. On August 22, 2008, FDA approved irradiation at a dose up to 4.0 kiloGray (kGy) as a food additive in fresh iceberg lettuce and spinach to make them safer and last longer without spoiling (USFDA, 2008).

There are 3 types of irradiation technology; electric beam (e-beam), X-ray, and gamma ray in decreasing order of energy levels. Although their energy sources, effect mechanisms, and penetration levels are different, they eventually cause cleavage of water molecules to create oxygen and hydroxyl radicals that cause DNA damage (Niemira & Fan, 2009).

Irradiation at a dose of 2.0 kGy was shown to control fungal and bacterial load on carrot slices at 5°C for 2 weeks without affecting sensory qualities of the carrot slices (Chaudry, Bibi, Khan, Khan, Badshah, & Jamil Qureshi, 2004). The combination of 7% calcium ascorbate and irradiation (0.5 or 1.0 kGy) enhanced microbial food safety while maintaining the quality of fresh-cut apple slices after 3 weeks at 10°C (Fan, Sokorai, Sommers, Niemira, & Mattheis, 2005).

A low-dose irradiation (0.51 kGy) followed by refrigerated storage (4°C) can effectively reduce or eliminate *Listeria monocytogenes* on chopped romaine lettuce (Mintier & Foley, 2006). In 2008, 1.2 kGy e-beam irradiation with a subsequent refrigeration for up to 15 days was shown to reduce *E. coli* O157:H7 (7 log CFU/g) load on contaminated ready-to-eat spinach leaves to under 1 log CFU/g while not sacrificing organoleptic properties of the spinach (Gomes, Moreira, Castell-Perez, Kim, Da Silva, & Castillo, 2008).

In a recent study, the researchers aimed to test the effect of irradiation on biofilm forming *E. coli* O157:H7 on green leafy vegetables, romaine lettuce, and baby spinach leaves (Niemira & Cooke, 2010). The leaves were dip inoculated with *E. coli* O157:H7 and stored at 4°C for up to 72 h to allow biofilm formation. Stomatal aggregations and biofilm formations of *E. coli* O157:H7 were confirmed via scanning electron microscope (SEM). At 0, 24, 48, and 72 h, the leaves were exposed to different doses of irradiation up to 1 kGy. Irradiation was more effective than 300 and 600 ppm chlorine treatment, which was only moderately effective against *E. coli* O157:H7 on baby spinach and romaine lettuce with maximum 1.3 and 1.8 log CFU/g reductions, respectively. Also, the pathogen counts after the irradiation treatment showed that the irradiation was becoming less and less effective in killing *E. coli* O157:H7 as the cells continue on

improving the biofilm structures during the incubation period before the irradiation treatment (Niemira & Cooke, 2010).

Although the irradiation is a promising method and approved by the FDA, the high cost of building the system makes it difficult for the fresh produce industry to widely use. The meat industry already incorporated irradiation in their routine process line. Produce processors may transport the produce to the meat process facilities for irradiation. However, this would cause time delay in marketing the fresh produce, which is vital in produce industry due to the short shelf life of the fresh produce. Additional contamination risks during the transportation of the produce should be calculated as well. Consumer stance towards the irradiated fresh produce should be investigated as well. When it comes to fresh produce, consumers may not be as accepting toward irradiation as they are with the processed meat products.

2.4. Bacteriophages

The numbers of foodborne related pathogen outbreaks show that the current routine methods employed by the industry to control the bacterial foodborne pathogens such as *Salmonella*, *Listeria*, *Escherichia coli*, and *Campylobacter* are not efficient (DuPont, 2007), and the marginal approaches used in scientific literature are expensive and not easily accepted by the consumers. It is safe to say that there is an urgent need for a novel and more effective sanitation agent and method to help keep fresh produce pathogen free.

One alternative method is the use of bacteriophages as natural antibacterial agents in food to eliminate pathogens. Bacteriophages (phages) are viruses that specifically interact with and infect bacterial cells. Phages are responsible of killing almost half of the bacterial population generated daily in the nature (Suttle, 2005). Considering the short generation time of the bacteria, phages are the most lethal enemies of the bacteria. The use of bacteriophages is expected to be

accepted readily by the consumers since phages are natural enemies of bacteria and found in nature with no known side effects on eukaryotic organisms (Hagens & Loessner, 2007; Strauch, Hammerl, & Hertwig, 2007).

2.4.1. History of bacteriophages. The discovery of phages is rather a debatable issue. With the beginning of the microbiological studies in Western world around the 1880s, sporadic findings over what seems to be phage or phage activity were reported by Hankin (1896) and Gamaleya (1898) (Sulakvelidze, Alavidze, & Morris, 2001). Today, it is accepted that bacteriophages were discovered in two independent research conducted by Twort (1915) and d'Herelle (1917) (Ackermann, 2011). The names bacteriophage and plaque were introduced by d'Herelle, who first used the phages to treat a human disease, severe dysentery in 1919 (D'Herelle, 2007; Sulakvelidze *et al.*, 2001). D'Herelle and his coworkers used the phage preparation to treat several patients with bacterial dysentery by ingesting one dose of phage preparation. The patients started to recover within 24 h of treatment. In another study, the researchers applied phages to the patients with staphylococcal skin disease (Bruynoghe & Maisin, 1921). Those patients, as well, recovered within 1-2 days of direct phage injection around the skin lesions. Later on, D'Herelle used various phage preparations to treat cholera and bubonic plaque in India (Summers, 1999).

Phages had been utilized in the Western world to treat bacterial infections before the first antibiotic, penicillin, was used clinically in 1940s (Peláez, 2006). Due to the wide range efficacy of antibiotics, they were immediately adopted and used widespread during World War II. Phage research could not find enough attention to survive in the West and was only continued in the former Soviet Union and Eastern European countries, especially in 2 institutes; Eliava Institute of Bacteriophage, Microbiology, and Virology (Tbilisi, Georgia) founded in 1923 and Hirszfeld

Institute of Immunology and Experimental Therapy (Wroclaw, Poland) founded in 1952. A comprehensive review of the topic is available by Sulakvelidze *et al*. (2001).

2.4.2. Types of bacteriophages. Phages are extremely diverse and abundant in nature; thus, present the most promising natural weapon against bacteria. Based on the interaction of the phages with their host bacterial cells, phages are divided into 2 groups; obligate and temperate phages, however, a third group (chronic phages) has been mentioned in the literature lately (Housby & Mann, 2009).

2.4.2.1. Obligate phages. Obligate phages are the best candidates for phage therapy studies. They are also called "lytic phages" as they have a lytic life cycle within their host cells. There are 2 different ways lytic phages kill their target bacteria; lysis-from-within (LWI) and lysis-from-without (LWO) (Delbruck, 1940). Which method to be employed is based on the multiplicity of infection (MOI), which is the ratio of the available virulent lytic phages over the available target bacterial cells. Lysis-from-within is employed when one or few lytic phages infect the susceptible bacterial cells. Upon infection, the phage genome is replicated inside the bacterial host cell by the cell's own DNA replication enzymes and proteins. When newly generated phages have been assembled, the membrane of the infected host cell is lysed to release the progeny phages. Lysis-from-without occurs when the MOI is equal to or more than the amount that bacterial host cells can accommodate (Delbruck, 1940). The multiple rush adsorption of the phages into the cell causes rapid swelling and shape deformation followed by the sudden destruction of the cell membrane. Neither the host bacterial cell nor the phage particles adsorbed survive after LWO (Delbruck, 1940).

2.4.2.2. Temperate phages. Temperate phages also known as "lysogenic phages" are capable of replicating themselves inside the host cell, and lysing the host to release the progeny

just like the lytic phages. The difference is that a lysogenic phage may incorporate its genome into the bacterial DNA; thus, forming a long term relationship with the host. A phage whose genome has been incorporated into the host DNA is called a prophage (Lwoff, 1953). The prophage genome is replicated with the host genome during mitosis and then distributed into the daughter cells. This is how a prophage is able to continue its presence in the host bacteria for generations. A prophage may quit lysogenic life cycle and convert back to a lytic life cycle by a process called prophage induction, which results in removal of prophage from the host genome upon stimulants such as radiation, pH, temperature, and heavy metals (Choi, Kotay, & Goel, 2010). During the removal of prophage from the host DNA, the prophage may take part of the flanking regions of the host DNA and these host DNA regions contribute to the genetic diversity among the bacteriophages and their hosts through transduction (Lennox, 1955).

2.4.2.3. Chronic phages. Chronic phages are filamentous phages that do not necessarily kill their bacterial hosts. They are able to release their progeny without needing to kill the host cell (Russel & Model, 2006). The fact that filamentous phages do not kill the host cells makes them unsuitable for using as an agent to control the pathogenic bacteria.

2.5. Bacteriophages as Biocontrol Agents

The utilization of phage based products in biologically controlling pathogens such as *S.* Enteritidis, *E. coli* O157:H7, and *L. monocytogenes*, during animal and plant derived food productions and processes have been investigated by many researchers using *in vivo* and *in vitro* studies. Phages are also shown to be effectively used for the sanitization of food preparation surfaces.

2.5.1. Bacteriophage use in food production and processing. Equipment and surface hygiene in food processing plants is highly crucial in maintaining pathogen-free facilities.

2.5.1.1. L. monocytogenes. *L. monocytogenes* is known for being a persistent pathogen in forming biofilms in food processing facilities. In a recent study, Phage P100 significantly reduced *L. monocytogenes* cells under two different biofilm matrices, namely 96-well plates and stainless steel coupons. The researchers tested 21 *L. monocytogenes* strains representing 13 different serotypes. The abilities of the strains differ in forming biofilms. The phage P100 significantly reduced the number of *L. monocytogenes* cells inside biofilm matrices by up to 5.4 log/cm^2 (Soni & Nannapaneni, 2010).

2.5.1.2. E. coli O157:H7. Abuladze *et al.* (2008) examined EcoShield™ (formerly known as ECP-100) for its efficacy to lyse *E. coli* O157:H7 strains on glass coverslips and gypsum boards. The 5-min phage cocktail (10^{10} PFU/ml) treatment significantly reduced the *E. coli* O157:H7 numbers recovered from the glass coverslips and gypsum boards by up to 99.99% and 100%, respectively (Abuladze, Li, Menetrez, Dean, Senecal, & Sulakvelidze, 2008).

In another study, a phage cocktail against *E. coli* O157:H7, referred to as BEC8, was successfully used in sanitizing common food preparation surfaces made of stainless steel, ceramic tile, and high density polyethylene chips (Viazis, Akhtar, Feirtag, & Diez-Gonzalez, 2011a). Three different *E. coli* O157:H7 strains were combined and spot inoculated on surfaces at approximately 4, 5, and 6 log CFU/chip, then the samples were air-dried and sprayed with sterile PBS or BEC8 (6 log PFU/chip) cocktail. *E. coli* O157:H7 counts were found to be below the detection limit of 10 CFU/chip within 1 hour at or above room temperature on all three surfaces tested (Viazis *et al.*, 2011a). These two studies show that phages can successfully be used for sanitization of non-porous food preparation surfaces.

2.5.2. Bacteriophage use in animals. Natural microflora in the intestines of food animals is the main reservoir of the pathogenic bacteria in food items. These pathogenic bacteria are not

harmful to their host animals and do not cause sickness, however, they are dangerous and can be fatal to humans. *E. coli* O157:H7, whose main reservoir is the cattle digestive system is considered to be one of the most deadly pathogens for humans and is routinely tested in food items. *E. coli* O157:H7 has also been detected in fecal samples by domestic and feral pigs, deer, wild birds, and other domestic livestock and wildlife animals (Cooley *et al.*, 2007; Jay, Cooley, Carychao, Wiscomb, Sweitzer, Crawford-Miksza *et al.*, 2007). Many approaches have been tested in several trials to eliminate the presence or inhibit the growth of this pathogen in foods. However, the problem still persists as we witness more outbreaks related to the consumption of food items contaminated with *E. coli* O157:H7. Therefore, part of the recent research has focused on the use of phage in controlling *E. coli* O157:H7 at pre-harvest stages as a natural biocontrol technique.

2.5.2.1. E. coli O157:H7. Orally administered bacteriophage T4-like CEV1 resulted in a 2 log unit reduction in intestinal *E. coli* O157:H7 level within 2 days in sheep (Raya, Varey, Oot, Dyen, Callaway, Edrington *et al.*, 2006). When the CEV1 was administered orally together with CEV2, an *E. coli* O157:H7-specific phage isolated from sheep, a 3 log unit reduction was observed in sheep' intestinal *E. coli* O157:H7 levels after 2 days (Raya, Oot, Moore-Maley, Wieland, Callaway, Kutter *et al.*, 2011). The study provides evidence that phage cocktails, composed of 2 or more types of phages, are more effective in killing target bacteria compared to single type phage application (Raya *et al.*, 2011). Single oral dose of previously characterized phage KH1 did not reduce the *E. coli* O157:H7 counts in sheep feces. Additional oral doses given on days 8, 9, and 10 also were not effective in improving the findings. Oral administration of phage SH1 alone or in combination with KH1 in mice for 3 consecutive days immediately reduced the *E. coli* O157:H7 counts in mice feces. Rectal administration of phage cocktail of

SH1 and KH1 to 6 months old cattle in combination with continuous phage (10^6 PFU/ml) ingestion through drinking water significantly lowered *E. coli* O157:H7 cell counts in swab samples (Sheng, Knecht, Kudva, & Hovde, 2006). The ineffectiveness of the single dose of KH1 in sheep might be due to the acquired resistance by *E. coli* O157:H7 cells. The use of KH1 together with the newly isolated phage SH1 restored the efficacy against the host *E. coli* O157:H7 in cattle.

Rectal administration of phages to the food animals is another way to reduce fecal shedding of pathogenic bacteria such as *E. coli* O157:H7. Efficiencies of oral and rectal administrations of an *E. coli* O157:H7-specific phage cocktail composed of 4 different strains were compared in terms of their effects on fecal shedding of *E. coli* O157:H7 for 83 days. Steers were experimentally inoculated with nalidixic acid-resistant (Nal^R) *E. coli* O157:H7 strain. Approximately 11 log PFU phage cocktail was administered in four groups of steers; oral, rectal, oral+rectal, and control. Orally administered steers shed the fewest *E. coli* O157:H7 positive samples. Mean shedding level (log CFU/g of feces) was the highest in rectal group (Rozema, Stephens, Bach, Okine, Johnson, Stanford *et al.*, 2009). The bacteriophages isolated from the control steers indicated that they can acquire the same amount of phages as the rectal group. This is promising as it shows not all of the animals have to receive phage treatment. Another study taken place in 2 commercial feedlots in Canada proved that there is a negative relationship between the presence of endemic phage infecting *E. coli* O157:H7 and the levels of shedding of *E. coli* O157:H7 by cattle. A significant negative correlation was found between isolation of phage and *E. coli* O157:H7 in fecal samples (Niu, McAllister, Xu, Johnson, Stephens, & Stanford, 2009).

2.5.2.2. Salmonella. In another study, *Salmonella* contamination during swine processing was reduced by using a *Salmonella*-specific phage cocktail (Wall, Zhang, Rostagno, & Ebner, 2010). Sixteen *Salmonella enterica* serovar Typhimurium-free market-weight pigs were split into 2 groups and orally administered a phage cocktail or control treatment. Pigs were then placed among *S.* Typhimurium infected pigs in a contaminated pen. Phage cocktail treatment resulted in 1.0-1.3 log unit reduction in intestinal *S.* Typhimurium in pigs compared to control pigs (Wall *et al.*, 2010). The use of phages to control *Salmonella* spp. in poultry has also been investigated by several researchers. The cecal *Salmonella enterica* serotype Enteritidis count in young chickens was reduced by 0.3-1.3 log units following the use of a cocktail of *S. enterica* Enteritidis-specific bacteriophages. Bacterial count could not be further reduced when the phage concentration increased to more than 7 log PFU/g of cecal content (Sklar & Joerger, 2001). The low level of reduction in *Salmonella* counts may be attributable to the development of phage resistance by the bacteria due to the use of a single type phage. This hypothesis was supported with the better reduction level in cecal *S.* Enteritidis counts (3.5 log unit) obtained in a similar study that used a cocktail of three *Salmonella*-specific phages instead of a single type phage (Fiorentin, Vieira, & Barioni, 2005). The presence of *Salmonella* could not be eliminated in either studies but they provided evidence that bacteriophage cocktail may be used in the poultry production for combating *Salmonella*, a major foodborne pathogen in the US. The counts of *S.* Enteritidis were significantly reduced in a day-of-hatch chicks experimentally challenged with 4 log CFU of the same bacteria and orally exposed to 2 different lytic phage cocktails isolated from commercial broiler houses (CB4Ø) and municipal waste water treatment plant (WT45Ø) (Andreatti Filho, Higgins, Higgins, Gaona, Wolfenden, Tellez *et al.*, 2007). CB4Ø and WT45Ø are composed of 4 and 45 different phages, respectively. Phage cocktails applied separately or together resulted in

significant reductions in *S.* Enteritidis counts recovered from cecal tonsils after 24 h of phage treatment.

Another study also aimed at controlling *S.* Enteritidis colonization in chickens with the use of a phage cocktail isolated from the sewage system of commercial chicken flocks. Nine-day old chickens were treated with 9 log PFU of a 3-strain phage cocktail by coarse spray or drinking water. Twenty-four hours later, chickens were challenged with 6 log CFU of *S.* Enteritidis and euthanized at day 20 for phage and *S.* Enteritidis isolation and count. Results showed that coarse spray was able to significantly reduce the pathogen count by 27.3%. Phages may be used as an alternative to antibiotics as aerosol spray or mixed with drinking water to reduce *Salmonella* infections in poultry (Borie, Albala, Sanchez, Sanchez, Ramirez, Navarro *et al.*, 2008). The successful results obtained in these animal studies are promising for the potential use of phages as antibacterial agents on other foods, such as fresh produce.

