IMPACT OF PRE-HARVEST ENVIRONMENTAL FACTORS ON THE SURVIVAL OF ENTEROHEMORRHAGIC *E.COLI* AND *SALMONELLA* ON LETTUCE

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Title

Impact of Pre-Harvest Environmental Factors on the Survival of Enterohemorrhagic *E.coli* and *Salmonella* on lettuce

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Enteric diseases linked to fresh produce consumption are on a rise. Pathogens can contaminate produce in the pre-harvest field and can survive for long time periods. Thus, this study quantified the survival of Enterohemorrhagic *E.coli* and *Salmonella* on pre-harvest lettuce under two relative humidity and seasonal conditions. The effect of relative humidity on pathogen survival depended on the seasonal conditions. The impact of chlorine stress on survival of the two pathogens after exposure to pre-harvest variables was also determined. A single EHEC strain developed resistance to chlorine after 3 days on lettuce plants. Gene expression analysis revealed the up-regulation of genes involved in osmotic and cell envelope stress. Up-regulation of a gene involved in oxidative stress was also observed which could possibly be responsible for imparting resistance to chlorine stress. Understanding these aspects will help develop effective post-harvest decontamination strategies to reduce consumer exposure to such pathogens on produce.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

EHEC……..Enterohemorrhagic *E.coli*

RH……..……Relative Humidity

DNA……..Deoxyribonucleic acid

RNA…….Ribonucleic acid

PBS……...Phosphate Buffered Saline

CFU…….….Colony Forming Unit

LB……...Luria-Bertani

BHI……....Brain Heart Infusion

PVPP……Polyvinylpolypyrrolidone

FDR……....False Discovery Rate

SDS……Sodium Dodecyl Sulphate

GLM……..General Linear Model

EPS……Exopolysaccharide

ROS……..…..Reactive Oxygen Species

MetSo……..Methionine Sulfoxide

Msr……..Methionine Sulfoxide Reductase

GBP…….General Bacterial Porin

PGA……..…Poly- β-1,6-N-Acetyl-D-Glucosamine

Gst……..Glutathione S Transferase

CHAPTER 1

Literature review

Enterohemorrhagic *E.coli* (EHEC) and *Salmonella* are two of the many enteric pathogens that inhabit the intestinal tracts of warm-blooded animals. Cattle [1, 2], sheep [3] and deer [4] are the major reservoir of EHEC, whereas *Salmonella* can be commonly found in the gastrointestinal tract of chickens [2] and cattle [5]. In humans, these pathogens are capable of causing severe gastroenteritis with an onset of symptoms such as nausea, vomiting, diarrhea and abdominal cramps. *Salmonella* are invasive and able to enter the blood causing systemic infections [6]. EHEC, on the other hand, produce shiga-toxins resulting in severe complications such as Hemolytic Uremic Syndrome, characterized by hemolytic anemia and renal failure [7]. These pathogens are transmitted to humans via the fecal-oral route either through direct contact with feces or contact with fecal material in food and water.

According to CDC 2011 estimates, non-typhoidal *Salmonella*, like *Salmonella* Typhimurium and *Salmonella* Newport, is the second leading cause of foodborne illnesses (following norovirus) and accounts for 11% of all foodborne diseases occurring annually in United States [8]. *Salmonella* is also the leading cause of hospitalizations (35%) and deaths (28%) each year. EHEC serotype O157 is among the top five foodborne pathogens resulting in the most deaths (8%). Besides the loss of life, EHEC O157 and *Salmonella* each cause the US economy an estimate $3.3 billion each year, for a total economic burden of $6.6 billion [9].

Historically, foodborne outbreaks were linked to products of animal origin but outbreaks of enteric pathogens linked to fresh produce consumption have increased in the past few decades [10-12]. CDC estimated that produce accounted for nearly half of all foodborne illnesses (46%) from 1998-2008. Several outbreaks due to consumption of contaminated fresh produce such as alfalfa seeds [13], raw clover sprouts [14], tomatoes [15], mangoes [16] and cilantro [17] have been reported. Among produce, leafy green vegetables such as spinach and lettuce have been most frequently implicated in outbreaks with enteric pathogens [18-21].

The increase in outbreaks due to consumption of contaminated fresh produce has raised significant concerns for human health especially for at-risk populations such as immune-compromised individuals, the elderly and young children. The minimal processing and raw consumption of fresh

produce also poses significant risks of exposing consumers to gastrointestinal illnesses. The rise in intake of fresh fruits and vegetables in pursuit of healthier eating habits and year round availability of a variety of fresh produce could also expose consumers to enteric pathogens on produce. Another important risk factor is the low infectious dose of enteric pathogens (for example, <50 cells for EHEC [22]), which means only a few surviving cells are necessary for causing severe illnesses. Therefore, it is important to understand how enteric pathogens survive on produce to eliminate the risk of exposing consumers to produce harboring enteric pathogens. Once our knowledge is expanded on the mechanisms of pathogen survival, effective control strategies could then be developed to eradicate their presence on the produce surface.

Fresh produce can become contaminated by pathogens through multiple means during pre-harvest production as well as during post-harvest processing, storage and distribution. Certain trace-back investigations have demonstrated contamination events in the pre-harvest field, most notably the US 2006 EHEC O157:H7 outbreak associated with spinach [18]. The most frequent vehicle of produce contamination in the field is the employment of contaminated water for irrigation, pesticide or fertilizer application [23, 24]. Other ways of contamination could be the usage of raw or improperly treated manure, insect vectors, direct deposition of fecal material on produce by wildlife, run-off from contaminated wetlands, proximity to animal sheds and improper personal hygiene of workers [23, 25].

Once produce contamination occurs, these pathogens are capable of surviving on the produce for long periods of time. Erickson et al. (2010) recovered EHEC O157:H7 after 27 days from field-inoculated lettuce [26]. The lettuce had been inoculated via spray inoculation with contaminated irrigation water. In another study, Islam et al. (2004) were able to recover *Salmonella* Typhimurium, 63 days post-inoculation from field-inoculated lettuce through application of contaminated compost [27]. The survival of EHEC O157 has also been assessed on greenhouse grown lettuce and spinach. EHEC O157 strain, mixed with avian pathogenic *E.coli* (which causes extra-intestinal infections in humans), was spot inoculated on spinach and lettuce plants. Both pathogens were detectable on plants, 10 days post-inoculation [28]. In yet another study, a cocktail of EHEC O157, *Salmonella enterica* and *Clostridium perfringens* was spot inoculated on hydroponically growing bell peppers, lettuce and cantaloupes in the greenhouse. All three pathogens were recovered from the produce types after 14 days of inoculation [29]. Thus, these studies

provide evidence that enteric pathogens can survive on fresh produce for variable time periods; however, little is known about the physiological state of these pathogens on pre-harvest produce and the effect of pre-harvest environmental factors on pathogen survival on produce.

Various environmental variables such as temperature, relative humidity (RH), UV radiation and a plant's native microbiota can play a role in influencing a pathogen's survival on produce in the pre-harvest environment [24]. A few studies have assessed the impact of specific pre-harvest variables on the survival of enteric pathogens. For example, *Salmonella enterica* survived better on lettuce under high humidity as compared to low humidity [29]. Likewise, the amount of UV exposure significantly impacted the survival of pathogens on lettuce. Pathogens inoculated on the lower side of the lettuce leaf (adaxial) survived better than pathogens inoculated on the upper side of the leaf (abaxial) [26, 29]. Native plant microflora has also shown to influence the survival of pathogens on produce in the pre-harvest environment. *E.coli* O157:H7 population decreased on lettuce in the presence of one of the most prevalent plant epiphyte, *Enterobacter ausburiae* [30]. Thus, the extent of pathogens survival can be influenced by environmental factors, enabling their entry in the food chain. Nonetheless, the physiology on enteric pathogens on produce in the pre-harvest field is largely unknown. More research is needed to elucidate how pathogens are responding to these environmental factors which enable their presence in the food chain.

Environmental variables pose physical, chemical and/or biological stress on pathogens present on produce. Pathogens are able to manage these stresses and survive. Bacteria activate mechanisms to be able to survive unfavorable conditions. One way pathogens can adapt to a new environment is via stimulation of systems that initiate transcriptional regulators, which respond to environmental changes through modulation of gene expression (Rodriguez-Romo and Yousef, 2005). Alterations in gene expression can indicate the up-regulation or down-regulation of specific stress responses. Such capabilities can be studied by quantifying genome-wide changes in mRNA expression (transcription). Various studies have focused on gene expression changes in enteric pathogens on produce. For example, Kyle et al. (2010) exposed EHEC O157 cells to lettuce leaf lysate and observed up-regulation of genes involved in oxidative stress, DNA repair and genes responsible for detoxification of noxious compounds [31]. Fink et al. (2012) studies interaction of *E.coli* O157 and K-12 with intact lettuce leaf

surface and observed activation of stress response genes related to biofilm formation and curli production [32]. However, these studies utilized post-harvest produce to study gene expression which does not provide information on how these pathogens behave in the pre-harvest environment.

Pathogens associated with pre-harvest lettuce are exposed to a variety of environmental stresses during post-harvest processing and handling. For lettuce and other leafy greens, this typically includes a decontamination treatment. Chlorine, in the form of sodium hypochlorite, is the most commonly used sanitizer in the fresh produce industry [33, 34]. Post-harvest decontamination steps are intended to reduce the risk of foodborne illnesses due to pathogens and the risk of spoilage microorganisms influencing the shelf life of the produce. It is possible that exposure to pre-harvest environmental factors could result in pathogens becoming better suited to future stressful environments; termed as cross-protection. In other words, exposure of these pathogens to pre-harvest variables could induce stress responses that make them resistant to chlorine decontamination, thus leading their entry in the food supply.

Many environmental stresses have been shown to induce cross-protection in enteric pathogens. In a study by Kyle et al. (2010), EHEC O157 was shown to up-regulate genes for oxidative stress in when exposed to lettuce leaf lysate. They also demonstrated that EHEC O157 showed enhanced resistance to chlorinated sanitizers [31]. In another study, *E. coli* O157:H7 exposed to a combination of acid and osmotic stress increased expression of heat shock proteins as well as a cell envelope stress response [35]. However, our knowledge about the physiology of enteric pathogens on pre-harvest produce is still limited. The stresses posed by pre-harvest environmental factors on enteric pathogens could be significantly different from those in the post-harvest environment.

These studies have used post-harvest produce purchased from grocery stores that is inoculated with bacterial cultures grown under optimal laboratory conditions. Therefore, to efficiently evaluate the true efficacy of chlorine or chlorine based sanitizers, it is important to take into account the influence of pre-harvest environmental factors on the pathogens' survival which could be a key factor in pathogens' survival.

Moreover, extensive research has been done on various aspects of pathogen contamination in the field, pathogen survival and persistence in soil, manure and on plants. Other areas of focus include

the ability of harvesting equipment and techniques to contribute to contamination, pathogen attachment mechanisms to plants and the efficacy of different sanitizers. Undoubtedly, these findings were important; but the mechanisms by which pathogens survive on produce needs more attention. As the physiological state of EHEC and *Salmonella* on pre-harvest produce is largely unknown, it is unclear what stress responses are activated and the extent to which they may be resistant to subsequent stresses.

Thus, the goal of this research was to determine if environmental factors affect the survival of EHEC and *Salmonella* on pre-harvest lettuce. This study used two seasonal conditions: June and March, mimicking the conditions present in Salinas Valley, California where most of the leafy greens are grown. We assessed the effect relative humidity and seasonal conditions in quantifying the survival of EHEC and *Salmonella* on lettuce. The second goal of this research was to determine if the exposure of EHEC and *Salmonella* to above environmental conditions will impact their ability to survive a chlorine decontamination wash. In addition, we also utilized transcriptional profiling to study which stress responses are activated or repressed by these pathogens in response to the pre-harvest variables. Understanding the survival of EHEC and *Salmonella* on pre-harvest produce will provide insights to develop effective post-harvest decontamination methods to reduce the exposure of consumers to these pathogens on produce.

CHAPTER 2

Introduction

In recent years, fresh fruits and vegetables have gained notoriety in association with enteric pathogens like Enterohemorrhagic *E. coli* and *Salmonella*. The number of outbreaks linked to produce consumption has increased in the past few decades [11]. During 1998-2008, produce accounted for more than a quarter of illnesses (46%) attributing leafy greens with most illness cases [36]. A variety of produce have served as vectors for enteric pathogens such as lettuce, spinach, melons, sprouts and tomatoes. Lettuce was regarded as the commodity responsible for causing the most outbreaks from 1973-1997.[11].