2.5.2.3. C. jejuni. Another dangerous foodborne pathogen in poultry is *Campylobacter jejuni*. Its colonization in poultry animals is common in developed countries. A research group led by Connerton first described the correlation of reduced presence of *C. jejuni* in cecal content of chicken with the increased number of *C. jejuni*-specific phages (Atterbury, Dillon, Swift, Connerton, Frost, Dodd *et al.*, 2005). In a follow up study, the researchers were able to drop cecal *C. jejuni* counts in experimentally contaminated chickens by 0.5-5.0 log units using 2 potent phages specific against *C. jejuni*, separately (Loc Carrillo, Atterbury, el-Shibiny, Connerton, Dillon, Scott *et al.*, 2005). Like the other studies, the host bacteria could not be eliminated completely in chickens and the phage resistant strains were present in phage-treated chickens. Preventive and therapeutic effects of *C. jejuni*-specific phage therapy on chickens were examined by Wagenaar *et al.* (2005). A single dose of *C. jejuni* was administered to the chickens

on the 4th day of a 10 day long phage treatment to measure the preventive effect of the phage therapy. The cecal *C. jejuni* count in the 5th day was 2 log units lower than that of control, which did not receive phage treatment. The difference between the treatment group and control group dropped to 1 log unit at the end of the trial. Therapeutic effect of a *C. jejuni*-specific phage was tested by administering a single dose of *C. jejuni* five days before the phage therapy. With the start of phage therapy, *C. jejuni* count in cecal content dropped by 3 log units, indicating the potential application of phage in animal food products (Wagenaar, Van Bergen, Mueller, Wassenaar, & Carlton, 2005).

2.5.3. Bacteriophage use on animal food products.

2.5.3.1. E. coli O157:H7. Control of pathogens on animal food products is a challenging area. Lately more and more studies using phages for surface sanitation of animal food product are released. One of the promising studies employed a phage cocktail of 3 virulent phages against *E. coli* O157:H7. The *E. coli* O157:H7-specific phages were tested for their ability to kill the pathogenic *E. coli* independently and as a mixture *in vitro* and *in vivo*. The phage cocktail reduced *E. coli* O157:H7 count by 5 log units at 37°C in 1 h, and completely eliminated the pathogenic bacteria on the surface of most beef samples experimentally contaminated with *E. coli* O157:H7 at a level of 3 log CFU/g. Presence of phage resistant *E. coli* O157:H7 was detected on the remaining beef samples after phage treatment. Further tests showed that the resistant bacteria reverted to phage sensitive state after 50 generations (O'Flynn, Ross, Fitzgerald, & Coffey, 2004). When the mixture of the 3 phages was tested against *E. coli* O157:H7 on beef samples incubated at 37°C for 1 h, the pathogen was eliminated completely in 7 out of 9 samples. It was pointed that phages may be used successfully as biocontrol agents on food since generally low amounts of pathogens are encountered in the environment.

A recent study tested the ability of UV-treated phage T4 to kill host *E. coli* cells on meat surfaces (Hudson, Bigwood, Premaratne, Billington, Horn, & McIntyre, 2010). Although UV irradiation produces replication deficient phages, which do not generate progeny (Shaw, Maurelli, Goguen, Straley, & Curtiss, 1983), UV treated T4 phages were still able to kill their target bacteria by LWO at 24 and 37°C in 2 h and 15 min (Hudson *et al.*, 2010). Replication deficient phages may be employed in controlling pathogens in foods to avoid potential horizontal gene transfer via phages.

2.5.3.2. L. monocytogenes. *L. monocytogenes* specific phage LH7 and antibiotic nisin additively reduced the cell counts of two *L. monocytogenes* species in Tryptic Soy Broth (TSB) and vacuum packed raw beef samples stored at 7 and 4°C, respectively. Reduced *L. monocytogenes* levels in TSB were recovered in time when the TSB samples were incubated at 30°C (Dykes & Moorhead, 2002). The combined effect of phage LH7 and nisin was more potent in TSB than on raw beef suggesting phage effectiveness can vary on different food items and should be evaluated beforehand. *Listeria*-specific Listex™ P100 phage was also used successfully to control *L. monocytogenes* counts on fresh catfish fillets (Soni, Nannapaneni, & Hagens, 2010). Raw catfish fillets were surface inoculated with *L. monocytogenes* mix of 2 serotypes (1/2a and 4b) at a level of 4.3 log CFU/g. Later, the fillets were treated with 7.3 log PFU/g P100 phage. Two-log unit reduction in *L. monocytogenes* counts was observed at all incubation temperatures, 4, 10, and 22°C. This phage was able to reduce *L. monocytogenes* counts by 1 log unit within 30 min. A 10 day shelf life study showed that the presence of phage maintained the overall low level of *L. monocytogenes* on catfish fillets.

Broad-range phages A511 and P100 were effectively used for biocontrol of *L. monocytogenes* on different ready-to-eat (RTE) foods; chocolate milk, mozzarella cheese brine,

hot dog, sliced turkey meat, smoked salmon, seafood, sliced cabbage, and lettuce leaves. Food samples were contaminated with 3 log CFU/g *L. monocytogenes* followed by 6-8 log CFU/g of phage treatment and stored at 6°C for 6 days. Pathogen levels in liquid foods rapidly dropped below detection level. On solid food items, bacterial counts dropped up to 5 log units. Higher dose of phage application (8 log PFU/g) resulted in lower pathogenic counts (Guenther, Huwyler, Richard, & Loessner, 2009). In a recent follow-up study, the researchers used the phage A511 to control the *L. monocytogenes* on soft ripened white mold and red-smear cheeses during their production and ripening phases. The surfaces of unripened cheeses were inoculated with 2-3 log CFU/cm^2 of different *L. monocytogenes* strains followed by single or repeated phage treatments at 8.5-9.0 log PFU/cm^2. Single dose phage treatment (8.5 log PFU/cm^2) resulted in reduction of 2.5 log units in *L. monocytogenes* strain Scott A (serovar 4b) counts on white mold cheese at the end of 21 day ripening period. Repeated application of 8.5 log PFU/cm^2 phage treatment did not improve the result while 9 log PFU/cm^2 phage treatment resulted in more than 3 log units reduction in *L. monocytogenes* counts (Guenther & Loessner, 2011).

2.5.3.3. Salmonella. Efficacy of phages was tested for controlling pathogens in dairy production as well. *Salmonella* phage SJ2 was used to eliminate *S.* Enteritidis experimentally introduced into raw and pasteurized milk before cheddar cheese manufacturing during 99 days. Phage treatment (8 log PFU/ml) of milk reduced the *S.* Enteritidis counts in cheese made from either raw or pasteurized milk by 1 to 2 log units compared to the samples not treated with phage. During the storage at 8°C, *S.* Enteritidis was eliminated completely in cheddar cheese made from phage treated pasteurized milk by day 89. However, cheese made from raw milk containing phage SJ2 still contained 50 CFU/g of *S.* Enteritidis by day 99 (Modi, Hirvi, Hill, & Griffiths, 2001). Two lytic phages were used against *S. aureus* in pasteurized whole milk during

curd manufacturing. When the lytic phage cocktail was introduced into milk at an MOI of 10^2, the experimentally inoculated *S. aureus* (6.5 log CFU/ml) was completely eliminated at 37°C (Garcia, Madera, Martinez, & Rodriguez, 2007). These studies demonstrated the possibility of phage use to control pathogenic bacteria in a variety of dairy products.

A study by Bigwood and coworkers (2008) utilized *S*. Typhimurium PT160 infecting phage P7 and *C. jejuni* infecting Cj6 at low and high MOI (10 and 10^4) against their hosts inoculated at densities of <100 and 10^4 CFU/cm^2 on raw and cooked beef. Significant reductions of the host *S*. Typhimurium PT160 were recorded in the orders of 2-3 log units at 5°C and >5.9 log units at 24°C (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008). Log reductions for *C. jejuni* could not be calculated at these temperatures as the *C. jejuni* counts dropped in phage-untreated control samples.

2.5.3.4. C. jejuni. The ability of the *C. jejuni* and the phages infecting *C. jejuni* to survive through the commercial poultry processing was demonstrated on experimentally contaminated chicken skin stored at 4°C (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003). This finding was important for proving the likelihood of using virulent phages in pathogen biocontrol on poultry products. Another study showed that lytic phages were able to reduce *Salmonella* Enteritidis and *C. jejuni* in a dose dependent manner on chicken skin. Phages used at MOI of 10^5 completely eliminated *S*. Enteritidis on chicken skin experimentally contaminated with 2 log CFU/unit of *S*. Entritidis (Goode, Allen, & Barrow, 2003). When used at MOI of 10^4, bacteriophage Felix O1, a member of *Myoviridae* with a broad host range in the genus *Salmonella*, was able to reduce *Salmonella enterica* serotype Typhimurium by 1.8-2.1 log units on chicken frankfurters contaminated with 300 CFU of the host bacteria (Whichard, Sriranganathan, & Pierson, 2003).

2.5.3.5. E. sakazakii. Although uncommon, *Enterobacter sakazakii* growth in reconstituted infant formula is a serious problem due to the high mortality rate associated with this pathogen. Two newly isolated phages were successfully used to control *E. sakazakii* growth in half-concentrated Brain Heart Infusion medium and reconstituted infant formula. Phages at concentration of 9 log PFU/ml were both able to reduce the pathogen counts to undetectable levels at 24°C, a 7 log unit reduction in infant formula (Kim, Klumpp, & Loessner, 2007).

2.5.4. Bacteriophage use on plants and produce at pre-harvest stage. Phage therapy studies on produce at pre-harvest stage are rare as the real world application of the phage products at farm level seems unrealistic. Most of the phage studies on produce cover the post-harvest scenarios. However, there are few phage efficacy studies to control pathogenic *Salmonella* populations in smaller level sprouting experiments.

2.5.4.1. Salmonella. A cocktail of 2 phage types resulted in higher killing rate of pathogenic *Salmonella* strains during seed sprouting compared to single phage use. The researchers isolated two phages; Phage A capable of lysing *S.* Typhimurium and *S.* Enteritidis, and Phage B, capable of lysing *S.* Montevideo. When used together, Phages A and B were able to suppress *Salmonella* growth more than when used separately on soaked broccoli seeds contaminated with all three types of *Salmonella* (Pao, Rolph, Westbrook, & Shen, 2004).

A recent study looked into the efficacy of two *Salmonella* phages (SSP5 and SSP6) against *S.* Oranienburg on alfalfa (*Medicago sativa*) seeds during sprouting. SSP5 and SSP6 caused about 5 and 2.3 log CFU/ml reductions in *S.* Oranienburg count *in vitro*, respectively. Although SSP5 lysed more cells, SSP6 had wider host range. *S.* Oranienburg count on contaminated alfalfa seeds showed a reduction of 1 log CFU/g only 3 h after SSP6 phage treatment. Later during sprouting at 25°C, phage treatment showed no inhibitory effect on

bacterial population even after a second phage treatment with SSP6 or SSP5. Surviving *S.* Oranienburg cells lost their temporary resistance against SSP5 and SSP6 in follow up trials (Kocharunchitt, Ross, & McNeil, 2009). The study showed the ineffectiveness of single phage usage in food safety due to the ability of the pathogenic bacteria to acquire resistance even if it is temporary.

2.5.5. Bacteriophage use on plants and produce at post-harvest stage. In the last decade, the number and the severity of foodborne outbreaks associated with the fresh produce have increased, thus elevating the public sensitivity to the issue. As a result, researchers try to find alternatives to the commonly used produce decontamination strategies to reduce the number of produce outbreak incidences.

2.5.5.1. Salmonella. Phages can play a significant role in regulating the microbial balance of fresh produce which gets easily contaminated with various foodborne bacteria, including *Salmonella* Enteritidis. A *Salmonella* Enteritidis-specific phage cocktail (SCPLX-1), developed by Intralytix, Inc. (Baltimore, MD) through combination of 4 different lytic phages, was tested on Red Delicious apple and honeydew melon slices, which were experimentally contaminated with *S*. Enteritidis. Although the phage cocktail was able to hold *Salmonella* counts around 2 log CFU lower than those of control samples on honeydew melon slices for up to 7 days at all 3 temperatures tested (5, 10, and 20°C), no effect was observed on apples. Follow up tests revealed that the phages could not resist the low pH (4.2) of the apples. The phage count dropped by 6 log in 3 h and almost no phage was detectable by 24 h (Leverentz, Conway, Alavidze, Janisiewicz, Fuchs, Camp *et al.*, 2001).

2.5.5.2. L. monocytogenes. In 2003, the same researchers developed another phage cocktail specific against *Listeria monocytogenes* and evaluated its efficacy on fresh-cut apple and

melon in a similar setting (Leverentz, Conway, Camp, Janisiewicz, Abuladze, Yang *et al.*, 2003). This phage cocktail was more effective in lysing the pathogen on melon surface than on apple, up to 4.6 log units vs. 0.4 log units. The authors concluded that the low efficacy of phage cocktail against *L. monocytogenes* on apples might also be due to the low pH. The amount of bacteria lysed was increased to 5.7 log units on honeydew melon and 2.3 log units on apple slices when the phage cocktail was used in combination with bacteriocin, nisin. Two different application methods for the delivery of phage cocktail were also evaluated to test the efficacy of the phage cocktail on melon slices. Spraying the phages over contaminated melon slices was more effective in reducing *L. monocytogenes* counts than pipette inoculation (Leverentz *et al.*, 2003).

2.5.5.3. E. coli O157:H7. A bacteriophage cocktail (ECP-100 or EcoShield™) that consists of three different phages in Myroviridae family was tested against *E. coli* O157:H7 significantly reduced the live bacterial counts of the pathogen of interest (Abuladze *et al.*, 2008). A 5 min phage treatment of hard surfaces, such as glass coverslip and gypsum board tainted with *E. coli* O157:H7, significantly reduced the bacterial counts by up to 4 log CFU. The researchers also tested the effect of the phage cocktail on spinach, tomatoes, broccoli, and red meat, and concluded that EcoShield™ phage cocktail was able to reduce the *E. coli* O157:H7 count significantly between 2-4 log CFU/g (Abuladze *et al.*, 2008).

In another study, EcoShield™ phage cocktail treatment was able to reduce *E. coli* O157:H7 counts on fresh-cut lettuce by 1.6 log CFU/cm^2 compared to untreated samples after 1 and 2 day treatments at 4°C (Sharma, Patel, Conway, Ferguson, & Sulakvelidze, 2009b). Antibacterial effect of the phage cocktail was seen as quickly as in day 0 samples, as well. Additionally, seven-day phage treatment of fresh-cut cantaloupe inoculated with *E. coli*

O157:H7 and stored at 4°C reduced the bacterial counts by 3.1 log CFU/ml (Sharma *et al.*, 2009b). This reduction was lower when the cantaloupe was held at 20°C. *E. coli* O157:H7 was able to grow at 20°C from day 0 to day 7 by 3.5 log CFU/ml in the presence of the phage cocktail.

The effectiveness of a phage cocktail against the target bacteria may be elevated by the application of phages in combination of essential oils. Whole baby spinach and baby romaine lettuce leaves were inoculated with a mixture of *E. coli* O157:H7 at 4-6 log CFU/leaf. The leaves were air dried for 1 h to allow bacterial attachment and treated with one of the following three treatments; BEC8 bacteriophage cocktail (approx. 6 log PFU/leaf), *trans*-cinnamaldehyde (TC), or both. BEC8 is composed of 8 lytic *E. coli* O157:H7-specific phages. No viable *E. coli* was rescued on the surface of either plant leaves with 4 log CFU bacterial load, treated with BEC8 or TC alone and incubated for 24 h at room temperature or higher. The phage and TC was not effective at lower incubation temperatures or against higher *E. coli* numbers. Combination of BEC8 and TC treatment for 10 min resulted in complete inhibition of *E. coli* O157:H7 in all samples and all temperature levels (Viazis, Akhtar, Feirtag, & Diez-Gonzalez, 2011b).

2.5.6. Commercialization of phage products. The number of commercially available phage-based therapeutics is increasing due to the promising results in the re-emerging field (Table 3). In 2005, OmniLytics, Inc. received the US Environmental Protection Agency (EPA) approval for its product "AgriPhage" effective against the plant pathogens, *Xanthomonas campestris* and *Pseudomonas syringae* (EPA registration # 67986-1). A European company, Exponential Biotherapies Inc. (EBI) Food Safety (Wageningen, The Netherlands) marketed its product "Listex™ P100" to control *Listeria* in meat and cheese products. The US FDA approved Listex™ P100 as a food biopreservative and granted it as GRAS (Generally

Table 3

Commercially available phage products.

Name	Susceptible Bacterium	Company	Notes
AgriPhage™	*Xanthomonas campestris* *Pseudomonas syringae*	Omnilytics www.phage.com	EPA Approved
BioTector	*Salmonella*	CheilJedang Corp. www.cj.co.kr	
EcoShield™	*Escherichia coli* O157:H7	Intralytix www.intralytix.com	FDA Approved
FASTPlaque-Response™	*Mycobacterium tuberculosis*	Biotech Laboratories/Lab21 www.biotec.com	
FASTPlaqueTB™	*Mycobacterium tuberculosis*	Biotech Laboratories/Lab21 www.biotec.com	
ListShield™	*Listeria monocytogenes*	Intralytix www.intralytix.com	FDA Approved
Listex™ P100	*Listeria monocytogenes*	EBI Food Safety www.ebifoodsafety.com	FDA Approved
MRSA/MSSA Blood culture test	*Staphylococcus aureus*	Microphage www.microphage.com	
MRSA Screening test	*Staphylococcus aureus*	Microphage www.microphage.com	
MicroPhage MRSA/MSSA test	*Staphylococcus aureus*	Microphage www.microphage.com	

Source: Adapted from Monk *et al*., 2010.

Recognized As Safe) (USFDA, 2006a) based partly on an oral toxicity study on rats that resulted in no side effects (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005) and the experience in Europe (Monk, Rees, Barrow, Hagens, & Harper, 2010). Bacteriophage Listex P100 (phage P100) was approved by the U.S. FDA and U.S. Department of Agriculture for controlling *Listeria monocytogenes* presence on both raw and ready-to-eat food products. In a recent study published in 2010, researchers inoculated fresh catfish fillets with approximately 4.3

log CFU/g *L. monocytogenes* before they introduced Listex P100 on the surface. Fifteen min of phage treatment reduced *L. monocytogenes* counts by less than 1 log CFU/g, while 30 min of treatment reduced the pathogen count by more than 1 log CFU/g regardless of the incubation temperature (Soni *et al.*, 2010). After 10 days of incubation at 4 or 10°C, *L. monocytogenes* was still present on the catfish fillets treated with Listex P100.