Since fresh produce is usually consumed raw and undergoes minimal processing, consumers are at a risk of being exposed to these pathogens. The increase in produce consumption and globalization has aided in the year round availability of fresh fruits and vegetables, thereby underlining the concerns on the safety of raw produce consumption. Human enteric pathogens like EHEC and *Salmonella* are among the top five foodborne pathogens responsible for high hospitalizations and outbreaks with fresh produce consumption have increasingly been liked to these pathogens. (Rangel et al 2005)[10].

A potential for contamination with enteric pathogen exists in the pre-harvest environment as well as during post-harvest handling. In pre-harvest field, produce contamination could occur through usage of contaminated irrigation water, application of un-composted manure, improper hygiene of workers, wildlife, run-off from nearby contaminated water sheds and insect vectors [23]. Trace back investigations of certain outbreaks have also documented the occurrence of contamination in the pre-harvest field, most notably the 2006 US EHEC O157:H7 outbreak. Once these pathogens are deposited on produce, they are capable of surviving and persisting in this non-host environment. Their extent of survival on pre-harvest produce could be significantly affected by several environmental factors like relative humidity, temperature and UV radiation. However, our knowledge about the effect of various pre-harvest variables on enteric pathogens on produce is still limited.

Various stresses are experienced by enteric pathogens on produce in the post-harvest processing and handling. One such stress is exhibited by pathogens during the decontamination wash. To minimize the risk of presence of pathogens on produce, the produce undergoes a decontamination wash. Chlorine in the form of calcium or sodium hypochlorite is the most commonly used sanitizer in the

fresh produce industry. Extensive studies have been performed on evaluating the efficacy of chlorine or chlorine based compounds for pathogen decontamination on produce [37, 38]. However, such studies utilized post-harvest produce inoculated with laboratory grown bacterial cultures, which is not indicative of stress experienced by pathogens in the pre-harvest environment. Since outbreaks of gastrointestinal illnesses have occurred after consumption of contaminated produce, it is evident that these pathogens are able to manage pre-harvest and post-harvest stresses, thereby entering the food supply. It is possible that the exposure to pre-harvest environmental factors could cross-protect these pathogens against the chlorine decontamination wash. It is therefore necessary to take into consideration the pre-harvest state of these pathogens for developing new decontamination methods.

Materials and methods

Bacterial isolates and growth conditions

A number of EHEC and *Salmonella* serotypes have been associated with produce outbreaks. Thus, two representative serotypes of each pathogen [15, 21] that have been linked to produce outbreaks were utilized in this study (Table 1). All isolates were stored at -80°C in Brain-Heart infusion (BHI) broth with glycerol. Each bacterial isolate was freshly streaked to Luria-Bertani (LB) agar from frozen stock and incubated for 24 h at 37°C. A single colony was transferred to 5 ml of LB followed by incubation at 37°C for 15 h. After 15 h, 100 µl LB culture was transferred to 100 ml LB broth with incubation at 37°C, and shaking at 215 rpm, for 15 h.

Lettuce cultivation conditions

Romaine lettuce seeds (*Lactuca sativa*) purchased from Living Whole Foods (Springville, UT) were seeded into sterile soil (Sungro Sunshine LC1 consisting of coarse perlite, dolomitic limestone, gypsum and Canadian sphagnum peat moss) in 4.5 inch plastic pots. Lettuce was grown in the North Dakota Agricultural Research Experiment Station greenhouse facility at 13°C-15°C during the night and 18°C-20°C during the day with a photoperiod of 14.5 hours. Plants were watered as needed.

Preparation of inoculum and lettuce inoculation

Following growth in LB for 15 h, cells were collected by centrifugation at 8000 rpm for 5 min (Avanti J-25 Centrifuge, Beckman Coulter). Supernatant was discarded and inoculum was prepared by

Table 1. List of isolates used in this study.

Isolate a	Pathogen	Serotype	Source	Year of Isolation
FSL R8-2543	*Salmonella*	Newport	Human sporadic	2008
FSL R8-4110	*Salmonella*	Newport	Bovine feces	2009
TW08264	EHEC	O157:H7	Japan sprouts outbreak (Sakai)	1996
TW014359	EHEC	O157:H7	US Spinach outbreak	2006
TW09184	EHEC	O26:H11	Human sporadic	2003
TW016501	EHEC	O26:H11	US Sprouts outbreak	2012
FSL P3-1552	*Salmonella*	Typhimurium	Soil	2012
FSL R6-0207	*Salmonella*	Typhimurium	Human sporadic	2006

[a]Isolates with 'FSL' are from the Food Safety Lab at Cornell University and isolates with 'TW' are from the STEC Center at Michigan State University

suspending the cell pellet in 50 ml Phosphate Buffered Saline (PBS) for a final concentration of approx. 10^9 cells/ml. After 28-35 days of growth, 8 pots of lettuce were inoculated with each isolate via spray inoculation in a biosafety cabinet. A hand-held TLC sprayer (model 422530-0050, Kontes Glass Company, Vineland, N.J) was used to deliver inoculum by spraying for 5 s (approx. 1 ml) onto the lettuce leaves of each pot (Lang M et al, 2004). The carrier gas was nitrogen at approximately 10 Psi. Inoculated plants were placed in a plastic tray filled with 2 cm water and kept in a greenhouse growth chamber (Conviron PGW40, Winnipeg, Manitoba, Canada).

Incubation conditions for inoculated lettuce

To quantify the impact of humidity and harvest season on pathogen survival, the inoculated lettuce plants were incubated under two levels of relative humidity (RH) (45% and 75%). Lettuce were grown under two harvest seasons mimicking the season in Salinas valley, California: June (14.8 h photoperiod, max temp 20°C, min temp 12.2°C) and March (12 h photoperiod, max temp 17. 2°C, min temp 6.7°C); for a total of 4 different environmental conditions (March 75% RH, March 45% RH, June 75% RH, June 45% RH). Climate data for these seasons was obtained from the Salinas Municipal Airport weather station for 2009-2011 from the National Climatic Data Center (http://www.ncdc.noaa.gov/). For

each environmental condition, 2 biological replicates of each strain were tested with 2 technical replicates per biological replicate. The inoculated lettuce plants were harvested on the day of inoculation as well as 1, 3 and 5 post-inoculation.

Harvest of lettuce leaves

For each biological replicate of each strain, plants from 2 lettuce pots were collected on each day of harvest. Each lettuce pot represents a technical replicate. One set of technical replicate was collected immediately after inoculation (day 0) while the rest were collected on days 1, 3 and 5 post-inoculation. Sterile scissors and tweezers were used to cut lettuce leaves approx. one inch above the soil.

Incubation and harvest of lettuce for chlorine survival assay

Lettuce was also inoculated to determine if pre-harvest environmental stresses affect chlorine resistance in these pathogens. For this experiment, the inoculated lettuce plants were incubated at 75% RH under June harvest conditions (14.8 h light, max temp 20°C, min temp 12.2°C). The experiment was replicated twice with two technical replicates per biological replicate for each strain. For each isolate, 2 technical replicates containing lettuce leaves from 2 pots were harvested on days 1 and 3 post-inoculation for each strain.

Preparation of chlorinated water

Chlorinated water (50 ppm) was prepared by adding 1 ml of XY-12 (sodium hypochlorite, Ecolab 42016) in 1.6 L of sterile distilled water. The concentration of chlorine was determined by a chlorine testing kit (Ecolab). A 0.5M Sodium thiosulphate solution was used to neutralize the chlorine solution. Both the solutions were pre-chilled at 5°C.

Chlorine survival assay

Leaves from two pots of lettuce were mixed in one sterile Whirl-pak bag using sterile tweezers. The lettuce was then weighed and approximately divided into half. To one bag, 500 ml of sterile water was added while 500 ml of chlorine solution was added to the other bag. The bags were closed and gently swirled in a circular motion for 2 minutes. To the bags with chlorine solution, 13 drops of the neutralizing solution was added and bag was gently shaken for 20 seconds. The leaves were transferred to new bags and diluted 1:10 with PBS.

Bacterial enumeration

Cut lettuce leaves were placed in sterile plastic bags, weighed and diluted 1:10 with PBS. Bags were homogenized in a laboratory homogenizer (IUL Instruments masticator, S.A) for 90 s. Cells were quantified by serially diluting the samples and plating in duplicate on MacConkey agar for *E.coli* and XLD agar for *Salmonella* using a Spiral plater, Autoplate Model 4000. Plates were incubated for 24 h at 37°C and colonies were counted using the Q count (Model 530, Spiral Biotech, M.A).

Statistical Analysis

The experimental design consisted of 4 strains each of two pathogens (EHEC and *Salmonella*) spray inoculated on greenhouse cultivated lettuce. Each experiment was replicated twice and each replicate consisted of two technical replicates of inoculated lettuce. Microbial data (CFU/ml) were divided by individual lettuce weights and log transformed (log CFU/g of lettuce) before statistical analysis. Mean and standard deviations were obtained from log cfu/g of lettuce for each harvest day. Statistically significant differences in survival were identified with the General Linear model (GLM) procedure of the Statistical Analysis System (SAS v.9.3). The Tukey's test was used to adjust for multiple comparisons. An adjusted p-value <0.05 was considered significant.

The model produced equation as follows:

Differences is survival over 5 days= μ + harvest season + biological replicate + technical replicate + error

For chlorine survival assay, the average log CFU/g of lettuce recovered from chlorine wash was subtracted from that recovered after water wash. This difference in survival was used in statistical analysis to identify which strains exhibited enhanced resistance to chlorine with the day of harvest as time factor. Tukey's test was used for comparisons and an adjusted p-value<0.05 was considered significant.

Results

We quantified the survival of 4 strains each of EHEC and *Salmonella* on lettuce on days 0, 1, 3 and 5 post-inoculation to determine the effect of two seasonal (June and March) and RH conditions (45% and 75%)(Fig.1 and 2). All EHEC and *Salmonella* strains showed decrease in cell number over 5 days regardless of season or RH. The average decrease in log CFU/g of lettuce recovered on the day of inoculation under all seasonal conditions is presented in Table 2. Due to the differences in log CFU/g

recovered from lettuce on the day of inoculation, log decrease in CFU/g of lettuce was used for all comparisons.

Effect of relative humidity on EHEC survival is dependent on the seasonal conditions

RH had a significant effect on the survival of EHEC strains which was dependent on the seasonal conditions ($p<0.0001$) (Fig. 1). Under June seasonal conditions, higher RH led to significantly lower survival of EHEC, while lower RH led to significantly greater survival. One day post-inoculation, an average decrease of 1.8 ± 0.5 log CFU/g of lettuce was observed under 75% RH (Fig. 1a) whereas an average decrease of 0.9 ± 0.4 log CFU/g of lettuce was observed under 45% RH (Fig. 1b). Three days post-inoculation, an average decrease of 3.1 ± 0.9 log CFU/g of lettuce was observed under 75% RH whereas an average decrease of 1.5 ± 0.8 log CFU/g of lettuce was observed under 45% RH. Five days post-inoculation, an average decrease of 3.6 ± 0.8 log CFU/g of lettuce was observed under 75% RH and an average decrease of 2.3 ± 0.8 log CFU/g was observed under 45% RH in the survival of EHEC.

For the March seasonal conditions, one day post-inoculation, average decrease of 1log ± 0.6 CFU/g of lettuce under 75% RH (Fig. 1c) and an average decrease of 1.4 ± 0.7 log CFU/g of lettuce under 45% RH (Fig. 1d) was observed. The total impact of RH on EHEC survival was also minimal after one day of inoculation. Three days post-inoculation, an average decrease of 1.6 ± 0.8 log CFU/g lettuce was observed under 75% RH. Under March 45% RH, three days post-inoculation, an average decrease of 1.8 ± 0.5 log CFU/g of lettuce was observed. Five days post-inoculation, for 75% RH, an average decrease of 2.2 ± 0.7 log CFU/g of lettuce was observed whereas an average decrease of 2.0 ±0.5 log CFU/g of lettuce was observed under 45% RH.

Table 2. Average cell density recovered from inoculated lettuce on the day of inoculation in log CFU/g under June and March seasonal conditions.

Pathogen	Season	Relative humidity	Log CFU/g of lettuce
EHEC	June	75%	8.3 ± 0.3
EHEC	June	45%	7.0 ± 0.3
EHEC	March	75%	7.0 ± 0.3
EHEC	March	45%	7.0 ± 0.4
Salmonella	June	75%	8.3 ± 0.4
Salmonella	June	45%	7.0 ± 0.3
Salmonella	March	75%	7.7 ± 0.3
Salmonella	March	45%	7.6 ± 0.3

Serotype differences among EHEC survival under June 75% relative humidity

Significant differences in survival ($p<0.0001$) were observed between EHEC serotypes (O157 and O26), three days post-inoculation under June 75% RH (Fig. 1a). Strains of serotype O26 had significantly higher survival with an average decrease of 2.3 ± 0.4 log CFU/g of lettuce as compared to strains of serotype O157 with an average decrease of 3.9 ± 0.6 log CFU/g of lettuce, three days post-inoculation. No significant differences in survival among EHEC serotypes were observed one day post-inoculation ($p=0.076$) or five days post-inoculation ($p=0.13$) under June 75% RH. Differences in survival among EHEC serotypes were insignificant under other seasonal and RH conditions.

Strain differences among EHEC survival on lettuce

Five days post-inoculation, the EHEC O157 spinach outbreak strain TW014359 had a significantly lower survival ($p=0.0009$) among all strains tested under March 45% RH, with an average decrease of 2.7 ± 0.2 log CFU/g of lettuce. The EHEC spinach outbreak strain also had significantly lower survival as compared to other EHEC strains under June 45% RH with an average decrease of 3.22 ± 0.8 log CFU/g of lettuce, five days post-inoculation ($p=0.006$).

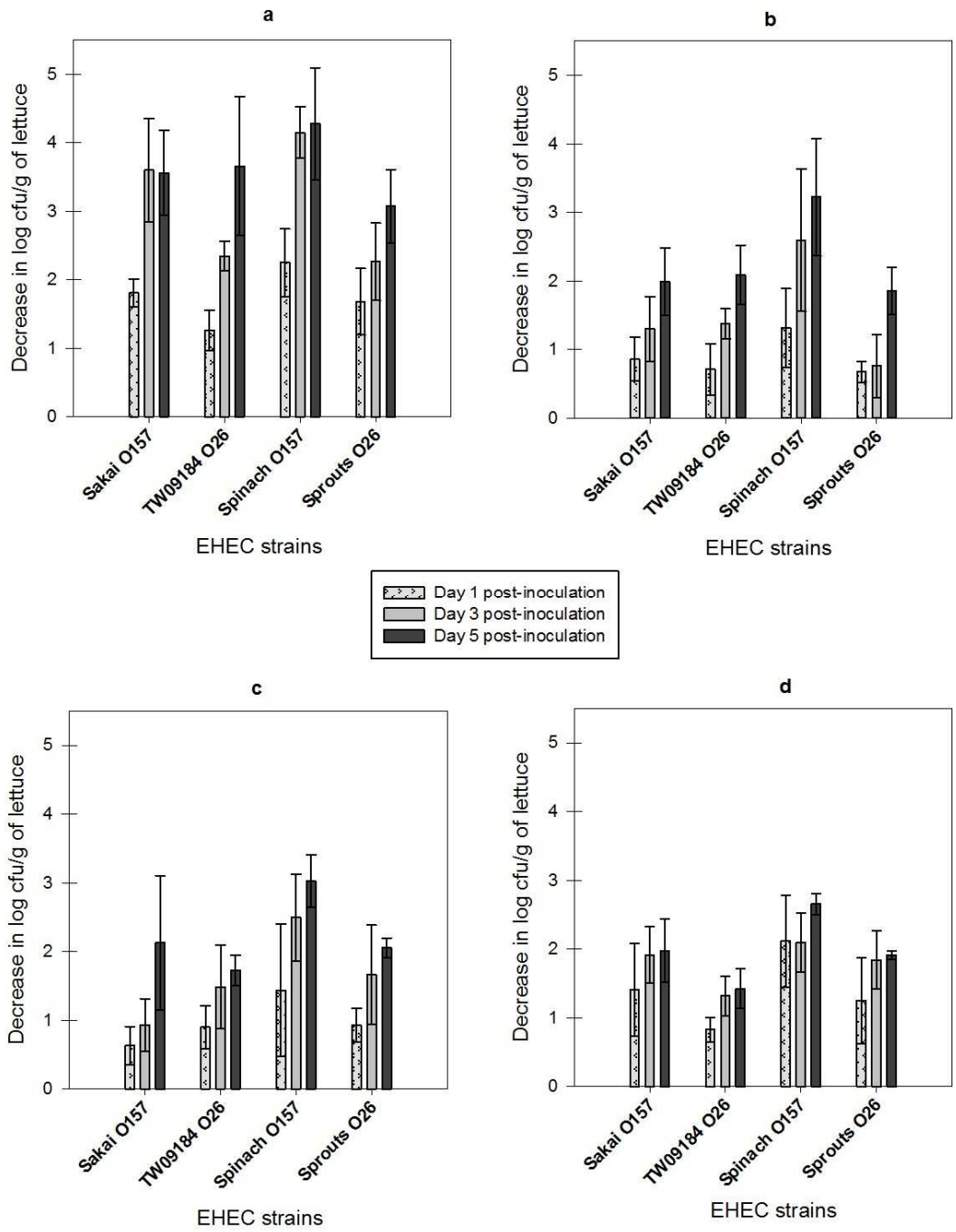

Fig. 1. Bar graphs representing log decrease in survival of EHEC on lettuce over 5 days under June 75% RH (a), June 45% RH (b), March 75% RH (c) and March 45% RH (d). Bars represent the average and standard deviation from two independent replicates and two technical replicates for each strain.

Differences in *Salmonella* survival dependent on relative humidity and seasonal conditions occurred only five days post-inoculation

Differences in *Salmonella* were not observed under either seasonal conditions one day post-inoculation ($p=0.55$) as well as three days post-inoculation ($p=0.09$) on lettuce. One day post-inoculation, an average decrease of 0.8 ± 0.3 log CFU/g of lettuce under June 75% RH (Fig. 2a), and an average decrease of 0.7 ± 0.3 log CFU/g lettuce under June 45% RH (Fig. 2b) was observed. One day post-inoculation, for March seasonal conditions, both 75% and 45% RH (Fig. 2c and 2d) led to an average decrease of 0.8 ± 0.3 log CFU/g of lettuce. Differences in survival of *Salmonella* was similar over three days post-inoculation, with an average decrease of 1.4 ± 0.5 log CFU/g of lettuce in June 75% RH and 1 ± 0.3 log CFU/g of lettuce in June 45% RH. Three days post-inoculation, under March seasonal conditions, an average decrease of 1.3 ± 0.6 log and 1.2 ± 0.3 log CFU/g of lettuce was observed for 75% RH and 45% RH, respectively.

Significant differences in *Salmonella* survival occurred on lettuce five days post-inoculation ($p=0.0045$), where the effect of RH was dependent on the season conditions. For the June season conditions, higher RH led to lower survival and lower RH led to greater survival of *Salmonella*, five days post-inoculation. Five days post-inoculation, an average decrease of 2.1 ± 0.6 log CFU/g of lettuce was observed under 75% RH whereas under 45% RH, an average decrease of 1.5 ± 0.3 log CFU/g of lettuce was observed. RH had no effect on the survival of *Salmonella* under March season conditions. The average log decrease in CFU/g of lettuce after five days was 1.68 ± 0.6 for March 75% RH and 1.62 ± 0.3 for March 45% RH.

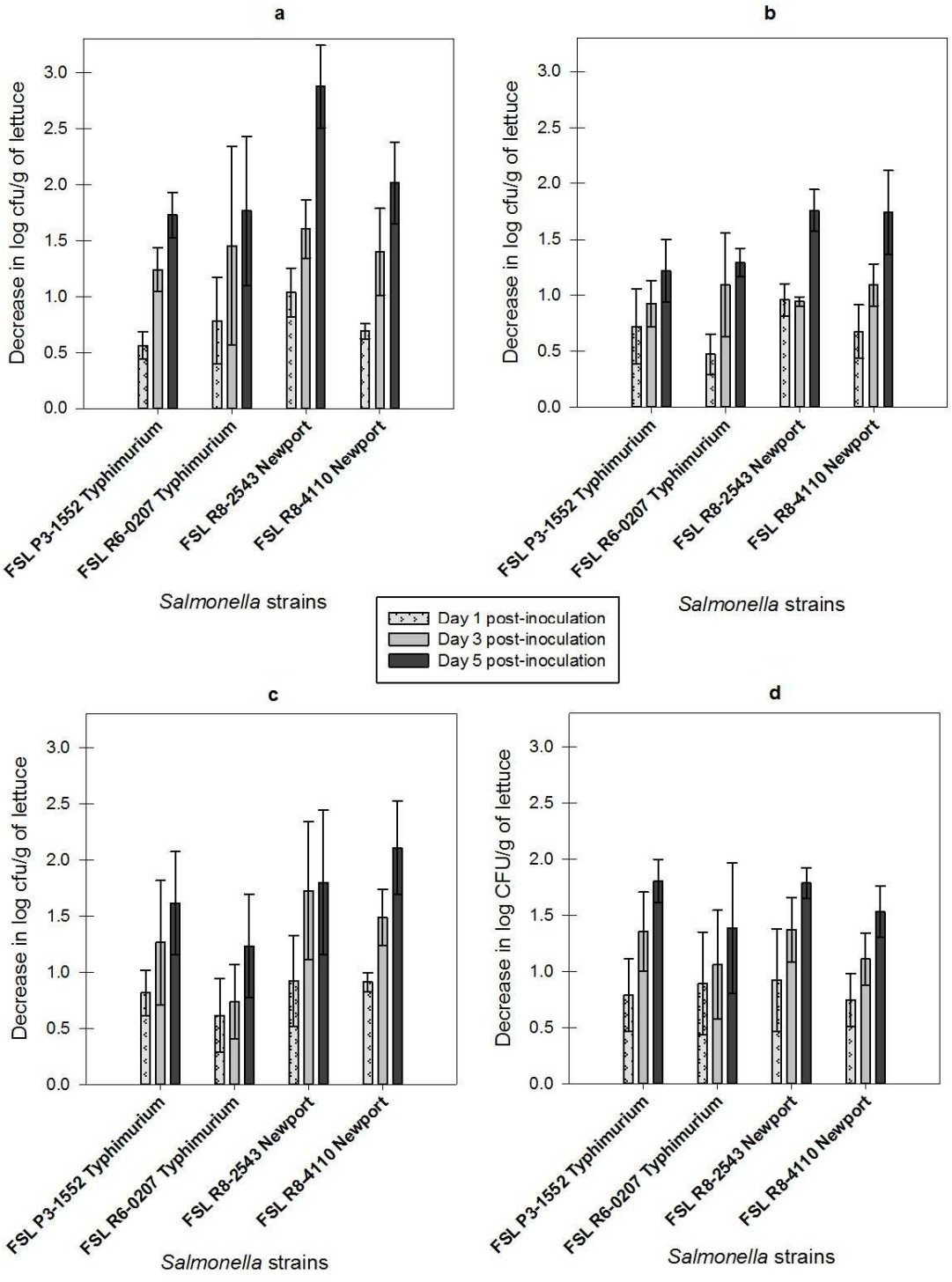

Fig. 2. Bar graphs representing log decrease in survival of *Salmonella* on lettuce over 5 days under June 75% RH (a), June 45% RH (b), March 75% RH (c) and March 45% RH (d). Bars represent the average and standard deviation from two independent replicates and two technical replicates for each strain.

Serotype differences were not observed among *Salmonella* under either seasonal or relative humidity conditions

Significant differences between *Salmonella* serotypes were not seen over the five days under any seasonal or RH conditions. However, strain difference in survival was observed for *Salmonella* FSL R8-2543 which had a significant lower survival from other *Salmonella* strains, five days post-inoculation ($p<0.0001$) under June 75% RH. An average decrease of 2.9 ± 0.4 log CFU/g of lettuce was obtained, five days post-inoculation.

EHEC O157:H7 spinach outbreak strain demonstrated enhanced resistance to chlorine

Each strain was tested for the ability to survive a decontamination wash with chlorine (sodium hypochlorite) after being exposed to June 75% RH for days 1, 3 and 5 post-inoculation. Inoculated lettuce was washed with chlorine solution and sterile water (control) for comparison for a time period of 2 minutes. The log CFU/g lettuce obtained from lettuce washed with chlorine was compared to that recovered from water wash and displayed as the difference in recovery between water wash and chlorine wash. One day post-inoculation, an average difference of 1 ± 0.3 log CFU/g of lettuce was obtained after chlorine wash for all EHEC inoculated on lettuce (Fig. 3). Differences in chlorine survival among strains were observed for three and five days post-inoculation. The EHEC O157 spinach outbreak strain demonstrated enhanced survival to chlorine wash resulting in an average difference of 0.4 ± 0.07 log CFU/g lettuce, on day three post-inoculation, which was not significantly different from the average difference of 0.4 ± 0.2 log CFU/g lettuce on day five post-inoculation ($p>0.05$). This indicates that longer incubation on lettuce led to greater survival after chlorine wash for EHEC O157 spinach outbreak strain.