Intralytix, Inc. (Baltimore, MD) received the FDA approval for ListShield™ (formerly LMP-102) to be used as a food additive in controlling *L. monocytogenes* in ready-to-eat meat and poultry products (USFDA, 2006b). Upon these approvals from the USFDA, phage usage in food safety applications gained even more momentum, which can be measured by the number of recent publications (Abuladze *et al.*, 2008; Bigwood *et al.*, 2008; Borie *et al.*, 2008; Guenther *et al.*, 2009; Hudson *et al.*, 2010; Kocharunchitt *et al.*, 2009; Merabishvili, Pirnay, Verbeken, Chanishvili, Tediashvili, Lashkhi *et al.*, 2009; Niu *et al.*, 2009; Raya *et al.*, 2011; Rozema *et al.*, 2009; Sharma *et al.*, 2009b; Soni & Nannapaneni, 2010; Soni *et al.*, 2010; Viazis *et al.*, 2011b; Wall *et al.*, 2010).

Lately, Intralytix, Inc. has also received regulatory clearance from the FDA for its phage-based food safety product EcoShield™. EcoShield™ has been given clearance as Food Contact Notification (FCN) specific for the use on red meat parts and trims prior to grinding. The FDA has designated the FCN clearance for this phage-based product as FCN No. 1018 (USFDA, 2011).

2.6. Modified Atmosphere Packaging (MAP) as a Food Preservation Technique

Today fresh produce travel long distances and maintaining the produce quality becomes a challenging issue. Storage temperature and environmental gas concentration of the fresh produce are controlled to reduce the physiological deterioration and prolong shelf life.

There are two methods of keeping the produce fresh; controlled atmosphere (CA) storage where the environmental gas concentration is monitored and adjusted constantly and modified atmosphere packaging (MAP) (Mattheis & Fellman, 2000). CA storage requires the use of expensive storage facilities and costly maintenance expenses. In a closed chamber, MAP is achieved by taking two factors into account; respiration rate of the produce and gas transfer rates through the packaging material. MAP is the method by which the produce is preserved longer in a sealed package under the presence of lower O_2 and higher CO_2 concentrations compared to the atmospheric air (Fonseca, Oliveira, & Brecht, 2002). MAP utilizes inexpensive plastic film packaging in which the atmosphere is adjusted based on the respiration properties of produce and the permeability of the plastic film used.

Modified atmosphere packaging (MAP) has been used to increase the shelf life of foods, meeting the market demand for fresh high-quality products available year-round and without the use of additives. In a study, green asparagus spears were stored under three different conditions until they were not fit for consumption: 1) refrigeration at 2°C, 2) MAP at 2°C, and 3) MAP at 2°C for 5 days, then at 10°C. MAP, combined with refrigeration at 2°C, showed the best results among the treatments in terms of retaining sensory, nutritional, and microbial quality, increasing the safety and extending the shelf life of green asparagus (Villanueva, Tenorio, Sagardoy, Redondo, & Saco, 2005).

In another study, five different packaging treatments, including two passive modified atmosphere packaging (MAP), two active MAP and a moderate vacuum packaging (MVP), were used for minimally-processed bunched onions. Various sealed-packaging treatments did not significantly influence microbiological populations, including mesophiles, psychrotrophs; and lactic acid bacteria. However, MVP with a gas-permeable plastic film retained the quality of

bunched onions better with reduced microbial decay and visual sensory aspects when compared with samples stored under different conditions (Hong & Kim, 2004). A study was conducted to evaluate the effect of an edible coating combined with modified atmosphere (MA, 60% O_2, 30% CO_2, and 10% N_2) packaging and gamma irradiation on the microbiological stability and physico-chemical qualities of minimally-processed carrots (Lafortune, Caillet, & Lacroix, 2005). Microbiological analysis revealed that uncoated carrots irradiated at 0.5 and 1 kGy under air and MA had lower aerobic plate counts (APCs) by up to 3.5 and 4 log CFU/g, and by 4 and 4.5 log CFU/g, respectively. For coated carrots, irradiation at 0.5 and 1 kGy under air and MA reduced the APCs by 4 and 4.5 log CFU/g, and by 3 and 4.25 log CFU/g, respectively. The effects of high O_2 and high CO_2 throughout the storage on the microbial and sensory qualities of fresh-cut bell peppers from two commercial California cultivars grown under different climatic conditions were studied (Conesa, Verlinden, Artés-Hernández, Nicolaï, & Artés, 2007). The results showed that 80 or 50 kPa O_2 combined with 15 kPa CO_2 inhibited the growth of spoilage microorganisms and Enterobacteriaceae in minimally processed bell peppers after 9–10 days at 5°C.

One disadvantage of MAP on pathogenic *E. coli* is that it can support acid resistance (Chua, Goh, Saftner, & Bhagwat, 2008). Subatmospheric oxygen level in MAP was found to trigger acid resistance when produce was stored at ≥15°C. Storage temperatures under 10°C did not cause an acid resistance. These results show that storage temperature of fresh produce packed under MAP should be closely monitored. A possible temperature abuse that could happen during the period from packing facility to the consumer could result in elevated acid resistance of the bacteria on produce. Acid resistance should be viewed as an important risk as it can help the pathogens pass the gastric acid challenge in the stomach.

The survival and growth of *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* inoculated onto shredded lettuce were determined under various modified atmosphere packaging conditions and at various storage temperatures. After the inoculation of pathogens, shredded lettuce was packaged in films with different permeability and stored at 5 or 25°C. *E. coli* O157:H7 and *Salmonella* counts decreased by 1 log unit after 10 days at 5°C, while *L. monocytogenes* population increased by 1 log unit, in all package films. The level of the pathogens increased by 2.44 to 4.19 log units after 3 day incubation at 25°C (Oliveira, Usall, Solsona, Alegre, Viñas, & Abadias, 2010). The results show that the MAP is not solely able to keep the pathogen counts lower. Permeability of the packaging film and the temperature need of the pathogens should be in constant consideration.

Packaging atmosphere conditions in combination with various storage temperatures of contaminated lettuce were studied to understand the combined effect on the *E. coli* O157:H7 virulence. Shredded lettuce inoculated with *E. coli* O157:H7 was packaged under treatment A (similar to commercial packaging conditions in gas-permeable film with N_2), treatment B (near-ambient air in gas-permeable film with microperforations), and treatment C (high CO_2/low O_2 condition in gas-impermeable film). Results showed that at 4°C, *E. coli* O157:H7 populations on lettuce decreased under all treatments, most reduction was determined under treatment B by 10 days. At 15°C, *E. coli* O157:H7 counts increased by at least 2.76 log CFU/g under all treatments. At 15°C, expressions of eae and iha virulence factor genes were significantly greater under treatment B than under treatments A and C on day 3. Treatment B at 15°C also promoted the expressions of stx2, eae, ehxA, and rfbE genes significantly on day 10. The results showed that storage under near-ambient air atmospheric conditions can promote expression of O157

virulence factors, and could increase the severity of *E. coli* O157:H7 infections associated with the leafy greens (Sharma, Lakshman, Ferguson, Ingram, Luo, & Patel, 2011).

2.7. Reason for Undertaking This Study

To the best of our knowledge, there is no information present as to how bacteriophages act under modified atmosphere packaging (MAP) conditions. Testing the efficacy of EcoShield™ bacteriophage cocktail on the survival of the pathogenic *E. coli* O157:H7 at refrigeration temperatures will provide new information to fill in this gap.

In this study, EcoShield™ phage cocktail was tested against *E. coli* O157:H7 on three green leafy vegetables, namely spinach, green leaf lettuce, and romaine lettuce alone or in combination with MAP. Spinach and the lettuces were chosen because of their associations in recent *E. coli* O157:H7 outbreaks.

CHAPTER 3

Materials and Methods

3.1. Bacterial Strains

E. coli O157:H7 strains RM1918, RM4406, RM4688, and RM5279 were obtained from the United States Department of Agriculture (USDA), Agricultural Research Services (ARS), Pacific West Area (PWA) (Albany, CA). All strains were previously associated with fresh produce outbreaks. They were cultured on Tryptic Soy Agar (TSA) (BD Difco™, Cat#236920, Franklin Lakes, NJ) and incubated at 37ºC. A single colony was inoculated into Tryptic Soy Broth (TSB) (BD Bacto™, Cat#211825, Franklin Lakes, NJ) and transferred daily into fresh TSB. Due to the difficulty in differentiating *E. coli* O157:H7 colonies on agar plates from other colonies formed by the indigenous microflora of the fresh produce surface, a nalidixic acid resistant (Nal^R) strain (*E. coli* O157:H7 RM4407 Nal^R) was later obtained from Dr. Manan Sharma at USDA, ARS, Environmental Microbial and Food Safety Laboratory (EMFSL) (Beltsville, MD). Since *E. coli* O157:H7 RM4407 Nal^R was found to be insensitive up to 50 µg/ml nalidixic acid, it was cultured in TSB that contained 50 µg/ml nalidixic acid (Sigma-Aldrich, Cat#N4382, St. Louis, MO).

3.2. Verification of *E. coli* O157 Strains with a Latex Test Kit

Authenticities of *E. coli* O157:H7 cultures were verified by drySPOT™ *E. coli* O157 Latex Test Kit (Oxoid, Cat#DR0120M, Cambridge, UK) according to the manufacturer's protocol. The kit includes blue color latex spots printed test cards. Two different latex spots are present. Test spots include latex particles coated with antibody specifically reactive with *E. coli* O157 serogroup antigen. *E. coli* O157:H7 strains were streak plated on TSA plates and incubated at 37°C overnight. A portion of a colony was picked up using a sterile inoculation loop and

emulsified in 50 µl of 0.9% NaCl solution on test area avoiding the latex test spots. The suspension was then dragged with the help of the inoculation loop and mixed with the latex test spots. Blue color of the latex spot was dissolved and the test card was rocked gently in a circular motion to observe agglutination. Another colony on the TSA plate was used to mix with the control latex spots the same way and observed for agglutination. The test was recorded as positive if agglutination was visible to the naked eye with the test latex, but not the control latex, within 60 seconds of mixing.

3.3. DNA Extraction from *E. coli* O157:H7 Cells

E. coli O157:H7 strains RM1918, RM4406, RM4688, and RM5279 were grown in TSB overnight and centrifuged at 15,700x*g* for 5 min at room temperature. Supernatant was discarded and the cell pellets were used for DNA extraction. One ml of DNAzol® reagent (Invitrogen, Cat#10503-027, Carlsbad, CA) was added to $5x10^6$ cells. Cells were lysed by pipetting up and down for about 20 seconds. The mixture was centrifuged at 15,700x*g* for 5 min at room temperature and the supernatant was transferred into a new sterile 1.5 ml microcentrifuge tube. Five hundred ml of 200 proof ethanol (Sigma-Aldrich, Cat#459836, St. Louis, MO) was added and the tubes were mixed by inversion several times followed by a 5 min wait at room temperature. Tubes were then centrifuged at 15,700x*g* for 5 min at room temperature and the supernatant was discarded. One ml of 100% ethanol was added into the pellet and the tubes were inverted several times. After the centrifugation at 15,700x*g* for 5 min, the supernatant ethanol was removed carefully with a pipette. The ethanol wash and the consecutive centrifugation step were repeated one more time with 1 ml of 100% ethanol. Ethanol was removed carefully using a pipette and the tubes were kept open for 1 min at room temperature to allow DNA air dry. To solubilize the DNA pellet, 1.5 ml of 8 mM NaOH was added to the tubes. DNA samples were

quantified by Genesys 10 UV spectrophotometer (Thermo Scientific, Cat#335903P, Rochester, NY) at 260 nm. Total amount of DNA in µg was calculated with the formula below:

$$\text{DNA } [\mu g] = (A260)\ ((50\ \mu g/ml) / (A260))\ (DF)\ (V),$$

where A260 is the absorbance of the DNA sample at 260 nm, 50 µg/ml is the conversion factor relating absorbance to concentration, DF is dilution factor, and V is the total volume in ml.

3.4. Verification of *E. coli* O157:H7 Strains with Polymerase Chain Reaction

Once the DNA concentrations were calculated, they were used as template in polymerase chain reaction (PCR) to verify the *E. coli* O157:H7 specific genes; stx1, stx2, uidA, eaeD, ehxA, and rfbE. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). The base sequence and product size information of the oligonucleotides used in the study is presented in Table 4.

Table 4

Oligonucleotides used to verify E. coli O157:H7 strains.

Gene	Primer	Sequence (5'→3')	Reference	PCR Size
stx1	F	CAG TTA ATG TGG TGG CGA AGG	(Cebula, Payne, & Feng, 1995)	348 bp
	R	CAC CAG ACA ATG TAA CCG CTG		
stx2	F	ATC CTA TTC CCG GGA GTT TAC G	(Cebula *et al.*, 1995)	584 bp
	R	GCG TCA TCG TAT ACA CAG GAG C		
uidA	F	GCG AAA ACT GTG GAA TTG GG	(Cebula *et al.*, 1995)	252 bp
	R	TGA TGC TCC ATC ACT TCC TG		
eaeA	F	ATT ACC ATC CAC ACA GAC GGT	(Fratamico & Strobaugh, 1998)	397 bp
	R	ACA GCG TGG TTG GAT CAA CCT		
ehxA	F	GTT TAT TCT GGG GCA GGC TC	(Feng & Monday, 2000)	158 bp
	R	CTT CAC GTC ACC ATA CAT AT		
rfbE	F	CAA GTC CAC AAG GAA AGT AAA GAT G	(Rashid, Tabata, Oatley, Besser, Tarr, & Moseley, 2006)	85 bp
	R	ATT CCT CTC TTT CCT CTG CGG		

Qiagen Multiplex PCR kit (Qiagen, Cat#206143, Valencia, CA) and Takara Ex Taq™ Hot Start Version (Takara, Cat#RR006A, Shiga, Japan) were used for the multiplex PCR reaction. The multiplex PCR protocol suggested by Qiagen was followed. Ten ng of DNA sample for each strain was used in multiplex PCR reaction. PCR conditions were as follows: first denaturation for 4 min at 94°C, then 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and elongation for 1 min at 72°C followed by final elongation step for 10 min at 72°C. Multiplex PCR reaction was performed with Eppendorf thermocycler (Eppendorf Mastercycler epgradient S, Hamburg, Germany). After the PCR reaction was completed, 10 µl of the PCR sample was mixed with 2 µl of gel loading dye (Invitrogen, Cat#15585-011, Carlsbad, CA), and the mixture was loaded on to 1% agarose gel (Invitrogen, Cat#16500-500, Carlsbad, CA) with DNA size marker (Takara, Cat#3409A, Shiga, Japan). The gel electrophoresis was performed at 100 volts for 40 min and the gel picture was taken by using Bio-Rad imaging equipment (Bio-Rad, Universal Hood II, Hercules, CA).

3.5. Bacteriophage Cocktail

EcoShield™ (formerly known as ECP-100), a bacteriophage cocktail (10^{10} PFU/ml in PBS, pH 7.4) specific against *E. coli* O157:H7, was provided by Dr. Alexander Sulakvelidze at Intralytix, Inc. (Baltimore, MD). The cocktail consists of three *E. coli* O157:H7-specific lytic phages (ECML-4, ECML-117, and ECML-134) in the Myoviridae family isolated from fresh and salt water environments. Phage cocktail was stored in dark at 4°C per the manufacturer's recommendation that the phage cocktail is highly unstable under light, and diluted as necessary in sterile 1.5% peptone water (w/v) (BD Difco™, Cat#218071, Franklin Lakes, NJ) immediately before application. Phosphate buffered saline (PBS) was first used for phage dilutions but

discontinued later as the salt residues caused dehydration of the leaf pieces during long incubation periods.

3.6. Verification of Bacteriophage Cocktail Titration

Although EcoShield™ phage cocktail was supplied as a standard 10^{10} PFU/ml suspension, the concentration of each new vial was always verified with a plaque assay. At all times, the stock phage cocktail suspension was handled under low light conditions. EcoShield™ phage titration was measured against *E. coli* O157:H7 according to the method described by Leverentz *et al.* (2004) with some modifications. Shortly, EcoShield™ phage cocktail was diluted serially in peptone water (w/v) down to 10^0 PFU/ml. Overnight grown *E. coli* O157:H7 was diluted serially in peptone water, and 100 µl of diluted *E. coli* O157:H7 (~7 log CFU/ml) culture was mixed with 1 ml of diluted phage cocktail in a sterile glass culture tube. Quickly, 3.5 ml of warm (50°C) soft lysogeny broth (LB) (Fisher Scientific, Cat#BP1427, Fair Lawn, NJ) agar that contains 0.75% agar (w/v) (Fisher Scientific, Cat#BP1423, Fair Lawn, NJ) was added to the glass tube followed by a brief, gentle vortex. Content of the tube was then quickly poured on top of an LBA plate that was prepared earlier. The LBA plates were incubated at 37°C overnight once the top agar layer solidified. Circular, clear plaques formed by the phages were counted the next day to calculate the concentration of the phage cocktail as log PFU/ml.

3.7. Screening the Efficacy of EcoShield™ Phage Cocktail against *E. coli* O157:H7 Strains in Liquid Laboratory Medium

The efficacy of EcoShield™ phage cocktail (10^7 PFU/ml) was tested against a standard concentration (10^4 CFU/ml) of each *E. coli* O157:H7 strains in Tryptic Soy Broth (TSB) (BD Bacto™, Cat#211825, Franklin Lakes, NJ). The test (phage) and control (peptone water) samples were incubated at 4 and 37°C for up to 48 h. Samples were serially diluted in sterile peptone

water as necessary and aliquots (100 µl) of appropriate dilutions were plated on Sorbitol MacConkey (SMAC) agar (BD Difco™, Cat#212123, Franklin Lakes, NJ) after 0.5, 2, 24, and 48 h of incubation. The plates were then incubated at 37°C overnight and the recovered colony enumerated and expressed as log CFU/ml.

Since the natural indigenous microorganisms of the green leafy produce compete with the *E. coli* O157:H7 for nutrients and the presence of high number of indigenous bacteria limits our ability to identify *E. coli* O157:H7 on the petri surface, it was necessary to use an antibacterial resistant EHEC strain. This is a well-known problem with the intervention studies that aims to reduce pathogen load on samples naturally containing high level of background microflora (Niemira, 2003). Studies showed that *E. coli* strains resistant to nalidixic acid (Nal) have similar growth properties and stress tolerance as their nalidixic acid sensitive parental strains and can be used as marker organisms in chemical interventions and growth studies (Blackburn & Davies, 1994; Taormina & Beuchat, 1999). Based on this information, a nalidixic acid resistant (up to 50 µg/ml) *E. coli* O157:H7 RM4407 strain was used in the following steps of the study.

The efficacy of the phage cocktail (10^7 PFU/ml) was tested against 10^5 CFU/ml of *E. coli* RM4407 Nal^R strain in TSB. The phage and control (peptone water) (BD Difco™, Cat#218071, Franklin Lakes, NJ) samples were incubated at 4, 10, and 37°C for up to 48 h. Samples were serially diluted in sterile peptone water, and aliquots (100 µl) were plated on SMAC agar after 0.5, 2, 24, and 48 h of incubation. The inoculated plates were incubated at 37°C overnight and the viable colony counts were expressed as log CFU/ml.

3.8. Efficiency of Bacteriophages on Young and Old *E. coli* O157:H7 Cultures

During the first phase of the liquid culture experiments with *E. coli* O157:H7 strains, the efficiency of the phage cocktail on EHEC strains fluctuated from one experiment to another. It

was hypothesized that as *E. coli* O157:H7 cultures were passaged continuously they were becoming less susceptible to the phage cocktail, which can be measured by the inhibited level of *E. coli* O157:H7 reduction. *E. coli* strains were cultured from single colonies 2 days apart from each other. When the oldest line reached 11th passage, *E. coli* O157:H7 (5 log CFU/ml) and the phage cocktail (7 log PFU/ml) were mixed in TSB and incubated at 4°C for 3 h. Samples were serially diluted in sterile peptone water as necessary and aliquots (100 μl) of appropriate dilutions were plated on TSA. The plates were then incubated at 37°C overnight and the viable colony counts were expressed as log CFU/ml to compare the recovered EHEC levels.

3.9. Leafy Green Vegetable Sample Preparation

After determining the most sensitive strain of *E. coli* O157:H7 to the phage cocktail, a new set of experiments were conducted on leafy greens. Pre-washed, bagged, and ready-to-eat fresh spinach (Fresh Express®) and conventional green leaf and romaine lettuces were purchased from a local grocery store, brought to the laboratory immediately, and stored at 4°C for up to 1 day until use. Partial oxygen and carbon dioxide concentrations inside the spinach bags were measured with handheld PBI Dansensor CheckPoint (Glen Rock, NJ) and recorded right before opening the bags for the experiments. All produces were handled with 70% ethanol-treated nitrile gloves. Outer leaves of the lettuces, damaged spinach and lettuce leaves, and the leaves that do not look edible and healthy were discarded. Lettuce leaves were washed gently under running tap water. Spinach and lettuce leaves were cut using a sterile knife and glass cutting board to about 2x2 cm^2 pieces. The pieces of the leaves were immediately placed into a sterile stomacher bag to reduce wilting due to water loss during preparation steps. The leaves were then placed side by side on aluminum foil sheets for bacteria and phage inoculation.

3.10. Determining the Efficacy of the Phage Cocktail against *E. coli* O157:H7 RM4407 Nal^R on Leafy Greens at 4 and 10°C

Newly started fresh culture of EHEC RM4407 Nal^R was grown in TSBN overnight at 37°C. The overnight grown EHEC culture was diluted in peptone water to bring the concentration down to ~7 log CFU/ml. Using a sterile micropipette, 10 µl from the diluted EHEC culture was inoculated gently on top side of the 2x2 cm^2 of leaf pieces that were placed on aluminum foil. Extra care was given to distribute the total volume homogenously in ~20 small droplets to cover the entire top surface of produce leaves without disturbing and damaging the intact surface of the leaf pieces. The inoculated droplets did not contact the open cut edges of the leaves. Contaminated leaf pieces were air-dried for 20 min to allow for bacterial attachment and then sprayed with 7 log PFU/ml EcoShield™ phage cocktail or peptone water (control). Spraying was chosen as a phage application method since the amount of the phages that actually end up on the samples cannot be standardized in dipping method (Personal communication with Dr. Alexander Sulakvelidze, 2008). A small fingertip sprayer (Bottle Crew, Cat#E25144, West Bloomfield, MI) that delivers ~100 µl was used to deliver the phage cocktail at a concentration of ~6.5 log PFU/cm^2 of leaf. The phage was sprayed from 10-12" above the surface, one stroke per leaf plus an extra stroke for the leaves on the edges.

The leaf pieces were incubated up to 7 days in dark at 4 and 10°C in sterile petri dishes humidified with sterile, wet filter papers. To determine the surviving EHEC counts, the leaf pieces were placed in glass tubes with 9 ml peptone water using sterile forceps, homogenized with a handheld blender (Kinematica AG, Polytron PT 1200 E, Lucerne, Switzerland). The blender was rinsed with a 3 step process; tap water, 100% denatured ethanol, and purified water to prevent cross-contamination between each sample. The homogenized leaves were further

diluted in 1.5% peptone water as necessary, and plated on MacConkey agar containing 25 µg/ml Nalidixic acid (MACN). After the overnight incubation at 37°C, violet color colonies were counted to express the surviving EHEC counts as log CFU/cm^2.

3.11. Effect of the Phage Cocktail against *E. coli* O157:H7 RM4407 Nal^R on Leafy Greens at 4 and 10°C under Different Modified Atmosphere Packages (MAP)

Inoculation of the EHEC RM4407 Nal^R on the leaf pieces and the treatment of phage cocktail were performed the same way as explained under chapter 3.10. The leaf pieces were incubated up to 15 days in dark at 4 and 10°C inside sterile petri dishes humidified with sterile, wet filter papers under two different atmospheric conditions, atmospheric air (A) and modified gas mixture (G). Modified gas mixture contained 5% O_2, 35% CO_2, and 60% N_2. Leaf samples inside the petri dishes were placed in vacuum pouches with zero oxygen transfer rate (Prime Source Vacuum Pouches, Cat#75001917, Kansas City, MO), and filled with the desired gas mixture by using a table-top vacuum packaging machine (Supervac, GK 125, Wien, Austria). The surviving EHEC counts were determined the same way as explained under chapter 3.10.

To enumerate the aerobic mesophilic and anaerobic indigenous microflora of the leafy greens, the homogenized leaves were further diluted in 1.5% peptone water as necessary, and plated on TSA and Reinforced Clostridial Medium (RCM, BD Difco™, Cat#218081, Franklin Lakes, NJ), respectively. Both media were incubated overnight at 37°C. RCM plates were placed in GasPak™ anaerobic chamber (BD, Cat#260629, Franklin Lakes, NJ). The oxygen content of the chamber was diminished by placing a CO_2 generator packet (BD, Cat#261205, Franklin Lakes, NJ) inside the chamber.

3.12. Recovered Phage Quantification from Fresh Produce

The quantity of live phage was measured against *E. coli* O157:H7 during 2-week storage at 4°C according to the method described in chapter 3.6 with some modifications (Leverentz, Conway, Janisiewicz, & Camp, 2004). Shortly, spinach leaf pieces containing only EcoShield™ phage cocktail were homogenized and diluted in peptone water the same way as explained in the chapter 3.9 above. Overnight grown *E. coli* O157:H7 in TSB was diluted serially in peptone water, and 100 µl of diluted *E. coli* O157:H7 (~7 log CFU/ml) culture was mixed with 1 ml of diluted homogenized leaf mixture, which contained the active phage particles, in a sterile glass culture tube. Quickly, 3.5 ml of warm (50°C) soft LB agar was added to the glass tube followed by a brief, gentle vortex. Content of the tube was then quickly poured on top of an LBA plate that was prepared earlier. The LBA plates were incubated at 37°C overnight once the top agar layer solidified. Circular, clear plaques formed by the phages were counted the next day to calculate the concentration of the phage cocktail as log PFU/cm^2.

3.13. Statistical Analysis

The experimental design used in this study consisted of 1) a 2x3x4 factorial design (phage treatment: phage and no phage; storage temperature: 4, 10, and 37°C; and time: 0.5, 2, 24, and 48 h) for liquid medium experiment, 2) a 2x2x2x6 factor factorial (produce type: spinach, and green leaf lettuce; phage treatment: phage and no phage; storage temperature: 4 and 10°C; and storage time: 0.5 h, 2 h, 1, 3, 5, and 7 days) for first phase fresh produce experiments, and 3) a 3x2x2x6x2 (produce type: spinach, green leaf lettuce, and romaine lettuce; phage treatment: phage and no phage; storage temperature: 4 and 10°C; storage time: 1, 3, 5, 7, 10, and 15 days; and packaging: air and modified gas) for fresh produce experiments under modified atmosphere

packaging. The dependent variables used as indicators of phage treatment effectiveness were EHEC counts in all experiments and aerobic and anaerobic counts for produce studies.

All experiments were repeated at least two times in duplicates. Microbial counts were log transformed to normalize data for statistical analysis. Means and standard deviations were calculated for dependent variables by treatment and used in tables and graphs. For inferential statistics, data was analyzed by Analysis of variance (ANOVA) using SAS® software (SAS Institute Inc., 2008). The Fisher's least significant difference test was used as post ANOVA test for pairwise comparison of treatment means. The significance of main treatment and interaction effects was judged at the 5% significance level.

CHAPTER 4

Results and Discussion

4.1. Verification of *E. coli* O157 Authenticity

Authenticities of *E. coli* O157:H7 strains RM1918, RM4406, RM4688, and RM5279 were verified by drySPOT™ *E. coli* O157 Latex Test Kit (Oxoid, Cambridge, UK) according to manufacturer's protocol as explained in chapter 3.2. The pictures of the test areas are shown in Figure 3. All strains resulted in agglutination in positive tests and no agglutination was observed in negative tests. A non-O157 *E. coli* was used as a control to confirm no agglutination forming. The amount of the cells used in the positive test may affect the level of agglutination observed. Since a small colony was used for the strain RM4688 the agglutination amount is lower compared to the other three strains.

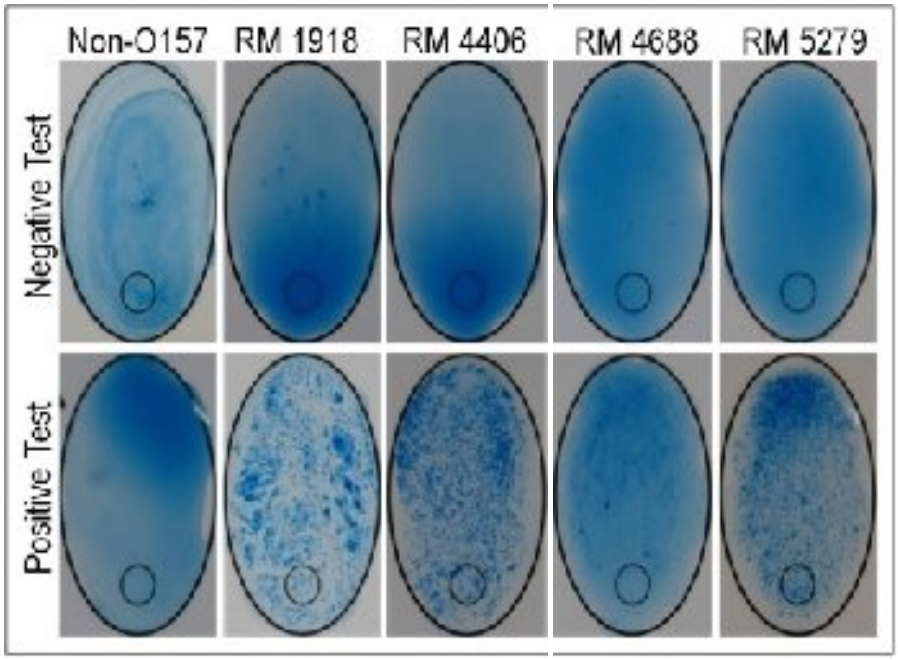


Figure 3. Verification of O157 antigen in *E. coli* O157:H7 strains by Oxoid *E. coli* O157 Latex Test Kit.

After the latex kit verification, a multiplex polymerase chain reaction (PCR) was performed to verify the presence of *E. coli* O157 specific genes, such as stx1, stx2, uidA, eaeA, ehxA, and rfbE. Figure 4 shows the image of the gel electrophoresis result after the multiplex PCR. Expected PCR product sizes of the genes are stx2 at 584 bp, eaeA at 397 bp, stx1 at 348 bp, uidA at 252 bp, ehxA at 158 bp, and rfbE at 85 bp. The sizes of these observed PCR bands were verified by comparing them to a known DNA marker. Control lane has everything but the template DNA.

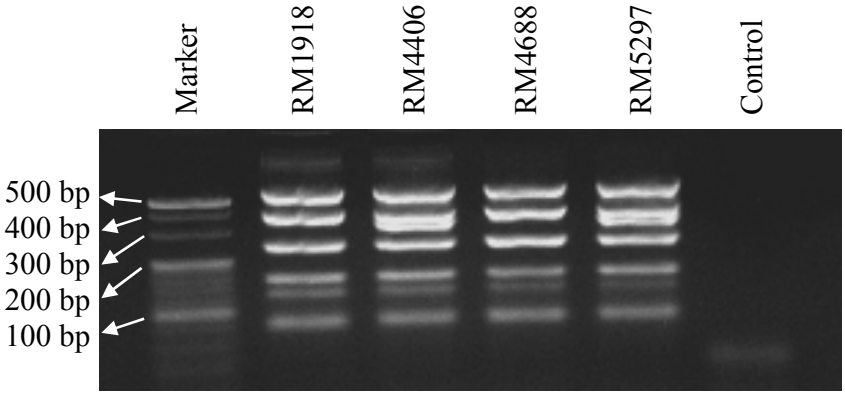


Figure 4. Result of multiplex PCR for EHEC characteristic genes in *E. coli* O157:H7 strains.

In Figure 5, the PCR band for stx1 (348 bp) was not present in lanes RM1918 and 4688. The 348 bp band was verified after another PCR when only stx1 primers were used (Figure 5). The multiplex PCR is a quick method for detecting multiple genes in a single reaction; however, the main challenge in a multiple PCR assay is the occurrence of unwanted primer dimers and nonspecific products. So, it is necessary to use primers whose annealing temperatures are closer as much as possible (Vidal, Kruger, Duran, Lagos, Levine, Prado *et al.*, 2005).

There are several multiplex PCR assays developed to detect *E. coli* O157:H7. These assays aim to detect the specific virulence factors in *E. coli* O157:H7; such as stx1, stx2, eaeA, and ehxA (Fagan, Hornitzky, Bettelheim, & Djordjevic, 1999; Fratamico & Strobaugh, 1998).

In addition to these genes, some PCR assays include more genetical targets specific to *E. coli* O157:H7 (Cebula *et al.*, 1995; Paton & Paton, 1998a). In this study, a 6-gene multiplex PCR was performed to verify *E. coli* O157:H7. Five of the genes were also used successfully by others (Feng & Monday, 2000). Due to the genetic variations in virulence in virulence factors, a 6th gene (rfbE) that encodes O antigen was also included in this study (Rashid *et al.*, 2006). All 6 genes were successfully detected with the multiplex PCR in all strains tested, and the authenticities of the *E. coli* O157:H7 strains were verified.

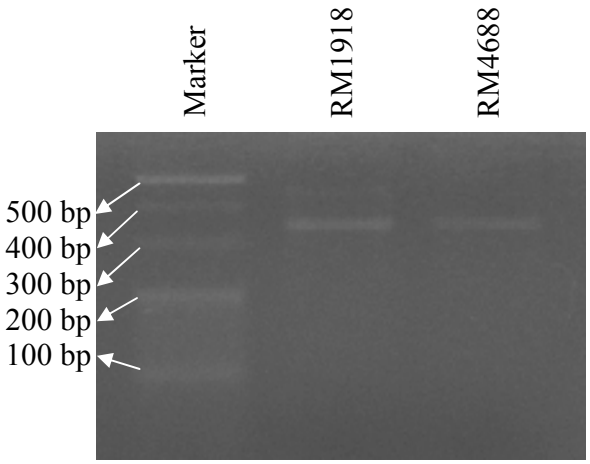


Figure 5. PCR gel picture for stx1 gene in *E. coli* O157:H7 strains RM4406 and RM5279.

4.2. Bacteriophage Cocktail Titration

The EcoShield™ bacteriophage cocktail contains 3 different phages that are specific against *E. coli* O157:H7. Knowing the actual concentration of the stock phage cocktail is important to adjust the experimental phage cocktail concentration. Phage titration was performed for every new bottle of stock phage cocktail. The overall stock phage cocktail concentration was found to be 9.5 log PFU/ml. A sample picture of the soft agar plates from the phage titration assay is shown in Figure 6.

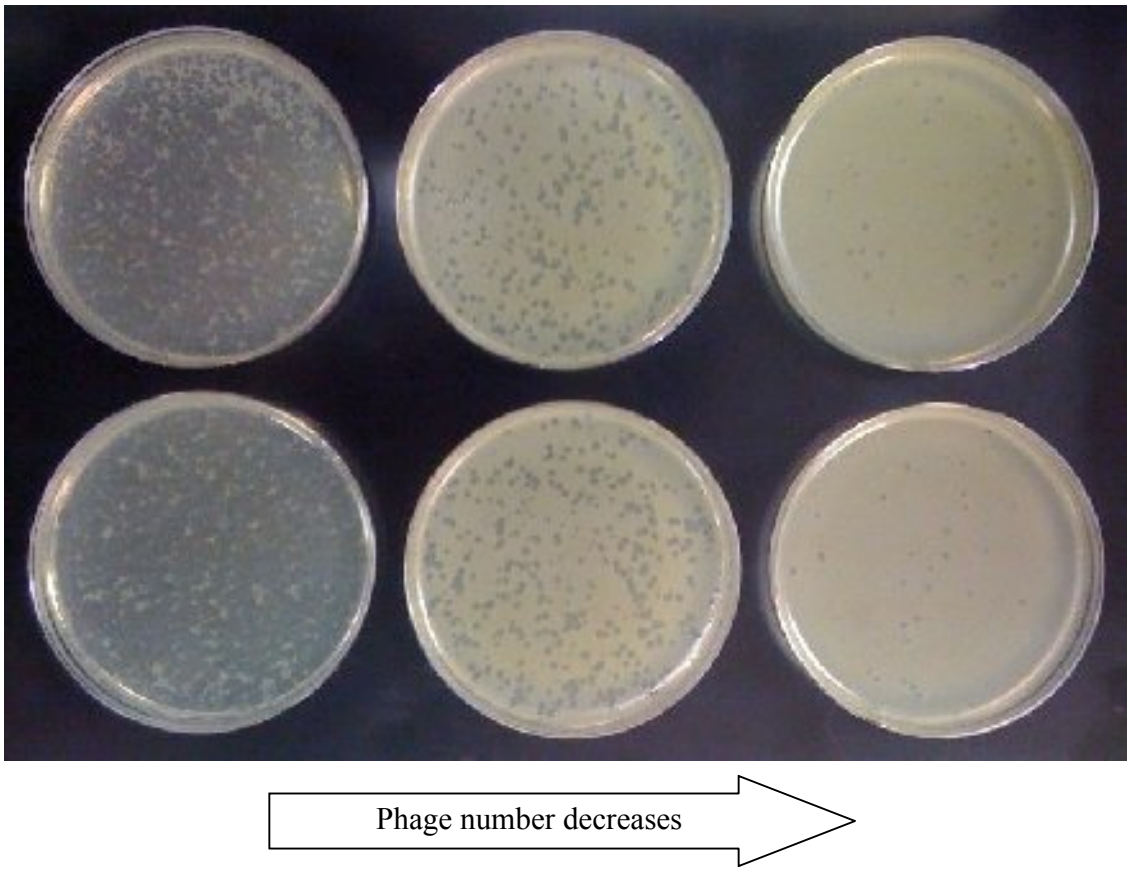


Figure 6. EcoShield™ phage cocktail titration on LBA soft agar.