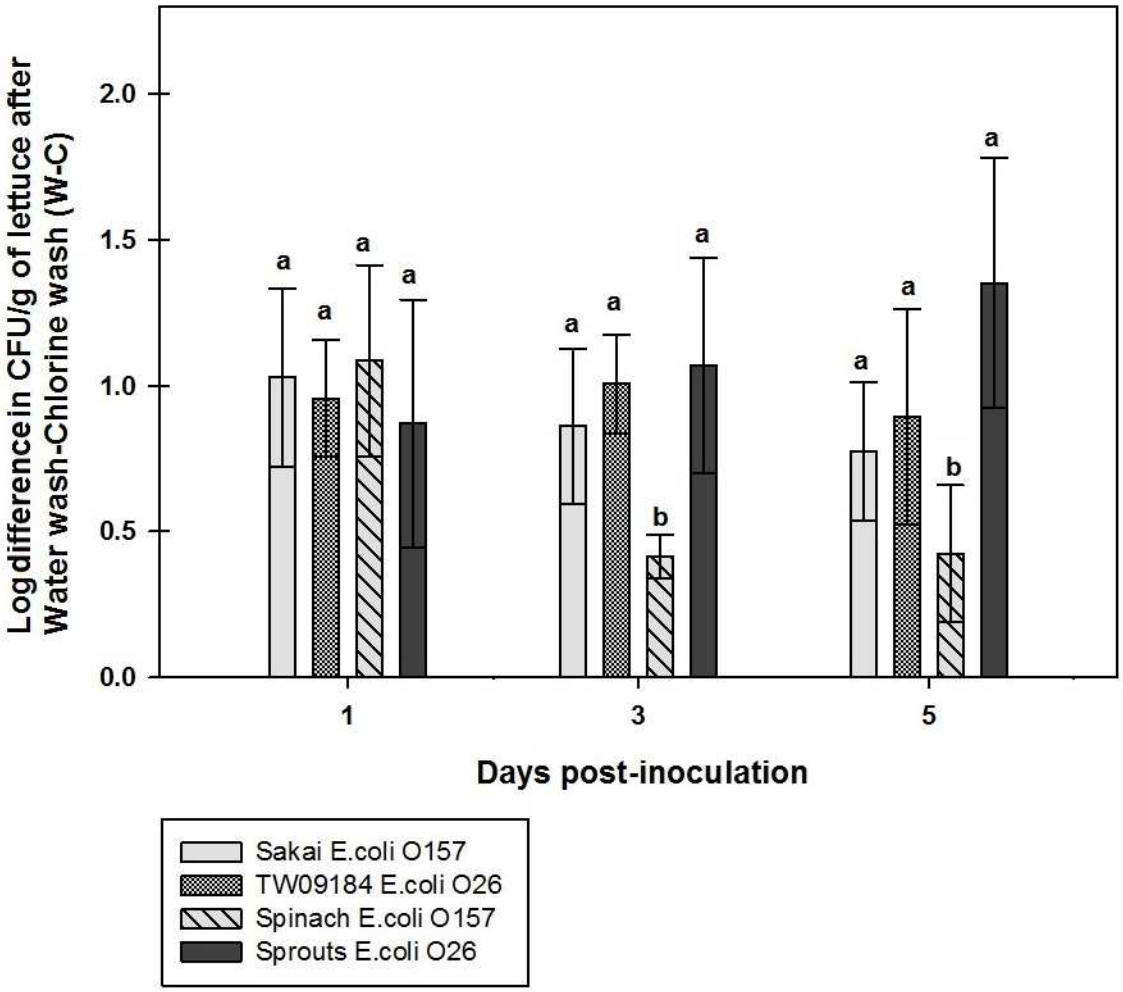

Fig. 3. Bar graphs representing difference in EHEC survival after chlorine wash over 5 days under June 75% RH. Bars represent the average difference in log CFU/g of lettuce recovered from water wash – chlorine wash and standard deviation from two independent replicates and two technical replicates for each strain.

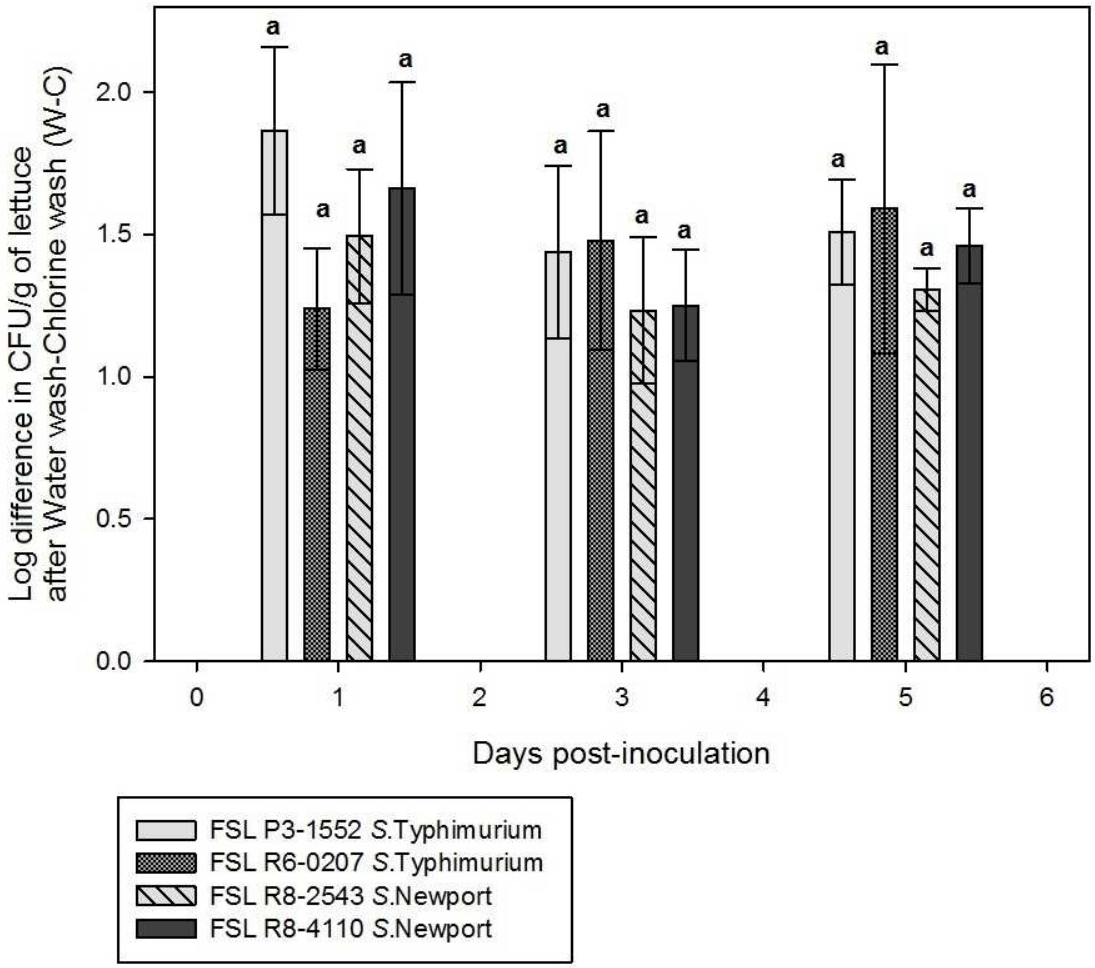

Fig. 4. Bar graphs representing difference in survival of *Salmonella* after chlorine wash over 5 days under June 75%. Bars represent the average difference in log CFU/g of lettuce recovered from water wash – chlorine wash and standard deviation from two independent replicates and two technical replicates for each strain.

Significant differences in survival were not observed for *Salmonella* after chlorine wash

For *Salmonella*, differences in survival were not observed after chlorine wash on any days of harvest post-inoculation (Fig. 4). An average difference of 1.6 ± 0.3 log CFU/g lettuce was observed for all *Salmonella* after chlorine wash, one day post-inoculation which was not significantly different from survival on days three and five post-inoculation. An average difference of 1.4 ± 0.3 log CFU/g lettuce and 1.5 ± 0.3 log CFU/g lettuce was observed after chlorine wash on days three and five post-inoculation, respectively.

Discussion

Survival of EHEC and *Salmonella* is influenced by pre-harvest environmental conditions

Our study demonstrated that pre-harvest environmental factors have an impact on the survival of enteric pathogens on produce. We quantified the survival of EHEC and *Salmonella* under two RH and seasonal conditions over a five day period. We observed that the effect of RH on the survival of these pathogens was dependent on the seasonal conditions. RH significantly impacted EHEC survival under June humidity conditions. Higher RH led to lower survival and lower RH led to significantly greater survival of EHEC on lettuce over five days. However, the effect of RH was minimal on EHEC survival under March seasonal conditions. Similar results were seen for the survival of *Salmonella* on lettuce, where the effect of RH was again dependent on the seasonal conditions. However, RH had a significant impact on *Salmonella* survival only five days post-inoculation. Higher RH led to lower survival and lower RH led to greater survival of *Salmonella*, five days post-inoculation. RH had no effect on the survival of *Salmonella* under March seasonal conditions.

All *Salmonella* and EHEC were recovered from inoculated lettuce for up-to five days post-inoculation indicating the long-term survival of these pathogens under the pre-harvest environmental factors. The survival of enteric pathogens on pre-harvest produce has been well documented. Erickson et al. (2010) were able to recover EHEC O157 from field-inoculated lettuce after 27 days via spray-inoculation with contaminated water [26]. Islam et al. (2004) were able to recover *Salmonella* Typhimurium, 63 days post-inoculation from field-inoculated lettuce through application of contaminated compost [27]. The survival of EHEC O157 has also been assessed on greenhouse cultivated produce. EHEC O157 strain, mixed with avian pathogenic *E.coli* (which causes extra-intestinal infections in humans), was spot inoculated on spinach and lettuce plants. Both pathogens were detectable on plants, 10 days post-inoculation [28]. In yet another study, a cocktail of EHEC O157, *Salmonella enterica* and *Clostridium perfringens* was spot inoculated on hydroponically growing bell peppers, lettuce and cantaloupes in the greenhouse. All three pathogens were recovered from the produce types,14 days post-inoculation [29]. Thus, it is clear that pathogens are able to manage pre-harvest environmental stresses and persist on produce for variable time periods.

The effect of certain per-harvest environmental factors on the survival of enteric pathogens has been assessed in a few studies. Stine et al. (2005) showed that relative humidity played a significant role on the pre-harvest survival of *Salmonella enterica* and EHEC O157 on greenhouse grown lettuce and cantaloupes [29]. *Salmonella enterica* was found to survive better under high humidity as compared to low humidity. Survival of EHEC was better under low humidity as compared to high humidity which is in agreement with the results of our study for EHEC survival. However, seasonal conditions also contributed to the overall survival of this pathogen in our study. In another research, the survival of *Salmonella* Montevideo was studied on the surface of tomato leaf as well the fruit [39]. The pathogen was shown to survive better under high RH on both tomato leaf and the fruit, in contrast to our results where *Salmonella* survival was low under high RH in June season. Although it should be noted that the study utilized environmental factors: relative humidity, temperature, photoperiod, *Salmonella* serotype and the method of inoculation, different than the ones used in our study.

The effect of light exposure on pathogens' survival on plant surfaces has also been studied [26, 29]. Stine et al. (2010) used a sodium light source in the environmental chamber to evaluate the effect of light on the survival of pathogens. They observed that pathogens without direct exposure to light and the ones that were kept in shade survived longer than pathogens with full light exposure. Therefore, the higher decline in pathogen cell number under June seasonal conditions in our study could likely be the result of difference in photoperiod from March seasonal conditions. UV light has been shown to affect pathogen survival on produce and other surfaces [40, 41]. Erickson et al. (2010) revealed that EHEC O157 sprayed on the lower side of the lettuce leaf resulted in greater survival of the pathogen than those sprayed on the upper side of leaves of field grown lettuce.

Another important result of our study was the existence of serotype specific differences in survival of EHEC and *Salmonella* strains. Since multiple strains of pathogens have the potential to contaminate produce in the field and have been implicated in many produce outbreaks, it is important to study their survival on produce. In this study, strains of serotype O26 survived better than strains of serotype O157 under June 75% RH, three days post-inoculation. Serotype differences in *Salmonella* have been investigated in their ability to attach to and colonize plant surfaces. Differences in attachment abilities of 5 *Salmonella* serotypes were tested to lettuce and cabbage leaves [42]. The results indicated that

Salmonella Tennessee produced the strongest biofilms in tryptic soy broth and showed attachment to lettuce in higher numbers than other serotypes (Newport, Negev, Thompson, Braenderup). Klerks et al. (2007) also found a differential interaction of *Salmonella* serotypes with lettuce. Among 5 serotypes tested, the serotype Dublin was found to endophytically colonize the lettuce leaves in comparison to other serotypes Newport, Typhimurium, Montevideo and Enteritidis. Taken together these results indicate that the survival and colonization abilities could be different among serotypes of a pathogen.

Differences were also observed in our study in the surviving capability of pathogens against chlorine decontamination. Chlorine is the most widely used sanitizer for decontamination of fresh produce. Various researchers have evaluated the efficacy of chlorine for produce wash [33, 34]. EHEC O157 spinach outbreak strain showed enhanced survival to chlorine stress after three and five days post-inoculation on lettuce. Significant differences in surviving capabilities were not observed for other strains of EHEC or *Salmonella*. The higher resistance of EHEC spinach outbreak strain shows that pre-harvest environmental induced certain stress responses in this strain, providing cross-protection against chlorine stress. Since chlorine is the most common sanitizer used in the fresh produce industry, the enhanced survival of enteric pathogens to chlorine is a significant concern. Cross-protection has been demonstrated in EHEC and *Salmonella* in various environmental conditions. Kyle et al. (2010) exposed EHEC O157 to lettuce leaf lysate and observed induction of genes involved in oxidative stress. Further testing showed that exposing the pathogen to lysate led to increased resistance to chlorinated sanitizers [31].

In conclusion, pre-harvest environmental factors have the potential to influence the survival of enteric pathogens on produce. Exposure to environmental variables in the field can induce resistance against subsequent stresses such as chlorine wash during post-harvest decontamination. Such enhanced resistance could lead to entry of these pathogens into the food chain, which is of great concern if pathogens have a low infectious dose. Understanding the survival of these pathogens in association with pre-harvest produce could help in development of effective and novel post-harvest decontamination treatments.

CHAPTER 3

Introduction

Contamination of fresh produce with human enteric pathogens can occur in the pre-harvest environment and certain outbreaks of contaminated fresh produce consumption have been traced back to the pre-harvest environment [18]. Research studies have provided evidence that once contamination occurs, these pathogens are capable of surviving on produce for long periods of time [26, 27, 43]. However, our knowledge about the physiological state of enteric pathogens on produce and the stress response mechanisms induced in these pathogens under pre-harvest environmental variables is largely unknown.

The physiological state of a bacterium influences its ability to adapt to and survive different stresses. Various environmental factors such as temperature, humidity, UV exposure and plant microbiota impose physical, chemical or biological stress on pathogens on produce [26, 29, 30]. Pathogens are able to manage these stresses and enter the food supply. One way pathogens can adapt to new environmental situation is through alterations in gene expression. Modulation in gene expression in response to a stress indicates the activation or repression of specific physiological response. The physiological state and the stress resistance capabilities of bacteria can be assessed by identifying genome wide changes in gene expression [44]. Whole-genome changes assessed through transcriptional profiling can provide an understanding of bacterial factors that may contribute to their survival on pre-harvest produce.

Enteric pathogens like EHEC and *Salmonella* are known to induce stress responses when exposed to environmental stresses. For example, *S*. Typhimurium and *S*. Enteritidis exposed to chlorine increased expression of genes involved in biofilm formation and genes encoding chaperone proteins [45]. EHEC O157 subjected to low temperature induced the expression of genes involved in acid resistance and, induced the expression of genes involved in oxidative stress in response to low pH [46]. Research evidence suggests that activation of such responses can increase the pathogen's ability to survive subsequent stresses. For example, Kyle et al. (2010) exposed EHEC O157 to lettuce lysate and found up-regulation of genes involved in oxidative stress response. When exposed to chlorine based sanitizers like calcium hypochlorite and hydrogen peroxide, the pathogen demonstrated enhanced ability in

surviving under these sanitizers. In another study, *S*. Typhimurium was exposed to mild acidic conditions (pH 5.8) and these acid-adapted cells developed thermo-tolerance when subjected to a temperature of 50°C. Moreover, these cells also developed enhanced tolerance towards other environmental stresses such as salt, hydrophobic dye crystal violet and polymyxin B [47]. Thus, elucidation of the physiological state of a pathogen can provide insights into which stress responses may play a role in survival under environmental stresses. In chapter 2, we studied the effect of pre-harvest environmental factors on the surviving capability of EHEC and *Salmonella* against a chlorine wash on lettuce. We found that EHEC O157 spinach strain exhibited enhanced survival against chlorine wash on days three and five post-inoculation. Transcriptional profiling could help us elucidate which stress response mechanisms this pathogen could be utilizing to better survive against oxidative stress posed by chlorine wash.

The physiological state of these pathogens on pre-harvest produce is not yet deciphered. It is unclear which stress response mechanisms are up-regulated and the extent to which pathogens may be resistant to subsequent stresses. Genomic based efforts have largely focused on identifying genetic responses of foodborne pathogens inoculated onto post-harvest produce [31, 32, 48]. Transcriptional profiling also has tremendous potential to improve our ability to understand the physiological state of pathogens on pre-harvest produce.

Whole-genome transcriptomics data can help determine factors that may contribute to the survival of enteric pathogens on pre-harvest produce. For example, *E.coli* and *Salmonella* have been shown to utilize adherence factors such as curli, fimbriae and exopolysaccharide to persist on the surface of leafy greens [49-51]. Transcriptome studies of EHEC O157 inoculated on post-harvest produce indicated the up-regulation of genes involved in curli fimbriae and expression of these genes contributed in attachment of EHEC O157 to the leaf surface [32]. Transcriptional profiling utilized in our study will help determine which environmental factors may influence the expression of adherence factors as well as other unknown factors that may be involved in survival on the lettuce leaf surface.

Since EHEC and *Salmonella* have been transmitted to humans via produce, it is clear that these pathogens are able to survive pre-harvest environmental stresses as well as stresses during post-harvest processing. Understanding the physiological state will expand our knowledge about how pathogens survive on produce which is important in developing effective mitigation strategies. Thus, the goal of this

project was to utilize transcriptional profiling to identify the physiological state of EHEC and *Salmonella* associated with lettuce plants under pre-harvest environmental conditions. The results obtained in this work could provide answers to the observation in the study described in chapter 2, that EHEC O157 spinach strain showed increased resistance to chlorine wash after incubation on pre-harvest lettuce. This work will help us to understand which genes are activated in response to pre-harvest environmental factors that could potentially lead to pathogen survival under chlorine decontamination treatment. Effective post-harvest decontamination methods could then be developed to reduce consumer exposure to these pathogens on produce.

Material and methods

Strains and growth conditions

All isolates (Table 3) were stored at -80°C in Brain-Heart infusion (BHI) broth with glycerol. Each bacterial isolate was freshly streaked to Luria-Bertani (LB) agar from frozen stock and incubated for 24 h at 37°C. A single colony was transferred to 5 ml of LB followed by incubation at 37°C for 15 h. After 15 h, 100 µl LB culture was transferred to 100 ml LB broth with incubation at 37°C, and shaking at 215 rpm, for 15 h.

Lettuce cultivation conditions

Romaine lettuce seeds (*Lactuca sativa*) purchased from Living Whole Foods (Springville, UT) were seeded into sterile soil (Sungro Sunshine LC1 consisting of coarse perlite, dolomitic limestone, gypsum and Canadian sphagnum peat moss) in 4.5 inch plastic pots. Lettuce was grown in the North Dakota Agricultural Research Experiment Station greenhouse facility at 13°C-15°C during the night and 18°C-20°C during the day with a photoperiod of 14.5 h. Plants were watered as needed.

Preparation of inoculum and lettuce inoculation

Following growth in LB for 15 h (stationary-phase), cells were collected by centrifugation at 8000 rpm for 5 min (Avanti J-25 Centrifuge, Beckman Coulter). Supernatant was discarded and inoculum was prepared by suspending the cell pellet in 50 ml Phosphate Buffered Saline (PBS) for a final concentration of approx. 10^9 cells/ml. After 28-35 days of lettuce growth, 12 pots were inoculated with each isolate via spray inoculation in a biosafety cabinet. A hand-held TLC sprayer (model 422530-0050, Kontes Glass

Company, Vineland, N.J) was used to deliver inoculum by spraying for 5 s (approx. 1 ml) onto the lettuce leaves of each pot (Lang M et al, 2004). The carrier gas was nitrogen at approximately 10 Psi. Inoculated plants were placed in a plastic tray filled with 2 cm water and kept in a greenhouse growth chamber (Conviron PGW40, Winnipeg, Manitoba, Canada).

Table 3. List of isolates used in this study.

Isolate a	Pathogen	Serotype	Source	Year of Isolation
FSL R8-2543	*Salmonella*	Newport	Human sporadic	2008
TW014359	EHEC	O157:H7	US Spinach outbreak	2006
TW016501	EHEC	O26:H11	US Sprouts outbreak	2012
FSL P3-1552	*Salmonella*	Typhimurium	Soil	2010

[a]Isolates with 'FSL' are from the Food Safety Lab at Cornell University and isolates with 'TW' are from the STEC Center at Michigan State University

Incubation conditions for inoculated lettuce

Inoculated lettuce plants were incubated under June season (14.8 h photoperiod, max temp 20°C, min temp 12.2°C) with 75% RH. The inoculated lettuce plants were harvested on days 1, 3 and 5 post-inoculation for cell collection and RNA extraction. For each strain, 2 biological replicates were inoculated for each day of RNA extraction.

RNA extraction

For each biological replicate of each strain, plants from 4 lettuce pots were collected in a sterile filter stomacher bag on each day of harvest using sterile scissors and tweezers. To each bag, 500 ml of physiological saline and 50 ml of ice-cold freshly prepared stop solution (10% acid phenol in ethanol) was added. Bag was sealed and kept on a rotator for 15 minutes at 200 rpm at 4°C. Homogenate was collected into a 250 ml centrifuge bottle. To pellet cells, homogenate was immediately centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was discarded, and cell pellet was suspended in 2 ml lysis buffer (20mM EDTA, 200mM sodium chloride) and transferred to a bead-beating tube containing ~1

cc acid washed 0.1 mm zirconium beads. For cell lysis, 3 ml acid phenol, 0.1 ml 20% Sodium dodecyl sulphate (SDS) and 100 mg polyvinylpolypyrrolidone (PVPP) were added and samples homogenized in a bead-beater (Biospec) for 3 minutes. Supernatant was collected and hot acid phenol-chloroform was immediately added and tube was held at 65°C for 1 h with periodic shaking. The supernatant was extracted with acid phenol-chloroform-isoamyl alcohol (125:24:1). RNA was precipitated in 2.5 volume of 100% ethanol, 1/10 volume 3M sodium acetate, pH 5.2 and 1/100 volume glycogen overnight at -80°C. RNA sample was treated with DNase using DNase (Promega), RQ1 Buffer (Promega) and 0.1M DTT (Thermoscientific) to remove genomic DNA. Extracted RNA was quantified using spectrophotometer (ND-1000) and the quality and integrity was analyzed using an Agilent 2100 bioanalyzer (Agilent Technologies, CA).

Library preparation

The rRNA was depleted using Ribo-Zero rRNA removal kit (Epicentre) and cDNA was synthesized using ScriptSeq complete kit (Epicentre) following the manufacturer's instructions. cDNA libraries were barcoded using the Epicentre indexing primers to allow for multiple samples to be run in the same sequencing lane. Sequencing was performed on Illumina Hiseq at the Biotechnology Resource Center at Cornell University. Each flow cell consisted of 12 samples per lane to obtain 100 bp single-end sequencing reads.

Genome Sequencing

The complete genome of EHEC O157:H7 TW014359 is available (Genbank #CP0013568.1). Genomic DNA from strains EHEC O26:H11 TW015601, *Salmonella* Typhimurium FSL P3-1552 and *Salmonella* Newport FSL R8-2543 were extracted with phenol:chloroform and DNA prepared for sequencing with the TruSeq kit (Illumina) following manufacturer's instructions. Libraries were sequenced on a MiSeq with 250 bp paired end reads at the Cornell University Biotechnology Resource Center. Draft genomes of EHEC O26:H11 TW015601, *Salmonella* Typhimurium FSL P3-1552 and *Salmonella* Newport FSL R8-2543 were assembled de novo using Velvet with a k-mer length of 91 [52]. Contigs for each genome were aligned to a completed reference genome using MAUVE [53]. Draft genomes were submitted to RAST [54] for annotation.