4.3. Efficacy of Bacteriophage Cocktail in Lysing *E. coli* O157:H7 Strains in Laboratory Medium

Liquid culture experiments showed that EcoShield™ phage cocktail was able to show its effect as early as in 30 min (Figure 7). Data is shown as mean ± SD. At 4°C, the phage cocktail lysed up to 5 log units *E. coli* O157:H7 cells resulting in complete lysis during the 48 h of incubation in TSB. At 10°C, *E. coli* O157:H7 continued to grow up to 6.33 log CFU/ml in control sample during 48 h of incubation. A complete lysis was detected at 10°C in 3 of 4 strains (RM4406, RM4688, and RM5279) treated with the phage cocktail. No surviving bacteria were detected in phage treated samples. The highest difference (6.08 log units) at 37°C between control and phage treated samples was achieved after 2 h incubation. Bacterial survival in phage

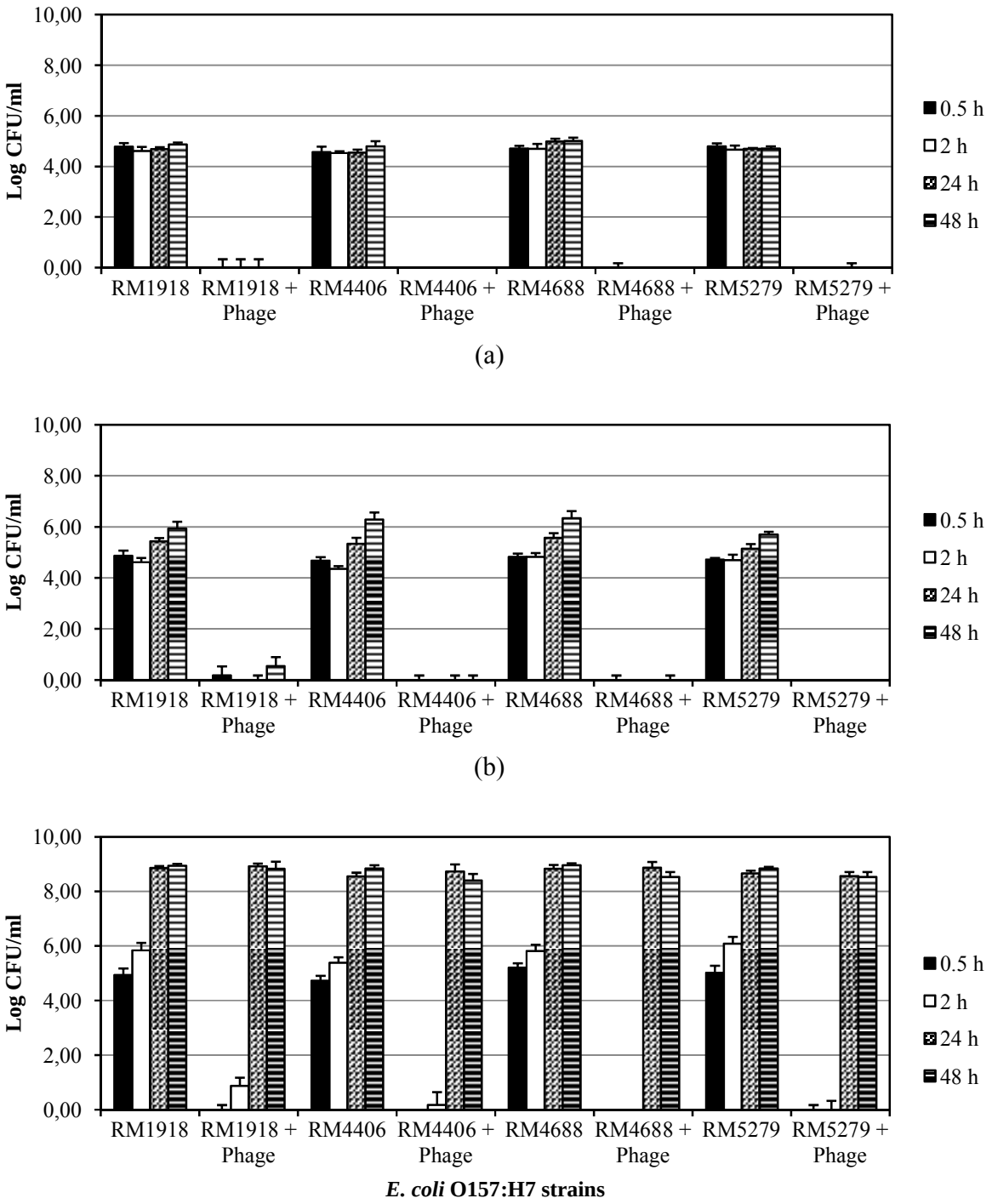


Figure 7. Efficacy of EcoShield™ against four *E. coli* O157:H7 strains in tryptic soy broth (TSB) at (a) 4°C, (b) 10°C, and (c) 37°C.

treated samples increased as the rapid growth rate of *E. coli* O157:H7 masked the lysis activity of the phage cocktail at 37°C.

The incubation time and temperature were significantly affecting the efficacy of the phage cocktail against all *E. coli* strains tested in this study ($P < 0.05$). However, type of the EHEC strain was not statistically significant ($P > 0.05$). Phage cocktail was equally effective against all EHEC strains used.

The liquid culture (TSB) results are in agreement with early efficacy studies that worked with EcoShield™ (Abuladze *et al.*, 2008; Sharma *et al.*, 2009b). Although these studies did not test the efficacy of the phage cocktail in a liquid laboratory medium, the levels of reduction they obtained are comparable to our findings.

4.4. Bacteriophage Becomes Less Effective Against Older *E. coli* O157:H7 Cultures

Figure 8 shows that there is a correlation between the age of the *E. coli* O157:H7 culture and the level of recovery after phage treatment. The log unit reduction in *E. coli* O157:H7 counts by the phage cocktail decreases as the culture ages. A linear regression analysis showed that each day 0.38 log CFU unit of *E. coli* O157:H7 bacteria are becoming phage insensitive. This finding is also partly supported by the findings of Hara-Kudo *et al.* (2000). The authors found that *E. coli* O157:H7 cells may lose their lipopolisaccharide O157 antigens on their cell surfaces if they are exposed to long-term sub-optimal environmental conditions (Hara-Kudo, Miyahara, & Kumagai, 2000).

During this study, it was determined that the susceptibility of *E. coli* O157:H7 strains to the EHEC-specific phage cocktail diminished by time as the cells are cultured daily for over a week (Figure 8). Therefore, a fresh bacterial culture was inoculated in TSB before starting a new experiment at each time.

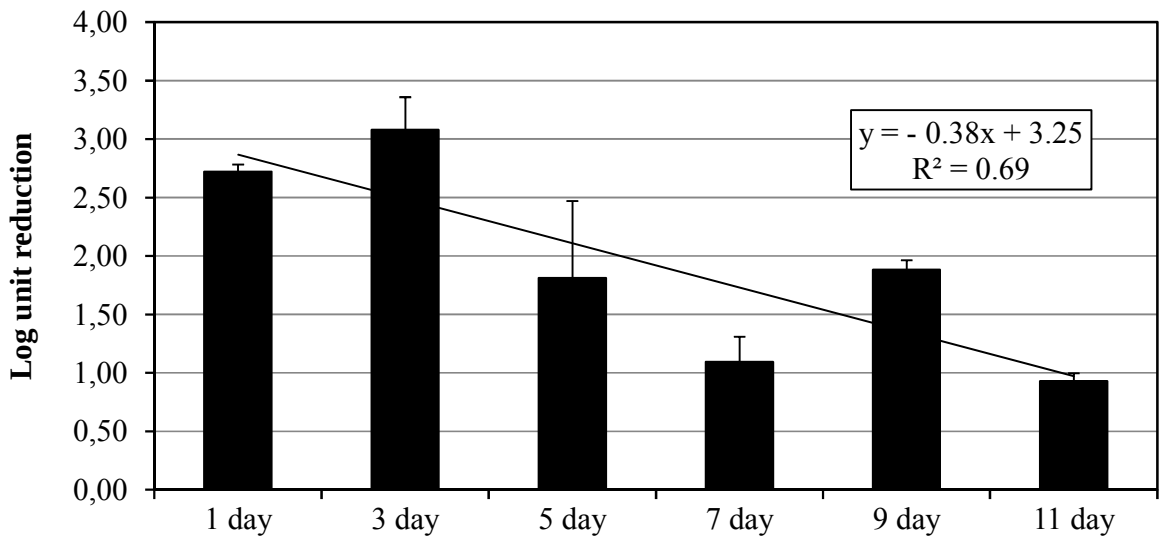


Figure 8. The efficiency of the bacteriophage cocktail diminishes as the *E. coli* O157:H7 cultures in TSB were passaged daily for over a week.

4.5. Bacteriophage Cocktail vs. *E. coli* O157:H7 RM4407 Nal^R in Laboratory Medium

The phage treatment significantly ($P < 0.05$) reduced *E. coli* O157:H7 counts in TSB after 2 h by 4.50, 5.29, and 6.02 log CFU/ml at 4, 10, and 37°C, respectively (Figure 9). Data are shown as mean ± SD. At 24 h, the inhibitory activity of the phage cocktail reached 4.75 and 5.75 log units at 4 and 10°C, respectively. However, the reduction level diminished to 0.89 log unit by 24 h at 37°C due to the fast growth rate of *E. coli* O157:H7 at 37°C. The inhibition levels were reduced by 48 h of incubation to 4.34, 4.48, and 0.19 log units at 4, 10, and 37°C, respectively. *E. coli* O157:H7 growth at 37°C almost completely masked the inhibitory activity of phage treatment by 48 h. The incubation time and temperature significantly ($P < 0.05$) influenced *E. coli* O157:H7 Nal^R counts. The inhibitory effect of the phage treatment on *E. coli* O157:H7 Nal^R strain is similar to our previous liquid culture experiment results shown in Figure 7, indicating that the phage treatment is highly effective in lysing different *E. coli* O157:H7 strains at 4 and 10°C.

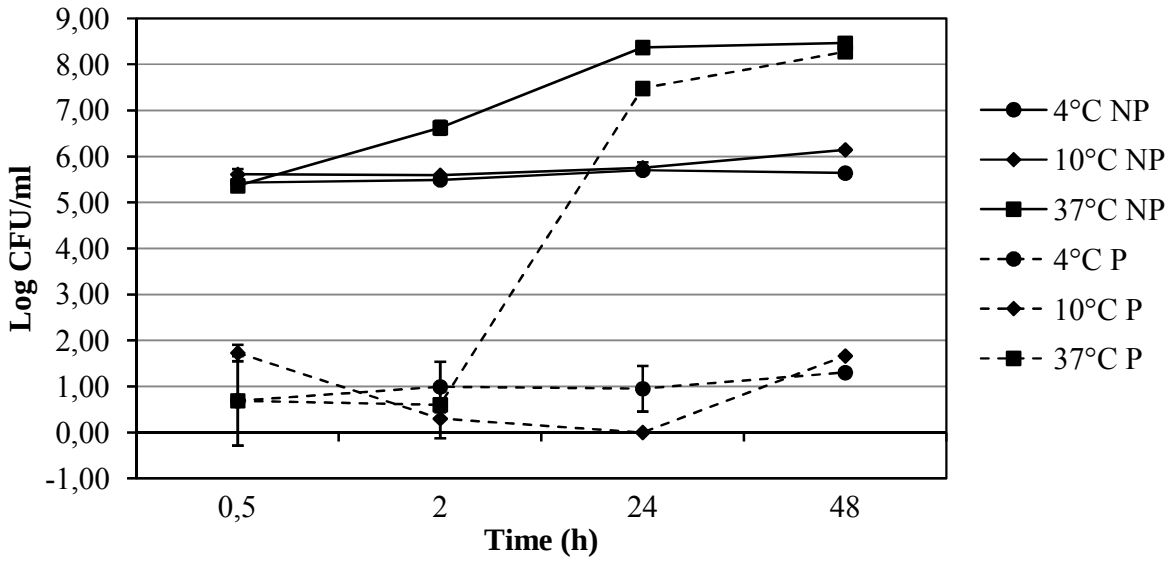


Figure 9. Effect of EcoShield™ on *E. coli* O157:H7 RM4407 Nal^R in TSB.

4.6. Efficacy of the Bacteriophage Cocktail against *E. coli* O157:H7 RM4407 Nal^R on Spinach and Lettuce

After verifying that EcoShield™ phage cocktail was able to lyse *E. coli* O157:H7 Nal^R cells and reduce their growth by up to 6 log units in liquid laboratory medium, the effectiveness of the phage cocktail against the same pathogen was tested on green leafy vegetables; such as spinach (Figure 10) and green leaf lettuce (Figure 11).

The phage cocktail was able to reduce *E. coli* O157:H7 by 2.38 and 2.49 log CFU/cm^2 at 4 and 10°C as short as in 30 min, respectively. The potent activity of the phage treatment against EHEC did not change through the 7-day storage period. Although a slight decrease in *E. coli* O157:H7 counts was observed in samples incubated at 4°C and a slight increase at 10°C after day 3, the relative difference between *E. coli* O157:H7 counts did not change between the phage-treated and untreated samples (Figure 10). Data is shown as mean ± SD. The preparation and the storage steps of this experiment took place in dark as recommended by the supplier of the phage cocktail. The level of reduction in EHEC count could have been lower should the storage took

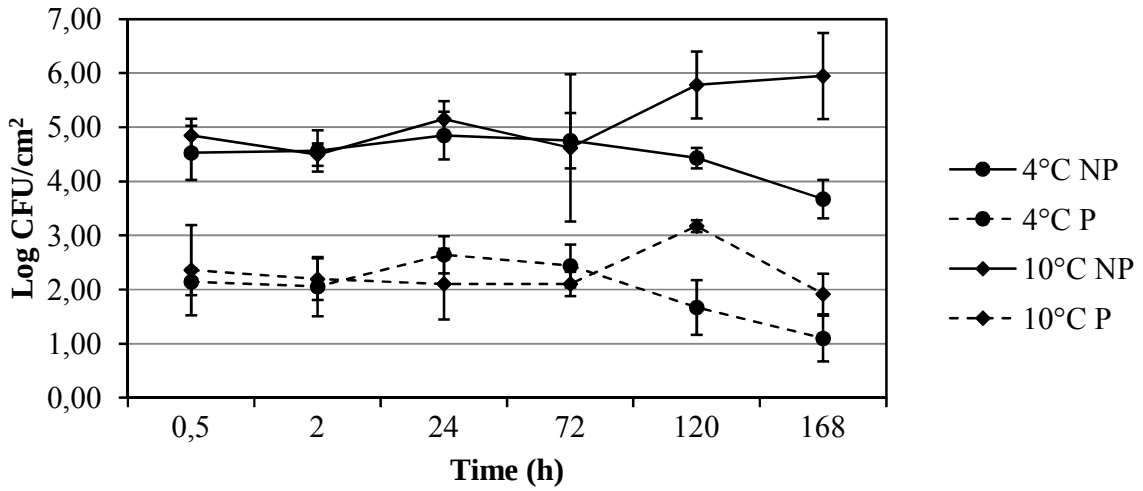


Figure 10. Effect of EcoShield™ on *E. coli* O157:H7 RM4407 Nal^R on spinach surface.

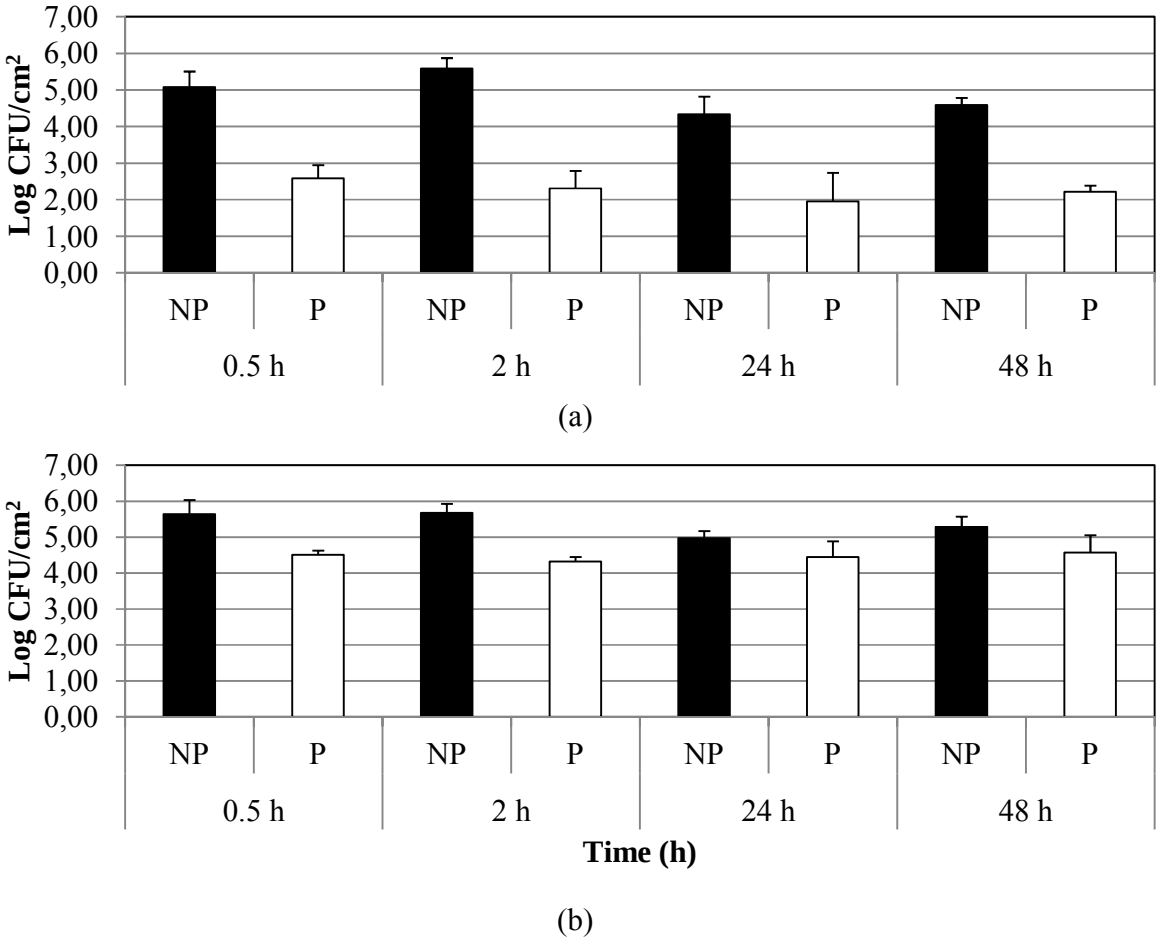


Figure 11. Phage cocktail vs. *E. coli* O157:H7 RM4407 Nal^R on green leaf lettuce at 4°C. (a) *E. coli* O157:H7; (b) Total aerobic mesophilic bacteria.

place under light. It is known that bacterial internalization is induced by light (Kroupitski, Golberg, Belausov, Pinto, Swartzberg, Granot *et al.*, 2009). Under light condition, bacteria internalize at a greater rate through chemotaxis as the increased stomatal activity and the more readily available nutrients attract the bacteria. Internalized bacteria are harder to remove and inactivate (Kroupitski *et al.*, 2009).

Linear regression analysis showed that phage treatment significantly reduced ($P < 0.05$) the recovered EHEC RM4407 Nal^R counts from fresh-cut spinach pieces by 2.60 log units. In our experimental design, the shortest phage treatment was 30 min and the inhibitory effect of the phage treatment was seen as early as 30 min after the phage cocktail application on spinach surface. This is in correlation with the findings of Abuladze *et al.* (2008), who showed a significant decrease in *E. coli* O157:H7 numbers recovered from the glass cover slips and gypsum boards after 5 min of EcoShield™ phage cocktail treatment. Together, these results show that the EHEC-specific phage cocktail used in this study is highly effective on both types of *E. coli* O157:H7; attached and in suspension.

In a comparable study, EcoShield™ phage cocktail treatment was able to reduce *E. coli* O157:H7 counts on fresh-cut lettuce by 1.6 log CFU/cm^2 compared to control samples after 1 and 2 day treatments at 4°C (Sharma *et al.*, 2009b). Antibacterial effect of the phage cocktail was reported as quickly as in day 0 samples, as well. The researchers treated 3.76 log CFU/cm^2 of EHEC with 5.98 log PFU/cm^2 of the phage cocktail on fresh-cut iceberg lettuce surface and obtained a 1.6 log unit decrease in the recovered EHEC numbers. In the current study, we were able to achieve 2.60 log CFU/cm^2 reduction in EHEC counts recovered from the spinach leaves on average. The multiplicity of infection (MOI) value, which is the ratio of phage particles vs. the viable bacterial cells per unit, was 100 fold in this study, which is equal to what Sharma *et al.*

(2009b) also used. The difference in log reduction could be due to the use of different EHEC strains. The strain used in this study might be more susceptible to the phage cocktail. Another possibility might be the dissimilarities between iceberg lettuce and spinach surfaces. Under high MOI, the phages might be less aggressive toward their target and host bacterial cells in order not to eradicate all available hosts to ensure their own survival should the life conditions worsen.

In the same study, Sharma *et al.* (2009b) also reported that seven-day phage cocktail treatment at 4°C decreased the EHEC counts by 3.1 log CFU/ml on the fresh-cut cantaloupe. The effect of the phage cocktail was not as strong when the incubation temperature was increased to 20°C as the EHEC grew 3.5 log CFU/ml during the 7-day incubation period in spite of the presence of the phage cocktail (Sharma *et al.*, 2009b).

A similar experiment was performed using green leaf lettuce. Phage treatment significantly ($P < 0.05$) lowered the recovered EHEC counts from green leaf lettuce surface (Figure 11-a). This is comparable to what was observed with spinach. Figure 11-a shows that the phage activity could be seen as early as in 30 min, similar to the spinach result. The experiment was repeated twice in duplicates. Phage cocktail was able to reduce *E. coli* O157:H7 RM4407 Nal^R cells by 2.49 and 3.28 log units within 30 min and 2 h at 4°C, respectively. The EHEC counts on the phage treated lettuce surfaces stayed low throughout the entire 48 h incubation period (2.38 log unit reduction).

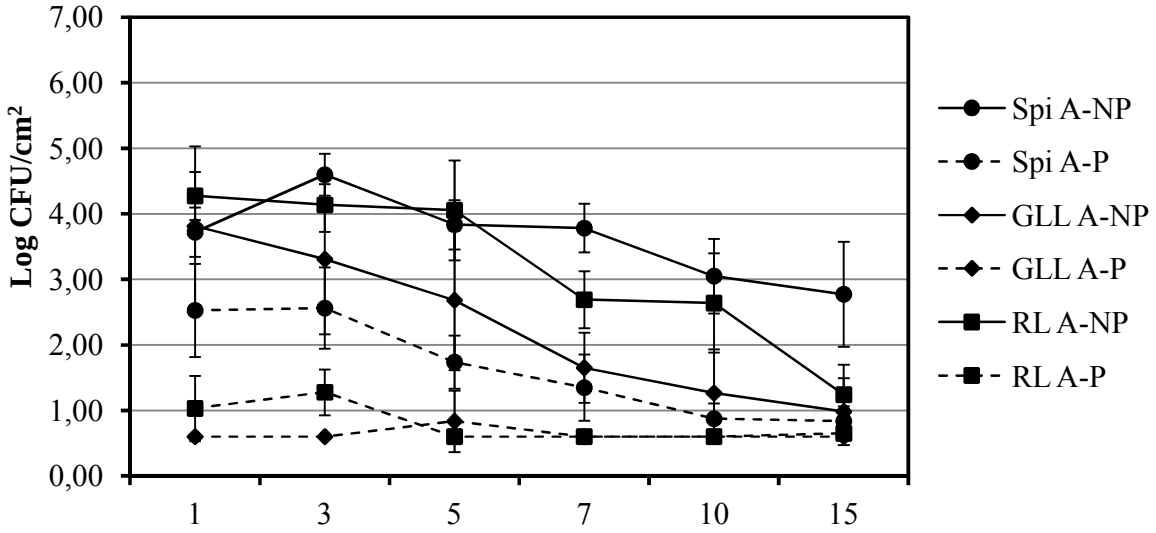
Besides the EHEC count in this study, general aerobic mesophilic microorganism count was monitored, as well. Figure 11-b shows that green leaf lettuce initially had 5.50 log CFU/cm^2 indigenous aerobic microorganism load. This is about 3-4 times more than the inoculated *E. coli* O157:H7 RM4407 Nal^R amount used in the study. In 30 min at 4°C, phage treatment killed

99.7% of the EHEC. The percentage dropped to 92.6% when the recovered EHEC colonies were counted with the indigenous aerobic bacteria.

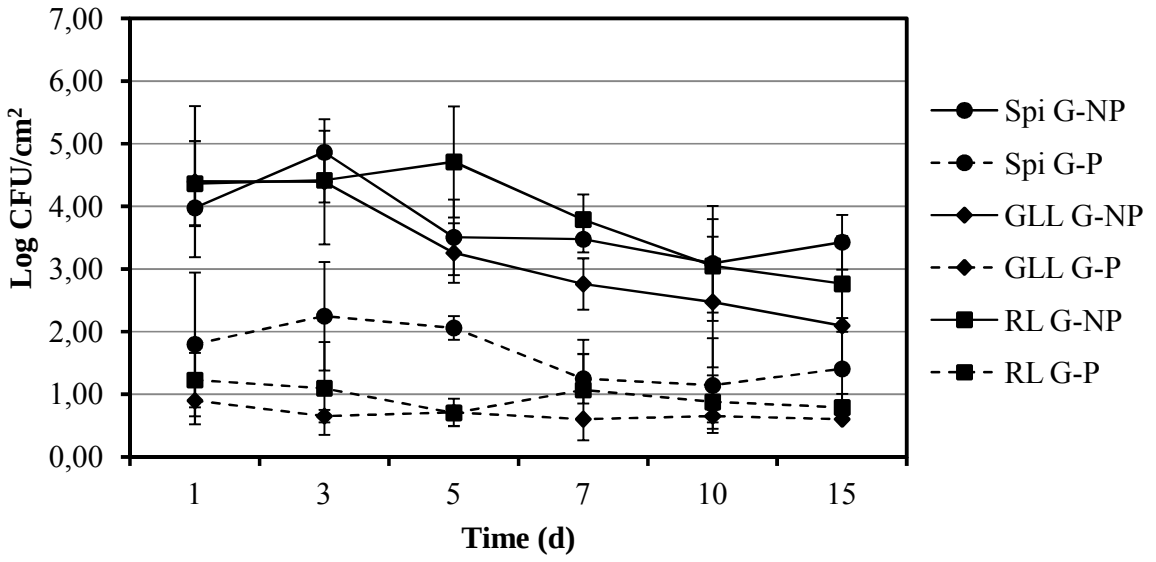
4.7. Bacteriophage Cocktail vs. *E. coli* O157:H7 RM4407 Nal^R on Spinach, Romaine, and Green Leaf Lettuce Surfaces Stored under Modified Atmosphere Packaging (MAP)

The main question that needed an answer in this research was how the efficiency of the lytic bacteriophages would be affected under different atmospheric conditions against *E. coli* O157:H7 colonized on the surface of fresh-cut green leafy vegetables stored at refrigeration temperatures. The leaf pieces of fresh-cut green leafy vegetables were inoculated with *E. coli* O157:H7 RM4407 Nal^R followed by spray inoculation of an EHEC-specific phage cocktail. The leaves were then packaged under 2 separate conditions; atmospheric air and modified air. Numbers of the surviving EHEC cells recovered from the fresh-cut green leafy vegetables stored under; A) atmospheric air and B) modified atmosphere (5% O_2, 35% CO_2, 60% N_2) packaging conditions can be seen in Figure 12 and Figure 13, respectively.

Phage treatment decreased the live EHEC counts as soon as day 1 and kept it low until day 15. Statistical data analysis showed that the phage cocktail significantly ($P < 0.05$) lowered the concentrations of *E. coli* O157:H7 RM4407 Nal^R in 1 day by 1.19 log on spinach, 3.21 log on green leaf lettuce, and 3.25 log units on romaine lettuce stored at 4°C under atmospheric air packaging (Figure 12-a). Experiment was repeated twice in duplicates for each produce. On the other hand, when stored under modified atmospheric gas, phage cocktail reduced the EHEC concentrations on spinach, green leaf lettuce, and romaine lettuce by 2.18, 3.50, and 3.13 log units, respectively (Figure 12-b). EHEC concentration on the phage-treated green leaf lettuce samples stored at 4°C was below the detection level of 0.60 log CFU/cm^2 in most of the sampling days during the 2 week study. Since the samples were stored in dark, the stomatal

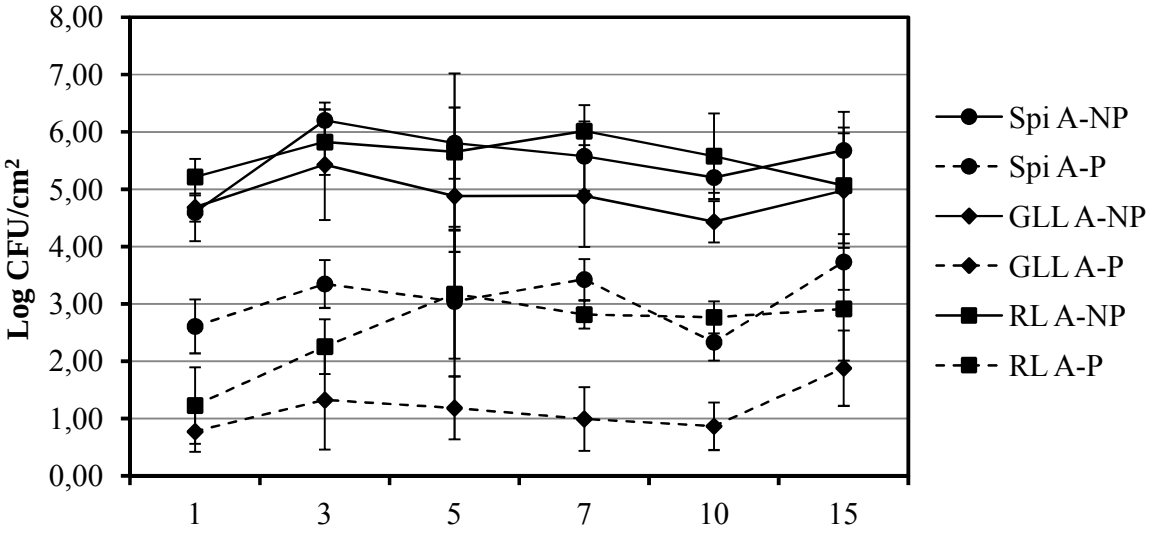


(a)

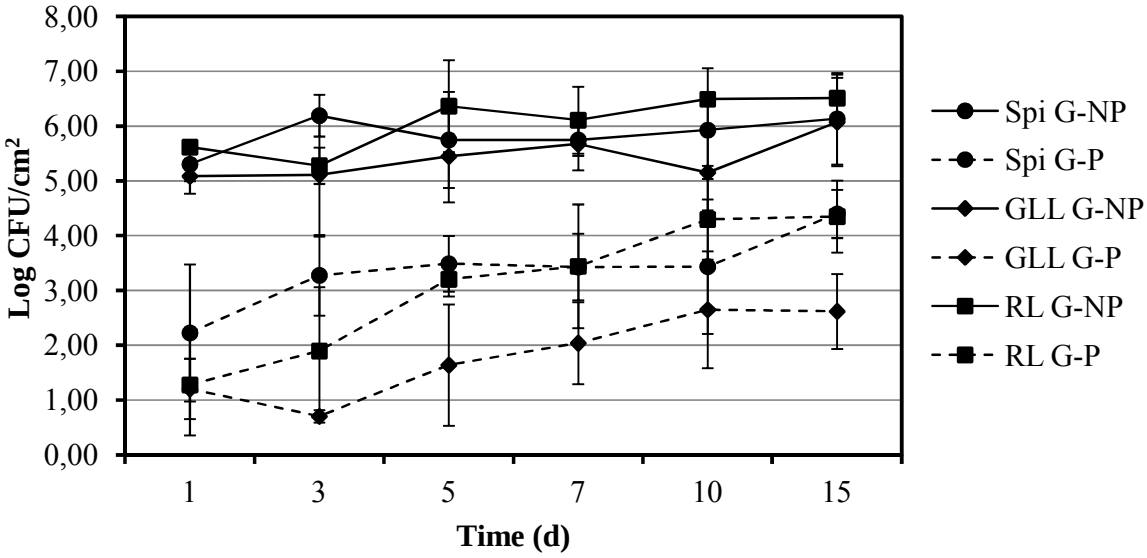


(b)

Figure 12. E. coli O157:H7 RM4407 Nal^R counts on green leafy produce contaminated with *E. coli* O157:H7 RM4407 Nal^R, treated with bacteriophage cocktail, and stored at 4°C under; (a) MAP with atmospheric air; (b) MAP with 5% O_2, 35% CO_2, 60% N_2 gas.



(a)



(b)

Figure 13. E. coli O157:H7 RM4407 Nal^R counts on green leafy produce contaminated with *E. coli* O157:H7 RM4407 Nal^R, treated with bacteriophage cocktail, and stored at 10°C under; (a) MAP with atmospheric air; (b) MAP with 5% O_2, 35% CO_2, 60% N_2 gas.

activity of produce samples was lower than that of under light resulting in reduced phage efficacy against EHEC. The stomatas draw the bacteria when they are active, helping them internalize produce surface more easily (Kroupitski *et al.*, 2009).

EHEC load on the control leaves sprayed with peptone water decreased at 4°C during the 15-day study by 0.94 log on spinach, 2.82 log on green leaf lettuce, and 3.04 log on romaine lettuce stored in sealed bags filled with atmospheric air. The log reductions after 15 days under modified atmosphere (high CO_2/low O_2) condition were 0.55, 2.30, and 1.60 log on spinach, green leaf and romaine lettuces, respectively. These numbers, although not exactly the same, are in agreement with the results of previous studies (Oliveira *et al.*, 2010; Sharma *et al.*, 2011). One study found out that *E. coli* O157:H7 counts on shredded lettuce decreased by 1 log after 10 day incubation at 5°C (Oliveira *et al.*, 2010). In another study with shredded iceberg lettuce stored at 4°C for 10 days, EHEC population declined by 1.70 log inside gas permeable package with perforations, 0.85 log inside gas permeable package with an initial N_2 flush, and 1.10 log inside gas-impermeable package filled with high CO_2/low O_2 (Sharma *et al.*, 2011). Our results are not in line with the findings of another study, which reported only 0.43 log decrease of the *E. coli* O157:H7 counts on romaine lettuce stored at 4°C for 9 days (Carey, Kostrzynska, & Thompson, 2009). However, in that study the lettuce samples were not incubated under MAP conditions. The particular *E. coli* O157:H7 strain used, type of the produce, storage atmosphere and temperature conditions, specific constituents and the concentrations of the indigenous microflora, and the initial bacterial load might be responsible for the discrepancies we observe between our study and the previously published literature (Lopez-Velasco, Davis, Boyer, Williams, & Ponder, 2010).