Identification of differentially expressed genes

Reads obtained from RNA sequencing for each sample were mapped to their respective reference genomes using BWA-MEM [55]. Read per gene were obtained from the number of reads mapped to a respective gene. Number of reads per gene for each sample were used to determine significant differential expression with BaySeq. For each bacterial strain, differentially expressed genes on lettuce plants over 1, 3, and 5 days post-inoculation were identified using Bayseq [56] implemented in R v.2.14.1. This approach is based on empirical Bayesian methods for identification of patterns of differential expression in RNA-seq count data based on a negative binomial distribution. Transcript counts for each gene on each day (1, 3, and 5) were determined to fit one of the following models: day 1 = day 3 = day 5 (not differentially expressed), day 1 ≠ day 3 = day 5 (differentially expressed on day 1 only), day 3 ≠ day 1 = day 5 (differentially expressed on day 3 only), day 1 = day 3 ≠ day 5 (differentially expressed on day 5 only), or day 1 ≠ day 3 ≠ day 5 (differentially expressed on all days). Genes with an FDR < 0.05 and fold change < 0.5 or > 2 were determined to be significant for a particular model. For a given gene, the fold change is calculated as dx/dy = (dxr1+dxr2)/(dyr1+dyr2), where x and y are different days post-inoculation and dxr1,dxr2,dyr1 and dyr2 are the count data for that gene from two biological replicates (denoted as r1 and r2) for each strain.

Results

***Salmonella* demonstrated minimal changes in gene expression over 5 days on lettuce**

Analysis of the transcriptional profile of two *Salmonella* strains revealed minimal changes in gene expression over five days on lettuce. In *Salmonella* Typhimurium strain FSL P3-1552, 13 genes were up-regulated whereas 4 genes were down-regulated on day five post-inoculation (Table 4). In *Salmonella* Newport strain FSL R8-2543, two genes were differentially expressed on day five post-inoculation: *ppdD* involved in Type IV pilus assembly and *yiaD* encoding an outer membrane protein and were up-regulated about 3 fold. Whereas 8 hypothetical genes were up-regulated by two fold on day one post-inoculation when compared to days three and five.

Increased expression of genes involved in multiple stress responses in EHEC O157 strain TW014359

Transcriptional changes of EHEC O157 strain TW014359 revealed that a total of 80 genes were differentially expressed on all days of sampling post-inoculation (Table 5). Multiple stress response genes involved in cell envelope stress, osmotic stress and oxidative stress were significantly up-regulated (Table 5, 6 and 7).

Increase in transcript levels was observed for several genes involved in response to cell envelope stress. A member of the Cpx pathway, *cpxP*, was strongly up-regulated on day three post-inoculation by 10 fold compared to day one and 24 fold compared to day five post-inoculation (Table 5). This gene is involved in exhibiting resistance to extra-cytoplasmic stress [57] and can also act as a chaperone [58]. A set of genes from the phage-shock *pspABCDE* operon were up-regulated significantly on day three-post inoculation as compared to other days (Table 5). Psp genes are induced during events like fluctuations in temperature, osmolarity or presence of proton ionophores [59] that pose stress to cell envelope. *pspB* located in the inner membrane displayed the highest transcript level, about 10 fold increase on day three as compared to day one and 26 fold as compared to day five post-inoculation. *pspC* was up-regulated by 10 fold on day three as compared to day one and 12 fold as compared to day five post-inoculation. *pspD* and *pspE* were up-regulated 5 fold on day three post-inoculation as compared to day one and16 and 12 fold respectively, as compared to day five post-inoculation. *degP*, encoding for a periplasmic protease, involved in chaperone activity during cell envelope damage, was up-regulated by 2 fold on day one post-inoculation as compared to day five (Table 5). The induction of *degP* was stronger on day three post-inoculation with about 10 fold increase in transcript level in comparison to day one and five.

In addition, induction was also observed in another important factor, *rpoH,* which helps bacterial cells to cope with cell envelope stress through chaperone and protease activity (Table 5). Differential expression was observed on day three with a 4 fold increase as compared to other days post-inoculation. *rpoH* is induced during events such as heat-shock, nutrient starvation, UV radiation, exposure to oxidants and other adverse conditions [60].

Table 4. Differentially expressed genes in *Salmonella* Typhimurium strain FSL P3-1552, five days post-inoculation. Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d5/d1[a]	d5/d3[b]	ANNOTATION
peg_2328	0.4	0.4	hypothetical protein
peg_2239	0.5	0.5	Mobile element protein
peg_81	0.6	0.5	mobile elment protein
yjeK	1.8	2.1	Lysyl-lysine 2,3-aminomutase
peg_1558	1.8	2.0	hypothetical protein
yadI	2.0	2.3	Putative PTS system IIA component yadI
lipA	2.2	2.3	Lipoate synthase
peg_3919	2.3	2.6	Benzoate transport protein
yrbF	2.5	2.6	Uncharacterized ABC transporter, ATP-binding protein YrbF
ribE	3.4	3.4	Riboflavin synthase eubacterial/eukaryotic
peg_2946	3.5	3.7	Phage endolysin

[a]Column 2 showing the fold change in genes on day five compared to day one post-inoculation
[b]Column 3 showing the fold change in genes on day five compared to day three post-inoculation

Table 5. Differentially expressed genes in EHEC O157 spinach outbreak strain TW014359 on days one, three and five post-inoculation. Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d1/d3[a]	d1/d5[b]	d3/d5[c]	ANNOTATION
pspB	0.1	2.2	26.3	Phage shock protein B
ecnB	0.1	3.5	37.8	Entericidin B precursor
pspD	0.1	1.8	16.7	Phage shock protein D
cpxP	0.1	2.8	23.9	P pilus assembly/Cpx signaling pathway, periplasmic inhibitor/zinc-resistance associated protein
groS	0.1	2.2	17.0	Heat shock protein 60 family co-chaperone GroES
pspC	0.1	1.6	12.0	Phage shock protein C
aceA	0.1	1.6	11.5	Isocitrate lyase
lpp	0.1	2.6	18.3	major outer membrane lipoprotein
degP	0.1	1.6	10.9	HtrA protease/chaperone protein
rplL	0.2	5.0	32.8	LSU ribosomal protein
pspG	0.2	2.7	17.5	Phage shock protein G
pspE	0.2	2.0	12.7	Phage shock protein E precursor
ybeL	0.2	2.0	13.0	hypothetical protein
sra	0.2	1.8	11.4	Stationary-phase-induced ribosome-associated protein
yhcN	0.2	3.1	19.3	probable exported protein
yebV	0.2	2.3	13.8	hypothetical protein
ECSP_2135	0.2	2.3	13.4	Death on curing protein, Doc toxin
aceB	0.2	1.7	9.9	Malate synthase
ychH	0.2	2.7	14.7	membrane protein YchH
sucD	0.2	2.4	12.7	Succinyl-CoA ligase [ADP-forming] alpha chain
ytfK	0.2	2.6	13.8	hypothetical protein
psiF	0.2	1.9	10.0	Phosphate starvation-inducible protein PsiF
yghA	0.2	2.2	11.2	hypothetical protein
ECSP_4040	0.2	5.3	26.9	hypothetical protein
rbsB	0.2	5.0	24.7	Ribose ABC transport system, periplasmic ribose-binding protein RbsB
osmC	0.2	3.5	17.3	Osmotically inducible protein C
hfq	0.2	2.4	11.3	RNA-binding protein Hfq
ytjA	0.2	1.5	7.4	possible membrane protein
yfiA	0.2	6.5	30.6	Ribosome hibernation protein YfiA
miaA	0.2	1.4	6.8	tRNA dimethylallyltransferase
ygaM	0.2	4.1	19.2	hypothetical protein
yhhA	0.2	3.2	14.9	hypothetical protein
cysK	0.2	3.7	16.8	Cysteine synthase
ihfB	0.2	2.2	10.3	Integration host factor beta subunit
dkgA	0.2	2.4	11.0	Methylglyoxal reductase, acetol producing/ 2,5-diketo-D-gluconate reductase A

Table 5. Differentially expressed genes in EHEC O157 spinach outbreak strain TW014359 on days one, three and five post-inoculation (continued). Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d1/d3[a]	d1/d5[b]	d3/d5[c]	ANNOTATION
rplJ	0.2	4.3	19.0	LSU ribosomal protein L10p
ycbK	0.2	2.5	11.1	exported protein
ydcH	0.2	5.0	21.5	YdcH protein
ECSP_1259	0.2	3.8	16.1	hypothetical protein
yqjD	0.2	2.6	11.0	Uncharacterized membrane protein YqjD
ydcY	0.2	1.8	7.7	hypothetical protein
bolA	0.2	2.3	9.8	Cell division protein BolA
yqjC	0.2	3.5	14.7	Periplasmic protein YqjC
yeaG	0.2	2.8	11.6	Serine protein kinase (prkA protein), P-loop containing
ycbL	0.2	1.9	8.0	Hypothetical metal-binding enzyme, YcbL homolog
acpP	0.2	2.2	9.0	Acyl carrier protein
ybjQ	0.2	2.7	10.8	hypothetical protein
lpxP	0.2	1.8	7.4	Lipid A biosynthesis lauroyl acyltransferase
zur	0.3	1.9	7.5	Zinc uptake regulation protein ZUR
ECSP_1560	0.3	1.5	5.6	Conidiation-specific protein 10
ybgS	0.3	1.9	6.8	Probable secreted protein
ydiZ	0.3	2.5	8.9	hypothetical protein
asr	0.3	4.4	13.8	Acid shock protein precursor
gst	0.3	1.3	3.9	Glutathione S-transferase
ptsH	0.3	1.2	3.7	Phosphotransferase system, phosphocarrier protein HPr
ECSP_1442	0.3	7.3	21.3	RelF inactive antibacterial toxin protein
ECSP_2134	0.3	2.1	6.2	putative regulator; Regulation (Phage or Prophage Related)
clpS	0.3	1.4	3.9	ATP-dependent Clp protease adaptor protein ClpS
hupB	0.4	1.1	3.2	DNA-binding protein HU-beta
ybhQ	0.4	1.2	3.4	Putative inner membrane protein
cspA	0.4	4.9	13.4	Cold shock protein CspA
tatE	0.4	2.1	5.3	Twin-arginine translocation protein TatE
ybaW	0.4	1.3	3.2	4-hydroxybenzoyl-CoA thioesterase family active site
yaiA	0.4	2.4	5.9	Protein YaiA
rraB	0.4	1.2	2.7	Ribonuclease E inhibitor RraB
yjdI	0.5	1.6	3.5	hypothetical protein
espM 1	0.5	1.9	3.9	Uncharacterized fimbrial chaperone YehC precursor
cfa	0.5	2.0	4.1	Cyclopropane-fatty-acyl-phospholipid synthase
mscL	0.5	1.3	2.6	Large-conductance mechanosensitive channel
ycfR	0.5	7.3	14.4	Putative outer membrane protein
ECSP_0475	0.6	1.4	2.2	ParD protein (antitoxin to ParE)

Table 5. Differentially expressed genes in EHEC O157 spinach outbreak strain TW014359 on days one, three and five post-inoculation (continued). Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d1/d3[a]	d1/d5[b]	d3/d5[c]	ANNOTATION
chaB	0.7	2.8	4.1	Cation transport regulator chaB
ECSP-0564	0.8	2.0	2.5	hypothetical protein
ECSP_1078	1.4	2.8	2.0	hypothetical protein
yahN	1.5	3.0	2.0	Threonine efflux protein
yhdL	1.7	3.1	1.9	hypothetical protein
ydiH	2.0	2.2	1.1	hypothetical protein
ECSP_5129	2.1	3.0	1.4	hypothetical protein
ECSP-0246	8.1	7.1	0.9	hypothetical protein

[a]Column 2 showing the fold change in genes on day one compared to day three post-inoculation
[b]Column 3 showing the fold change in genes on day one compared to day five post-inoculation
[c]Column 4 showing the fold change in genes on day three compared to day five post-inoculation

Lpp, encoding a major lipoprotein, involved in maintaining cell envelope integrity was up-regulated 3 fold on day one and 18 fold on day three post-inoculation compared to day five (Table 5). *lpxP*, induced in response to cold shock was up-regulated by 2 fold on day one compared to day five post-inoculation. This gene encodes an inner membrane protein which is involved in maintaining cell envelope integrity and was up-regulated by 7 fold on day three post-inoculation as compared to day five (Table 5).