It was determined that phage-treated samples stored at 10°C under air had significantly ($P < 0.05$) lower EHEC counts after 1 day by 1.99 log in spinach, 3.90 log in green leaf lettuce, and 3.99 log units in romaine lettuce (Figure 13-a). Experiment was repeated twice in duplicates. When samples were stored under modified atmospheric gas condition at 10°C, the EHEC concentrations on spinach, green leaf lettuce, and romaine lettuce fell down by 3.08, 3.89, and 4.34 log units, respectively (Figure 13-b). A previous study reported an at least 2.76 log CFU/g increase in *E. coli* O157:H7 when stored at 15°C. (Sharma *et al.*, 2011). The 5°C difference in the storage temperature seems to mask the effect of phage against the pathogen of interest.

Multiple regression analysis of the entire data showed that the phage treatment significantly ($P < 0.05$) reduced the EHEC counts by 2.64 log units on average. The EHEC counts in all samples stored at 4°C started to descend steadily after day 3; however, EHEC counts on the phage-treated samples were always lower than the controls at each time point throughout the study. Incubation at 10°C significantly ($P < 0.05$) increased the EHEC counts recovered from the produce leaves by 1.84 log units on average compared to those incubated at 4°C. The concentration of *E. coli* O157:H7 on control leaf samples did not drop during the 15 day incubation period at 10°C (Figure 13-a&b). The standalone phages are not affected by the oxygen and carbon dioxide levels in the packages as the phages are not active without their hosts. The phages are able to show their capabilities the same level as their host cells stay active. *E. coli* O157:H7 is a facultative anaerobe that can survive and function normally under atmospheric air, and low oxygen and high carbon dioxide environments. Hence, the phages that infect their EHEC hosts are not suspected to be affected by different O_2/CO_2 concentrations. As expected, once inside of their hosts they will take over the host replication machinery to replicate their genomic material for their progeny. The indigenous microorganisms of the fresh produce

surface may compete against *E. coli* O157:H7 host bacteria for the limited nutrients that are available on the surface. The indigenous microorganisms on produce samples, especially those that form biofilm structures, might have formed physical obstacles which might have prevented the phage particles to lyse their host EHEC cells.

Through visual observations, it was understood that leaf pieces stored at 10°C became spoiled faster than those stored at 4°C. Also, high CO_2 and low O_2 (modified atmosphere packaging) conditions resulted in slower spoilage of the leaf pieces although this modified atmosphere storage resulted in an average of 0.40 log unit increase ($P < 0.05$) in EHEC counts compared to air storage. High CO_2 concentration might be blocking the growth of spoilage bacteria, which in turn minimizes the competition and resulting in slightly higher EHEC counts than atmospheric air packs.

The partial oxygen and carbon dioxide concentrations were measured during the MAP studies (Figure 14). The oxygen level in air packages dropped by 2.2% and 5.9%; whereas, CO_2 levels increased by 1.1% and 3.7% at 4 and 10°C, respectively (Figure 14-a). The decrease in the oxygen concentrations and the increase in the carbon dioxide concentrations are most likely the result of the slow respiration rate of the produce samples and the presence of indigenous bacteria (such as *Lactobacillus* spp.) on produce. In modified air filled packages stored at 4°C, O_2 level elevated by 1.8%, while CO_2 level depleted by 18.1%. When the samples were stored at 10°C, O_2 level decreased by 1.4% and CO_2 level declined by 14.1% (Figure 14-b). The reduction in the CO_2 levels might be the effect of the short-time light exposure during gas content measurements, performed by bringing the sealed packages out of the dark storage environment.

The two different O_2 and CO_2 concentrations used in this study did not significantly ($P > 0.05$) alter the efficiency of the phage cocktail to lyse the EHEC cells. This can also be

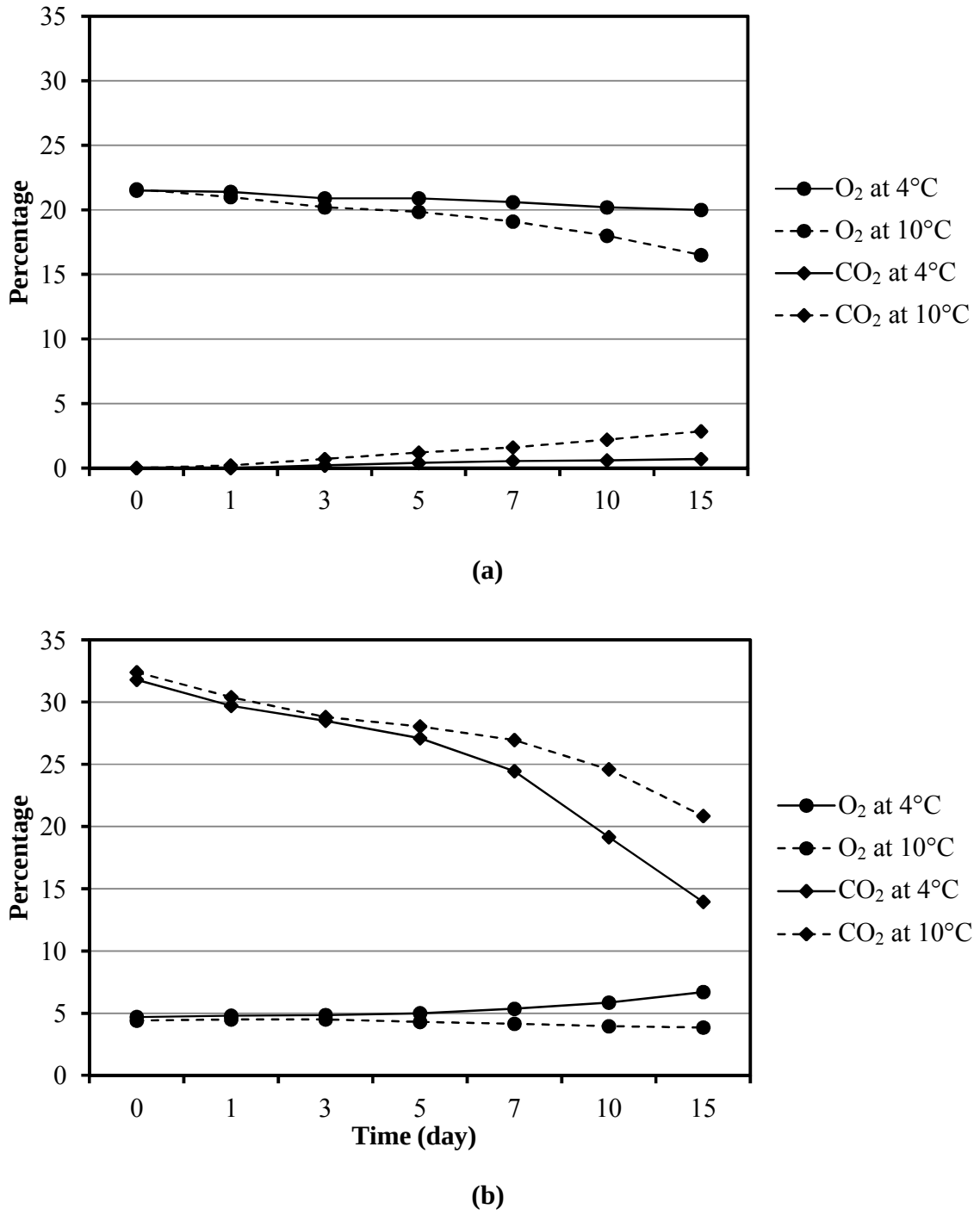


Figure 14. Partial percentages of O_2 and CO_2 during MAP experiment. (a) MAP with atmospheric air; (b) MAP with 5% O_2, 35% CO_2, 60% N_2 gas.

interpreted as the different O_2 and CO_2 concentrations did not have an effect on the growth and survival of the EHEC cells. Several previous studies have also showed similar results. One study reported no effect on EHEC populations by the storage atmosphere that is composed of 3% O_2 and 97% N_2 (Abdul-Raouf, Beuchat, & Ammar, 1993). Another study concluded that EHEC growth on shredded iceberg lettuce was not affected by any of the 4 different MAP conditions at 13 and 22°C (Diaz & Hotchkiss, 1996). Lastly, a recent study noted that the various O_2 and CO_2 levels developed inside the different packaging film materials during 10 day of incubation at 5 and 25°C did not change the survival or the growth of EHEC on shredded romaine lettuce (Oliveira *et al.*, 2010).

Overall, these results are not supporting our hypothesis that the phage cocktail will be less effective under low oxygen, high carbon dioxide atmosphere in lysing the EHEC strain used in the study compared to that of under atmospheric air condition. Previous studies showed that the aerobic and anaerobic microflora, such as aerobic mesophilic bacteria, psychrotrophic bacteria, Pseudomonadaceae, Enterobacteriaceae, Micrococcaceae, lactic acid bacteria, and yeasts on produce surfaces would act differently under low O_2 storage conditions (Babic, Roy, Watada, & Wergin, 1996). This would create variations in the level of indigenous bacteria, which would compete against the EHEC strain for the limited resources to survive, resulting in lower numbers of EHEC under modified air (5% O_2, 35% CO_2, 60% N_2) packaging. The fact that *E. coli* O157:H7 is a facultative anaerobic species makes it easy for the pathogen to accommodate to low and high levels of oxygen concentration available in the ambient air.

4.8. Recovered Phage Quantification from Fresh Produce

The stability and the effectiveness of the EHEC-specific phage cocktail on the surface of fresh-cut spinach pieces were tested at 4°C in the absence of the host EHEC. The active phage

cocktail amount present on the produce surface did not change during the 17-day storage at 4°C (Figure 15). The initial inoculum level of 6.5 log PFU of EHEC-specific phages persisted at the same level throughout the entire storage period. The results are the mean of 3 replicates ± SD. No significant correlation was found between the phage count and the incubation time ($P > 0.05$). The increasing number of the aerobic and anaerobic microflora on the produce surface and the deteriorating condition of the produce during 2 weeks storage did not seem to have an effect on the EHEC-specific phage cocktail used in the study. This suggests that the phage cocktail may provide protection against an EHEC contamination that may occur after the packaging and sale; such as consumer-originated contamination cases arisen from poor hygiene conditions.

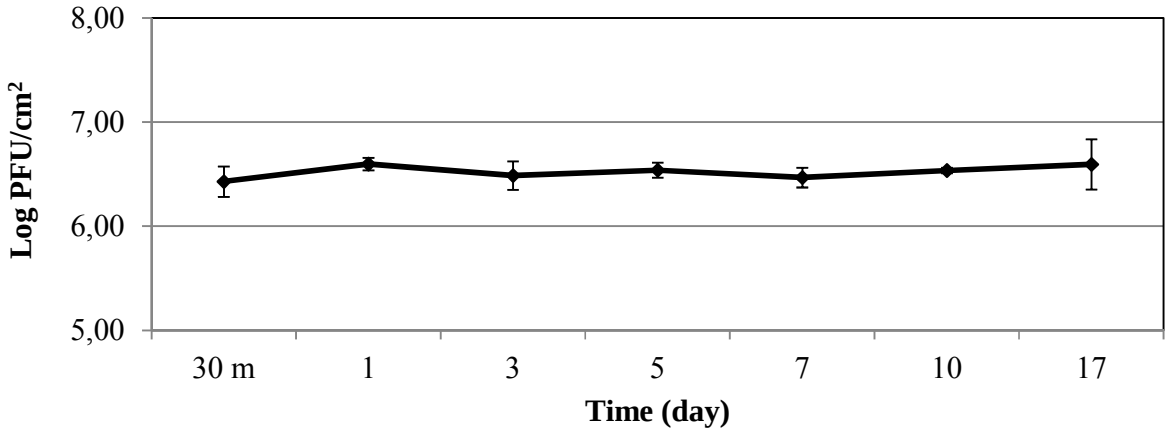
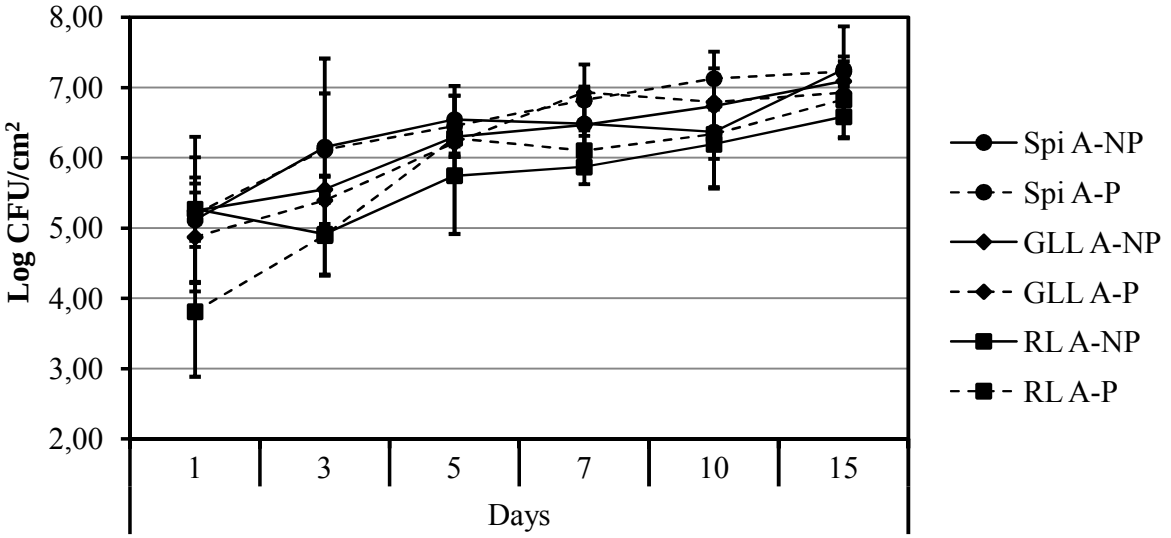


Figure 15. Recovered bacteriophage quantification from fresh produce stored at 4°C.

4.9. Aerobic Mesophilic Microorganism Counts on Spinach, Green Leaf, and Romaine Lettuce Stored under MAP

Another variable that was measured during the MAP experiments was the total number of general aerobic mesophilic microorganisms on the fresh-cut green leafy vegetables. Experiments were repeated twice in duplicates for each produce. Aerobic indigenous microflora on leafy green vegetables stored at 4°C can be seen in Figure 16, while Figure 17 shows the microbial



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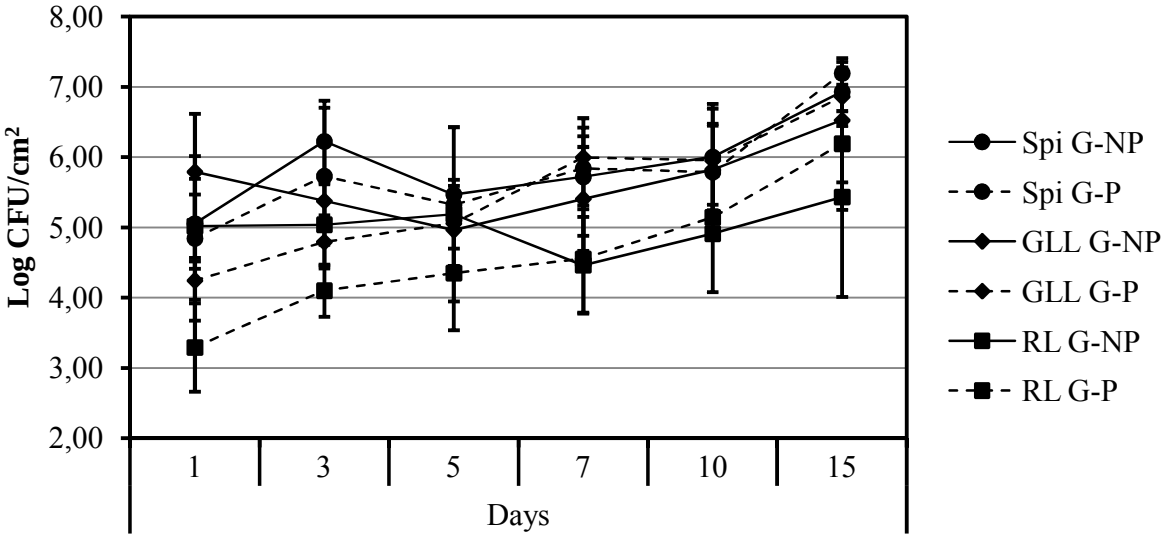
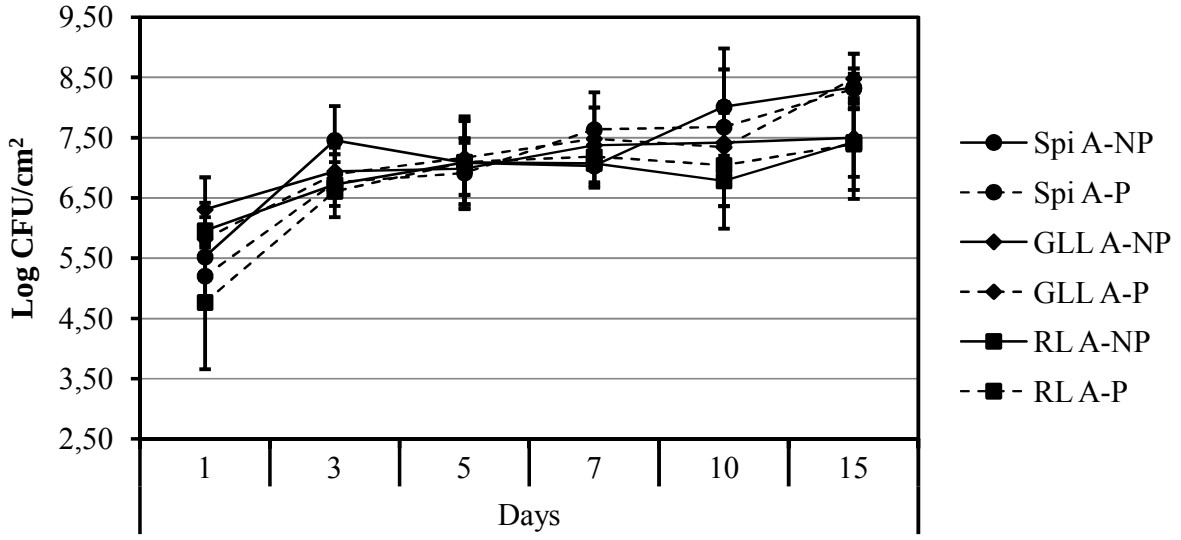


(b)

Figure 16. Aerobic mesophilic microorganism counts on green leafy produce contaminated with *E. coli* O157:H7 RM4407 Nal^R, treated with bacteriophage cocktail, and stored at 4°C under; (a) MAP with atmospheric air, (b) MAP with 5% O_2, 35% CO_2, 60% N_2.



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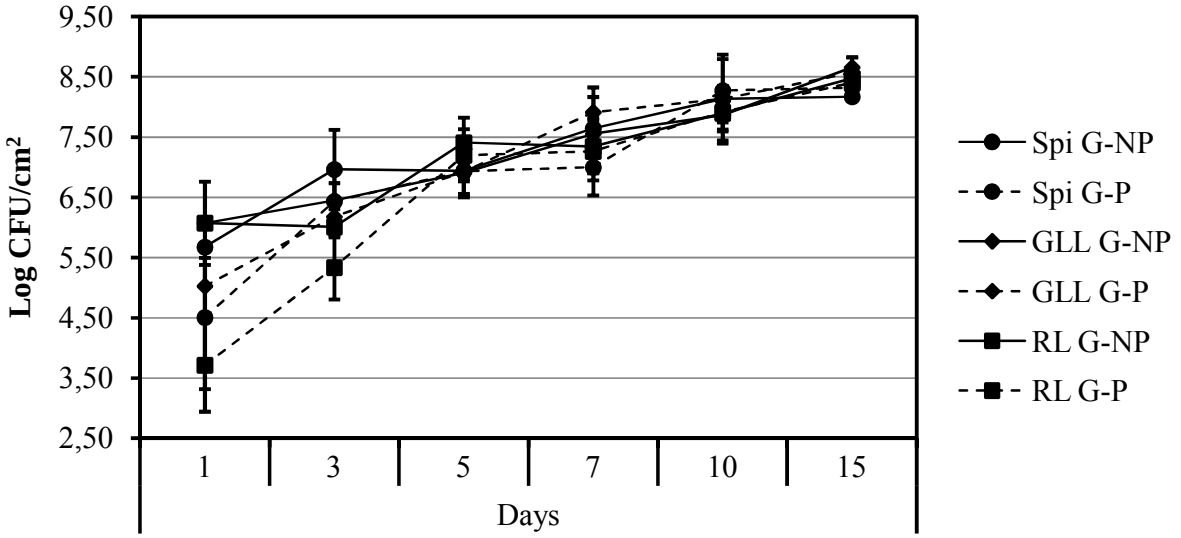


(b)

Figure 17. Aerobic mesophilic microorganism counts on green leafy produce contaminated with *E. coli* O157:H7 RM4407 Nal^R, treated with bacteriophage cocktail, and stored at 10°C under; (a) MAP with atmospheric air, (b) MAP with 5% O_2, 35% CO_2, 60% N_2.

load in samples stored at 10°C. Further taxonomical identifications of the recovered bacteria were not performed as it was not among the aims of the study.

Statistical analysis showed that there is no correlation between the phage treatment and the total aerobic microbial load on the leafy greens ($P > 0.05$). The oxygen and carbon dioxide concentrations of the packaging gas also did not alter the level of indigenous microflora. On the other hand, the storage temperature and the time significantly ($P < 0.05$) increased microbial load.

Romaine lettuce appeared to have the least amount of aerobic indigenous microflora compared to green leaf lettuce and spinach. However, the difference seemed to be close toward the end of the 15-day storage at refrigeration temperatures (Figure 16 and Figure 17). Although not significant, it is quite interesting to note that the aerobic indigenous microorganism load on the spinach, which was the only produce thoroughly pre-washed and bagged that was used in this study, was relatively higher in comparison to those of lettuce samples.

This can be interpreted as the sanitizing techniques employed by the industry are not effective in reducing the microbial load of the produce, and the sanitizing agents are used mainly to keep the wash water clean to prevent cross contamination between produce batches (Behrsing *et al.*, 2000). The spinach used in this study was from Fresh Express, who announced to be using LA-PAA mixture, which is 7 times more effective than traditional chlorine wash (Ho *et al.*, 2011), as sanitizing agent to reduce the total bacterial count of their produce. The reason we observed more bacteria on bagged spinach than on conventional lettuces might be that the LA-PAA mixture may not be as effective in reducing microbial load as it was claimed. Another reason may be the increased shelf life of the pre-washed and packaged fresh produce. The

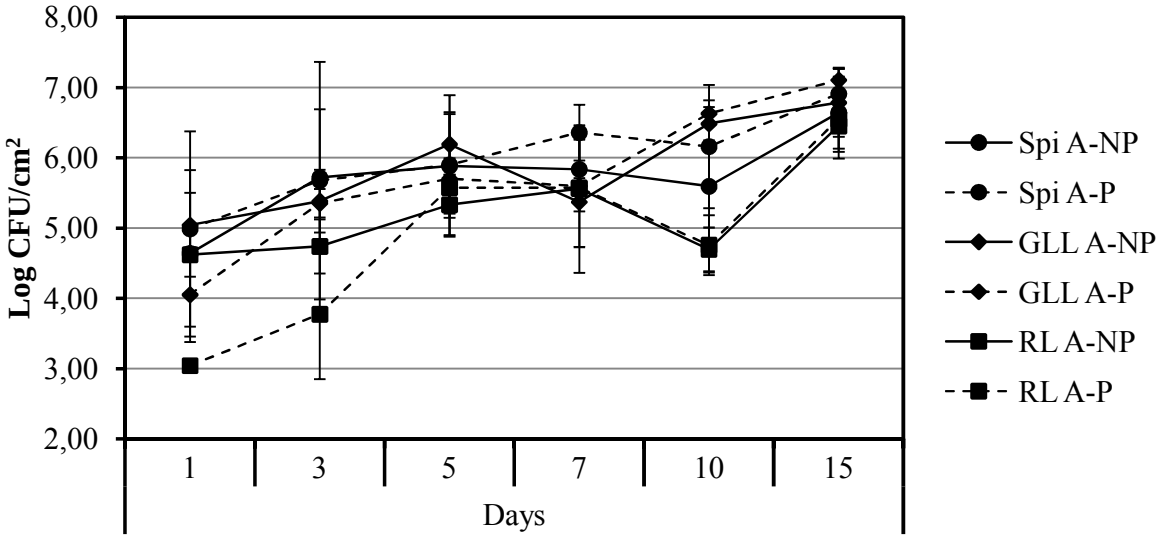
remaining indigenous microorganisms may flourish during the extended shelf life of these pre-washed produce resulting in higher counts of aerobic bacteria.

4.10. Anaerobic Microorganism Counts on Spinach, Green Leaf, and Romaine Lettuce Stored under MAP

The anaerobic indigenous microflora on the fresh-cut green leafy vegetables was also enumerated during the MAP study. Similar to the aerobic mesophiles, the recovered anaerobic microorganisms were not further identified taxonomically. Anaerobic indigenous microflora level on the leafy green vegetables stored at 4°C can be seen in Figure 18, while Figure 19 shows the microbial load in samples stored at 10°C. Experiments were repeated twice in duplicates for each produce.

Statistical analysis showed that there is no correlation between the phage treatment and the anaerobic indigenous microorganism load on the leafy greens tested ($P > 0.05$). The oxygen and carbon dioxide concentrations of the packaging gas also did not affect the level of indigenous anaerobic microflora. Nonetheless, the storage temperature and time significantly ($P < 0.05$) increased the anaerobe counts.

Similar to the aerobic counts, romaine lettuce appeared to have the least amount of anaerobic indigenous microflora compared to the green leaf lettuce and the spinach. However, the numbers increased the 15-day of storage at refrigeration temperatures (Figure 18 and Figure 19). The anaerobic counts were relatively higher on pre-washed and packed spinach stored under same conditions as lettuce samples. This may also be due to the inefficiency of the sanitizing agents used by the fresh produce industry. Like aerobic bacteria, anaerobic bacteria may survive after the chlorine or LA-PAA mixture washes.



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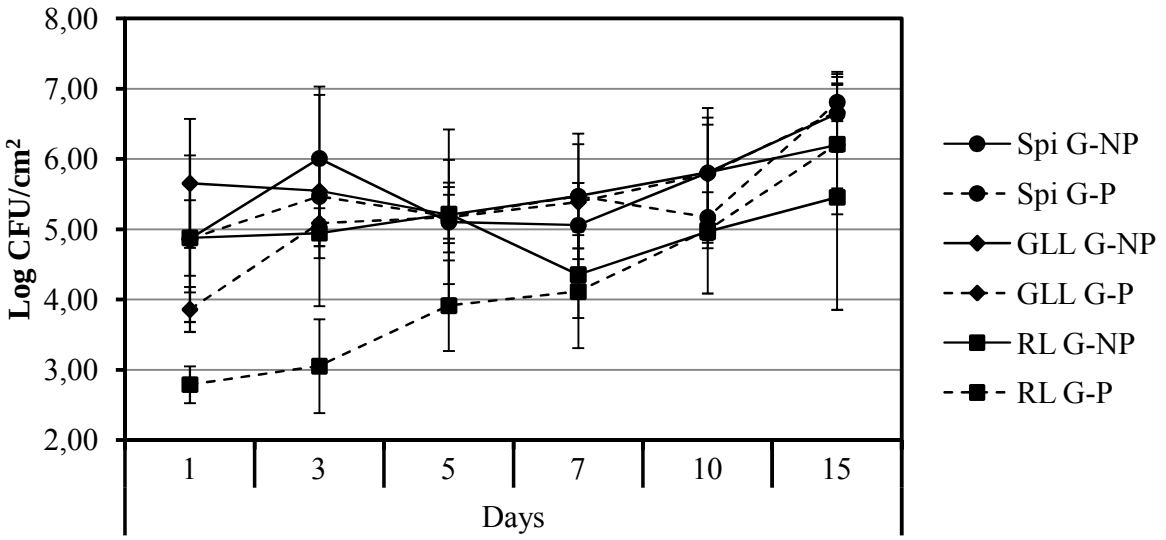

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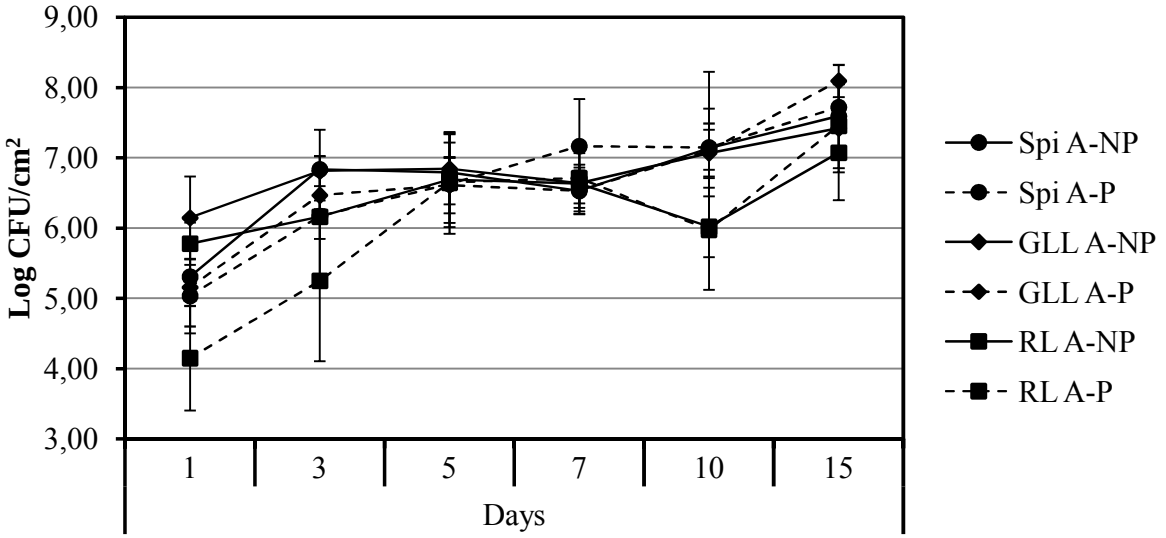
Figure 18. Anaerobic microorganism counts on green leafy produce contaminated with *E. coli* O157:H7 RM4407 Nal^R, treated with bacteriophage cocktail, and stored at 4°C under; (a) MAP with atmospheric air, (b) MAP with 5% O_2, 35% CO_2, 60% N_2.



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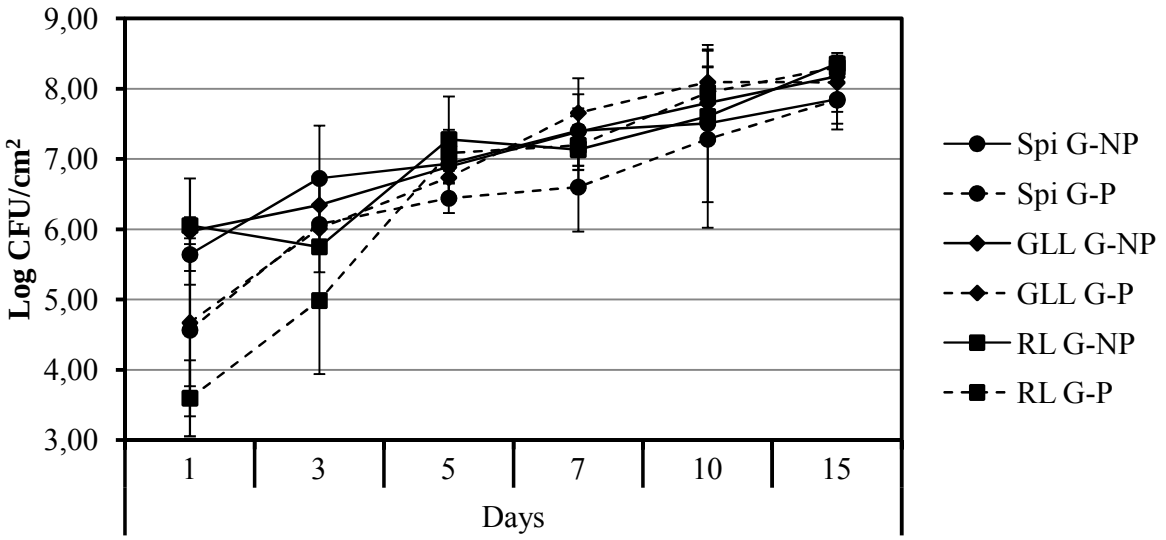

(b)

Figure 19. Anaerobic microorganism counts on green leafy produce contaminated with *E. coli* O157:H7 RM4407 Nal^R, treated with bacteriophage cocktail, and stored at 10°C under; (a) MAP with atmospheric air, (b) MAP with 5% O_2, 35% CO_2, 60% N_2.

CHAPTER 5

Conclusions

In this study, the effect of an EHEC-specific phage cocktail on the survival and growth of an *E. coli* O157:H7 strain on the fresh-cut green leafy vegetables stored under different atmospheric conditions was investigated. At 4°C, phage cocktail significantly ($P < 0.05$) lowered the *E. coli* O157:H7 count by 1.19, 3.21, and 3.25 log units on spinach, green leaf lettuce, and romaine lettuce packaged under atmospheric air after 24 h storage. The reduction levels were 2.18, 3.50, and 3.13 log units in the same order under the modified atmosphere conditions tested. There was no significant ($P > 0.05$) difference in the recovered EHEC counts from the produce stored under 2 different oxygen and carbon dioxide concentrations, atmospheric air and modified (5% O_2, 35% CO_2, 60% N_2) air. Although *E. coli* O157:H7 populations on all produce samples started decreasing after 3-day storage at 4°C, the EHEC numbers recovered from the phage treated samples were always lower than those of the control samples throughout the storage.

The EHEC-specific phage cocktail significantly ($P < 0.05$) reduced the EHEC levels by 1.99 log on spinach, 3.90 log on green leaf lettuce, and 3.99 log units on romaine lettuce leaves stored at 10°C under atmospheric air for 24 h. The amount of reduction in the recovered *E. coli* O157:H7 counts was not significantly different under modified atmosphere conditions with the log reduction values of 3.08, 3.89, and 4.34 log units, respectively. The numbers of EHEC recovered from the samples incubated under different atmospheric conditions were not significantly ($P > 0.05$) different from each other. This may mean the ambient oxygen and carbon dioxide concentrations are not the determining factors for the phage efficacy.

The phage treatment did not change the numbers of indigenous aerobic mesophiles and the anaerobes. The oxygen and the carbon dioxide concentrations inside the packages also were

not determining factors for the amounts of aerobic and anaerobic microflora of the leafy greens based on the statistical analysis. For both populations, the storage time and temperatures were significant, indicating the importance of the storage and shelf conditions of the fresh produce.

To the best of our knowledge, this study is the first to test phage efficiency against the target bacteria on leafy green vegetables stored under modified atmosphere packaging condition that is composed of lower O_2 and higher CO_2 than the atmospheric air. Overall, it was shown that the efficiency of an *E. coli* O157:H7-specific bacteriophage cocktail under 5% O_2, 30% CO_2, and 60% N_2 is not significantly different from that of atmospheric air condition. The results of this study suggest that the phages may be employed onto produce to successfully control/reduce pathogenic bacterial presence and/or growth under modified atmosphere packaging.

Since small (2x2 cm2) leaf pieces of the leafy green vegetables were used in this study, it would be necessary to use whole leaves in future studies to better understand the efficiency of the phage cocktail under MAP conditions. The application method of the phage cocktail onto the produce may also be changed when whole produce is used. Introducing the phage cocktail to the wash tank might provide higher bacterial lysis as the wash water may carry the phage particles to the deeper areas of the produce compared to the surface exposure obtained in spray method. Additionally, to improve the effectiveness of the phage cocktail used in this study, produce samples could be first washed with hypochlorite or organic acid wash solutions and sprayed with specific phage cocktail to combat pathogenic growth on produce.

Although the bacteriophages are ubiquitously present in practically every food items, drinks, and the air, the use of phages that are specific for bacteria in food safety might face strong resistance from the public. The notion of added viruses inside the food items, such as fresh leafy green vegetables, for food safety may not be perceived as a safe method. The

consumers should be educated about the phages to overcome their potential reservations toward the issue since phages are natural antibiotics that are highly effective in controlling target pathogens.

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