Up-regulation was also observed in the genes encoding for proteins responsible for protection against osmotic stress. Up-regulation in three osmotically inducible genes *osmB*, *osmC* and *osmE* was observed, whose protein products function to protect the cell from stress caused due to fluctuating extracellular water and solute concentration. The inner membrane lipoprotein *osmB* was up-regulated by 5 fold on day three post-inoculation as compared to days one and five (Table 6). *osmC* was up-regulated 5 fold on day three compared to day one and17 fold as compared to day five (Table 5). *osmE*, encoding for an inner membrane lipoprotein was up-regulated 3 fold on day three post-inoculation when compared to other days (Table 6). Another prominent member responsible for maintaining osmotic balance of the cell, encoded by *mscL*, was found to be up-regulated 3 fold on day three post-inoculation as compared to days one and five (Table 5).

Table 6. Differentially expressed genes in EHEC O157 spinach outbreak strain TW014359, three days post-inoculation. Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d3/d1[a]	d3/d5[b]	ANNOTATION
ECSP_1446	0.4	0.3	Holliday junction resolvase / Crossover junction endodeoxyribonuclease rusA
fliQ	0.4	0.4	Flagellar biosynthesis protein FliQ
setB	0.5	0.5	Sugar efflux transporter B
ECSP_0778	0.5	0.4	DNA for 3-methylaspartate ammonia-lyase, glutamate mutase
ydiT	0.5	0.4	Ferredoxin-like protein FixX
priC	0.5	0.4	Primosomal replication protein N prime prime
yjfL	0.5	0.6	Membrane protein with DUF350 domain
ymcA	0.6	0.5	Putative outer membrane lipoprotein YmcA
ECSP_3294	1.8	2.0	hypothetical protein
lpoA	2.2	2.7	LppC putative lipoprotein
ECSP_3682	2.4	2.2	hypothetical protein
mutL	2.5	3.1	DNA mismatch repair protein MutL
mdtJ	2.5	2.8	Spermidine export protein MdtJ
dtd	2.5	2.7	D-tyrosyl-tRNA(Tyr) deacylase
mdtA	2.6	2.5	Multidrug transporter MdtA
sseA	2.7	3.2	3-mercaptopyruvate sulfurtransferase
lspA	2.7	2.5	Lipoprotein signal peptidase
yigP	2.8	3.3	Protein YigP (COG3165) clustered with ubiquinone biosynthetic genes
ddpF	2.8	2.9	Oligopeptide transport ATP-binding protein OppF
osmE	2.9	2.6	Osmotically inducible lipoprotein E precursor
ECSP_2670	3.0	2.9	hypothetical protein
znuC	3.0	2.8	Zinc ABC transporter, ATP-binding protein ZnuC
ECSP_2632	3.2	3.5	putative repressor protein
espJ	3.2	2.9	unknown protein encoded within prophage CP-933U
deoD	3.2	3.0	Purine nucleoside phosphorylase
yobH	3.3	3.3	putative exported protein
yrdB	3.3	3.1	hypothetical protein
bssS	3.3	5.0	hypothetical protein
	3.3	3.6	hypothetical protein
yrbK	3.3	3.9	Uncharacterized protein YrbK clustered with lipopolysaccharide transporters
gabT	3.5	3.7	Gamma-aminobutyrate:alpha-ketoglutarate aminotransferase
cysQ	3.5	5.0	3'(2'),5'-bisphosphate nucleotidase
igaA	3.5	4.8	IgaA: a membrane protein that prevents overactivation of the Rcs regulatory system
prpC	3.6	3.7	2-methylcitrate synthase
pfkB	3.7	4.5	6-phosphofructokinase class II

Table 6. Differentially expressed genes in EHEC O157 spinach outbreak strain TW014359, three days post-inoculation (continued). Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d3/d1[a]	d3/d5[b]	ANNOTATION
gefL	3.7	4.7	RelF inactive antibacterial toxin protein
ydgD	3.7	4.7	possible peptidase
trxA	3.8	4.1	Thioredoxin
yidQ	3.8	2.6	Outer membrane lipoprotein YidQ
yniA	3.8	7.7	Ribulosamine/erythrulosamine 3-kinase potentially involved in protein deglycation
ybdD	4.0	6.2	Hypothetical small protein yjiX
malT	4.0	5.7	Transcriptional activator of maltose regulon, MalT
ynfB	4.0	4.3	putative secreted protein
rpsP	4.1	4.3	SSU ribosomal protein S16p
ECSP_0555	4.2	4.8	adherence and invasion outermembrane protein (Inv,enhances Peyer's patches colonization)
yibT	4.3	3.4	hypothetical protein
uspC	4.3	5.8	Universal stress protein C
ECSP_1778	4.3	4.7	Conidiation-specific protein 10
ygaF	4.4	3.8	L-2-hydroxyglutarate oxidase
ECSP_5128	4.5	13.4	hypothetical protein
ECSP_2820	4.7	3.4	Helix-turn-helix motif
osmB	5.0	6.9	Osmotically inducible lipoprotein B precursor
ybjR	5.0	8.1	N-acetylmuramoyl-L-alanine amidase
coxT	5.3	6.7	putative DNA-binding protein
ydcA	5.4	6.7	hypothetical protein
ECSP_1558	5.8	8.3	Protein YciE
sodC	5.8	7.1	Superoxide dismutase [Cu-Zn] precursor
yqjK	6.3	13.1	Inner membrane protein YqjK
rpoH	6.4	3.6	RNA polymerase sigma factor RpoH
yedX	6.8	6.9	5-Hydroxyisourate Hydrolase (HIUase)
smpA	9.5	14.7	Outer membrane lipoprotein SmpA, a component of the essential YaeT outer-membrane protein assembly complex

[a]Column 2 showing the fold change in genes on day three compared to day one post-inoculation
[b]Column 3 showing the fold change in genes on day three compared to day five post-inoculation

In addition to the induction of genes encoding proteins involved in osmotic and cell envelope stress, up-regulation was also observed for genes encoding proteins involved in response to oxidative stress. Superoxide dismutase C (*sodC*), was significantly up-regulated 7 fold on day three post-inoculation as compared to day one and five (Table 6). The protein encoded by this gene functions during

the presence of reactive oxygen species and converts superoxide radicals to hydrogen peroxide and water. Glutathione S-transferase (*gst*) is also involved in protection from oxidative stress [61]. A 4 fold increase in transcript level was observed in *gst* on day three post-inoculation as compared to days one and five. A universal multiple stress resistance gene *ycfR* was found to be up-regulated 7 fold on day one compared to day five and 14 fold on day three in comparison to day five post-inoculation. *ycfR* has been shown to be up-regulated in response to oxidative stress when cells were treated with chlorine [62]. Protein encoded by this gene is also involved in biofilm formation in *E.coli* K-12 as well as in EHEC serotype O157 [32, 63].

Multiple genes encoding proteins involved in attachment mechanisms such as formation of extra-polymeric substances, biofilm formation, or flagella formation were up-regulated significantly. One component that is shown to be involved in attachment to surfaces and was up-regulated in this pathogen was *wcaD*, which encodes for production of an exopolysaccharide (EPS), colanic acid (Table 7). This gene was up-regulated on day three post-inoculation, with a 2 fold increase in transcript level, compared to day five. *bssS*, regulator of biofilm formation, was found to be up-regulated by fivefold on day three post-inoculation as compared to other days. *fliQ*, a part of flagellar export apparatus, was found to be up-regulated by 2.5 fold on day one and five post-inoculation when compared to day three (Table 8).

In addition to the induction of genes encoding for proteins involved in attachment mechanisms, two genes encoding proteins involved in coping with nutrient starvation were found to be up-regulated. A 10 fold increase was observed in phosphate starvation inducible protein F (*psiF*) on day three post-inoculation as compared to day five. *phoE*, a member of bacterial porin family was observed to be up-regulated by 2 fold on day three in comparison to day five post-inoculation. This outer membrane porin has been shown to be induced in response to phosphate limitation [64]. Induction in universal stress protein C (*uspC*) was observed on day three post-inoculation as compared to other days by a 5 fold

Table 7. Differentially expressed genes in EHEC O157 spinach outbreak strain TW014359, five days post-inoculation. Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d5/d1[a]	d5/d3[b]	ANNOTATION
phoE	0.3	0.5	Outer membrane pore protein E precursor
pheA	0.5	0.5	Chorismate mutase I / Prephenate dehydratase
yiaA	0.5	0.5	Inner membrane protein YiaA
yhbS	0.4	0.5	Acetyltransferase
yccA	0.5	0.5	Putative TEGT family carrier/transport protein
yfgD	0.4	0.5	Arsenate reductase
yeaQ	0.5	0.5	Transglycosylase associated protein
wcaD	0.4	0.5	Colanic acid polymerase WcaD
ydjK	0.5	0.5	Putative transport protein YdjK, MFS superfamily
ydhU	0.5	0.5	Thiosulfate reductase cytochrome B subunit
ybaP	0.6	0.5	possible ligase
sbcB	0.2	0.4	Exodeoxyribonuclease I
ECSP_2649	0.4	0.4	hypothetical protein
ydjC	0.6	0.4	Cellobiose phosphotransferase system YdjC-like protein
yicS	0.5	0.4	Putative secreted protein
dusC	0.5	0.4	tRNA-dihydrouridine synthase C
sulA	0.5	0.4	Cell division inhibitor
ftsB	0.4	0.4	Cell division protein DivIC (FtsB), stabilizes FtsL against RasP cleavage
rpoE	0.4	0.4	RNA polymerase sigma factor RpoE
yhcO	0.4	0.3	probable ribonuclease inhibitor YPO3690

[a] Column 2 showing the fold change in genes on day five as compared to day one post-inoculation
[b] Column 3 showing the fold change in genes on day five compared to day three post-inoculation

increase in transcript level. This gene has also been shown to be induced by phosphate starvation [65] and its protein product plays a role in aggregate formation as well as flagellar motility in *E.coli [66]*. *malT*, encoding a protein involved in maltose catabolism and transport was found to be up-regulated by 4 fold on day three post-inoculation, compared to day five (Table 6).

Two multidrug transporters *mdtA* and *mdtJ* were up-regulated by 3 fold each on day three post-inoculation as compared to day one and five. *mdtA*, a membrane fusion protein, works as a part of *mdtABC* efflux system whereas *mdtJ* is a spermidine efflux transporter (Table 6).

Increased expression of genes involved in attachment in EHEC O26 strain TW01506

In contrast to EHEC O157 strain TW041539 which showed induction of genes encoding for proteins involved in osmotic and cell envelope stress responses, the transcriptional profile of EHEC O26 revealed the induction of several genes involved in attachment of the pathogen to various surfaces. *yjbE*, a member of the *yjbEFGH* operon and predicted to encode a protein present in the periplasm, was found to be up-regulated by 2 fold on day three and five post-inoculation compared to day one. Research studies have predicted that this operon is involved in producing proteins responsible for the production of EPS such as colanic acid or PGA that are utilized in attachment [67]. *yadM*, encoding a fimbrial adhesion, was up-regulated by 2.8 fold on day one than days three and five post-inoculation (Table 8). This adhesion could contribute to the pathogen's ability to attach to and colonize the surface of the lettuce leaf [68].

fliQ, part of flagellar apparatus, was more than 2 fold higher in transcript level on day one post-inoculation when compared to other days post-inoculation (Table 8). Up-regulation of a major flagellar gene, *flhD*, was 2.7 fold on day one post-inoculation as compared to other days (Table 8). *flhD* is the principal factor for flagellar biogenesis and swarming motility in *E.coli* [69]. Another gene encoding protein involved in regulation of biofilm formation and motility, *ydeH*, was observed to be up-regulated by 2.4 fold on day one post-inoculation than other days. *ydeH* has been shown to produce proteins that promote biofilm formation by enhancing production of PGA [70]. Up-regulation by 7.2 fold was observed in another adhesion factor, *ecpD*, encoding proteins responsible for fimbrial formation which is utilized in biofilm formation to enable the pathogen to adhere to abiotic and biotic surfaces [71](Table 8).

Various genes encoding proteins involved in response to oxidative stress were also induced on day one post-inoculation. One of the superoxide dismutase present in *E.coli*, encoded by *sodC*, which alleviates oxidative stress, was found to be 2.4 fold higher in transcript level one day one when compared to other days. Up-regulation in the transcript level of the gene *mltC* was observed by 2.2 fold on day one post-inoculation. This gene has been shown to encode a protein product in response to superoxide stress under the control of SoxRS, a well characterized signal transduction system involved in oxidative stress [72]. A 2.7 fold increase in transcript level was observed for a gene encoding for a methionine sulfoxide

reductase (Msr) (Table 8). The gene peg_5227 has not been annotated. Msr are one of the enzymes that eliminate reactive oxygen species and relieve stress caused due to oxidation of methionine [73].

In addition to the induction of genes encoding proteins involved in attachment and response to oxidative stress, induction was observed for two genes producing proteins involved in nutrient utilization. *gatD*, showed a 2.5 fold up-regulation on day one post-inoculation in comparison to other days and encodes for a naturally occurring hexitol called galactitol. Galactitol is utilized as a carbon source by some *E.coli* strains [74]. Up-regulation was also found in *phoU* by 2.9 fold on day one as compared to days three and five. The protein product of *phoU* is responsible for sensing environmental phosphate and is involved in phosphate signal transduction [75].

Table 8. Differentially expressed genes in EHEC O26 strain TW015601, one day post-inoculation. Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d1/d3[a]	d1/d5[b]	ANNOTATION
yjbE	0.5	0.5	YjbE secreted protein
peg_3974	1.1	2.3	putative membrane protein
crcB	1.2	2.1	CrcB protein
dsdX	1.2	2.5	D serine permease DsdX
peg_5227	1.3	2.7	Free methionine R sulfoxide reductase
peg_2857	1.3	2.5	transcriptional control
yadM	1.3	2.8	Fimbrial protein YadM
peg_669	1.3	2.4	hypothetical protein
cspB	1.3	2.9	Cold shock protein CspB
peg_1498	1.4	2.6	Alpha-fimbriae chaperone protein
peg_2677	1.4	2.3	PilT
psuG	1.4	2.1	Pseudouridine kinase
xapR	1.4	2.1	Xanthosine operon regulatory protein XapR, LysR family
umpG	1.4	2.0	tRNA psuedouridine 13 synthase
peg_1230	1.4	2.5	Type III secretion inner membrane protein
peg_374	1.4	2.1	hypothetical protein
glyA	1.5	3.1	hypothetical protein
flhD	1.5	2.7	hypothetical protein
cobS	1.5	3.0	Adenosylcobinamide-phosphate guanylyltransferase
stcC	1.5	2.3	Fimbriae usher protein StcC
yqeF	1.5	2.1	Acetyl-CoA acetyltransferase
chbR	1.5	3.0	Chitobiose-specific regulator ChbR, AraC family
yejK	1.5	2.6	hypothetical protein
yqeJ	1.5	3.8	YqeJ protein
ydjO	1.5	2.3	hypothetical protein
ydiA	1.5	2.4	Neopullulanase
nudG	1.6	2.0	hypothetical protein
espX7	1.6	2.0	Putative secreted effector protein
ydeH	1.6	2.4	hypothetical protein
peg_5010	1.6	4.1	hypothetical protein
pntA	1.6	3.0	hypothetical protein
yneF	1.6	3.1	hypothetical protein
dicC	1.6	2.8	unknown protein encoded by prophage
peg_5349	1.6	2.6	putative DNA-binding protein
hycA	1.6	2.5	Formate hydrogenlyase regulatory protein HycA
ydhL	1.7	2.3	hypothetical protein

Table 8. Differentially expressed genes in EHEC O26 strain TW015601, one day post-inoculation (continued). Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d1/d3[a]	d1/d5[b]	ANNOTATION
yhfU	1.7	2.8	hypothetical protein
yhiS	1.7	2.6	hypothetical protein
peg_1176	1.7	2.3	Beta-glucoside bgl operon antiterminator, BglG family
sodC	1.7	2.4	Superoxide dismutase precursor {Cu-Zn]
yhaB	1.7	2.8	hypothetical protein
peg_5346	1.8	3.4	unknown protein encoded by prophage
peg_4736	1.8	3.3	PTS system, cellobiose-specific IIB component
fliQ	1.8	2.5	Flagellar biosynthesis protein FliQ
peg_3134	1.8	2.3	hypothetical protein
peg_743	1.8	2.1	predicted outer membrane lipoprotein YfeY
cbtA	1.8	2.4	conserved domain protein
peg_1330	1.9	2.6	inner membrane protein
peg_4628	1.9	2.0	hypothetical protein
ygeN	2.0	3.5	ORF_f143
ymfT	2.0	2.6	hypothetical protein
cydA	2.0	3.1	hypothetical protein
peg_5150	2.0	4.4	hypothetical protein
peg_4860	2.1	0.8	hypothetical protein
gltP	2.1	2.9	hypothetical protein
dinJ	2.1	2.2	DNA-damage-inducible protein J
peg_1685	2.1	1.1	hypothetical protein
yfbT	2.1	4.2	Entericidin A precursor
mltC	2.2	2.2	hypothetical protein
peg_2918	2.2	1.6	orf; unknown fucntion
peg_2893	2.2	2.3	YeeU protein (antitoxin to YeeV)
ygeH	2.2	2.9	PTS system, sorbose-specific IIA component
paaD	2.3	1.7	Phenylacetic acid degradation protein PaaD, thioesterase
peg_1363	2.3	1.6	hypothetical protein
yhhK	2.3	1.5	hypothetical protein
ycdZ	2.3	2.4	hypothetical protein
argE	2.3	0.8	hypothetical protein
peg_3598	2.4	1.8	Phage tail length tape-measure protein 1
gatD	2.5	1.7	Galactitol-1-phosphate 5-dehydrogenase
peg_153	2.5	1.9	putative rhamnosyl transferase
peg_4329	2.5	1.6	hypothetical protein
papP	2.6	3.7	hypothetical protein

Table 8. Differentially expressed genes in EHEC O26 strain TW015601, one day post-inoculation (continued). Genes showing a >2 fold up-regulation or down-regulation and a False Discovery Rate adj p-value<0.05 were considered to be differentially expressed.

GENE	d1/d3[a]	d1/d5[b]	ANNOTATION
peg_1114	2.6	1.8	HokE protein
phoU	2.9	3.3	hypothetical protein
yqgB	3.1	1.8	Hypothetical protein YqgB
ansP	4.2	3.4	hypothetical protein
peg_4647	4.2	3.1	hypothetical protein
peg_2314	4.5	2.1	hypothetical protein
ecpD	7.2	1.6	hypothetical protein

[a]Column 2 showing the fold change in genes on day one compared to day three post-inoculation
[b]Column 3 showing the fold change in genes on day one compared to day five post-inoculation

Discussion

Whole-genome transcriptional profiling of EHEC strains revealed the up-regulation of a number of genes encoding for proteins involved in cell envelope stress response, nutrient limitation, oxidative stress and production of attachment factors. However, the transcriptional analyses of the two *Salmonella* strains revealed minimal changes in gene expression over 5 days on lettuce.

EHEC strains exhibited a high number of differentially expressed genes. Most of the genes were up-regulated on day three for EHEC O157 spinach strain whereas in EHEC O26 sprouts strain, most of the genes were induced on day one post-inoculation. For the EHEC spinach strain, the set of genes highly up-regulated were those involved in protection of the cell envelope from a variety of stresses. Since the cell envelope is continuously in contact with the external medium, it is the initial target of physical (osmolarity) or chemical (fluctuations in pH) stresses that may alter envelope components [76]. The gram negative cell envelope controls the passage of molecules into and out of the cell and provides an ion-permeability barrier for establishment of proton motive force across the inner membrane. Extra-cytoplasmic stress response systems such as the Cpx pathway and the Phage shock protein (Psp) response system are induced in response to osmotic stress and mis-localization of cell envelope proteins [77]. The induction of Cpx occurs through the sensing of misfolded periplasmic proteins [77, 78] and in response, protein folding and degradation factors such as a protease *degP* and one of the chaperones *cpxP* is generated. Both *cpxP* and *degP* are utilized by the activated Cpx pathoway to encode proteins

that suppress toxic effects associated with protein misfolding. *cpxP* encodes protein that function to prepare substrates for *degP* and further processes *degP*'s proteolytic products. Induction of these genes indicates that the pathogens experienced some stresses on the leaf surface that influenced the cell envelope integrity. Fluctuations in pH, temperature or osmotic shock could result in protein misfolding and damage, causing toxicity in the cells.

Up-regulation was also observed in the genes encoding proteins involved in coping with osmotic shock in EHEC spinach strain. The operon encodes proteins responsible for repairing damaged inner membrane of the cell and maintaining the osmotic balance within the cell. *osmB* and *osmC* have been shown to be induced by osmotic changes [79, 80]. Gunasekera et al. (2008) also observed an up-regulation in *osmC* in *E.coli* in response to osmotic stress when cells were subjected to different salt concentrations. Fink et al. (2012) observed the induction of *pspABCDE*, *osmBY* and *otsAB* following incubation of *E.coli* K-12 cells on the lettuce leaf tissue. The induction of these genes indicated that pathogens experienced osmotic shock which could have caused damage to the cell envelope on lettuce. Lettuce and other plants possess numerous stomata that open and close with the turgor activity of guard cells[81]. Since guard cells activity is influenced by the efflux and influx of different ions such as potassium and chloride ions, and numerous guard cells are present on the plant surface, it is possible that this uptake and efflux of ions could be inducing osmotic stress in EHEC.

In response to extra-cytoplasmic changes such as fluctuations in pH, temperature, osmolarity or presence of toxic compounds and to maintain inner cytoplasmic ionic composition, bacterial cells possess specific ion channels and transporters. Certain ion channels or porins exists in the outer membrane that allows the diffusion of solutes across the cell membrane. Various transporter systems exist that play osmoregulatory role by allowing the uptake and efflux of ions or the passage of osmoprotectants such as glycine betaine or proline during osmotic upshock. *mscL* or mechano-sensitive channel of large conductance is one of the two major mechano-sensitive channels in *E.coli*. It is the largest gated channel and predicted to release excess cell turgor to maintain internal homeostasis. It senses biophysical properties of the membrane such as lipid bilayer deformation or hypo-osmotic shock [82, 83]. Up-regulation of this channel has also been documented in *E.coli* O157 on lettuce phyllosphere by Fink et al. (2012).

lpp, up-regulated during cell envelope stress, encodes for a major outer membrane lipoprotein necessary for stabilization and integrity of the bacterial cell envelope and physically tethers outer membrane to the peptidoglycan layer [84]. Cells mutants lacking *lpp* have been found to be hypersensitive to toxic compounds and release periplasmic proteins to extracellular medium [85, 86]. Stress induced in the bacterial cell due to factors such as heat shock or osmotic shock could result in protein misfolding or damage. Protein degradation thus plays a crucial role in maintaining cell stability and quality control. Misfolded, damages, truncated or stress-induced aggregates of proteins are recognized and degraded by cellular mechanisms. *clpS* encodes a chaperone protein that is located in the cytosol and works as an adapter of the ClpAP protease complex aiding in degradation of aggregated protein substrates [87]. *clpS* also shows *oxyR* dependent induction of expression by hydrogen peroxide.

Various stress response mechanisms involved in oxidative stress were observed in EHEC O157 strain as well as in EHEC O26. Presence of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), can lead to oxidative modification of biological membranes and intracellular components, thus leading to cell damage. ROS have been shown to cause damage to DNA, RNA, protein and lipids[88]. *E.coli* utilizes antioxidant enzymes such as superoxide dismutases (SOD) and hydroperoxidases that are involved in ROS scavenging and DNA repair [89]. Superoxide dismutases convert superoxide radicals to hydrogen peroxide and water. *sodC* encodes for one of the three superoxide dismutases in *E.coli* containing copper-zinc as metal cofactors and is a periplasmic enzyme [90]. The role of *sodC* in oxidative stress response has been well documented [62]. *osmC* has also been shown to encode a protein with peroxidase activity with a strong preference towards hydrogen peroxide [91, 92]. Gunasekera et al. (2008) observed the up-regulation of *sodC* due to heat and osmotic stress. It is therefore possible that in our experiment, up-regulation was seen in *sodC*, due to osmotic stress experienced by the pathogens on lettuce. Transcriptional analysis of EHEC O157 by Wang et al. (2009) under oxidative stress (sublethal concentrations of chlorine and hydrogen peroxide) demonstrated increased expression of *sodABC* as well as *osmBCE*. Since these genes were turned on in response to oxidative stress and up-regulation in some of these genes was also observed in our study, it is therefore predicted that the pathogens were experiencing either osmotic stress, oxidative stress or a combination of both on lettuce phyllosphere.

Presence of multiple stresses can induce cross-protection against a variety of stresses. Osmotic stress on lettuce could be influencing the transcription of gene (*sodC*) encoding proteins involved in response to oxidative stress. One study observed the up-regulation of *sodC* in EHEC O157(strain EDL933) on day one post-inoculation as compared to day three on lettuce leaf surface [32]. The pathogen was spray inoculated on lettuce and incubated at 100% RH for 3 days at 25°C with a photoperiod of 16 days. Research has shown that release of reactive oxygen species is a defense strategy in plants against bacterial pathogens [93]. Once a plant recognizes a pathogen's presence, a cellular response in the form of oxidative burst is initiated [94]. Kyle at al. (2010) showed the presence of ROS such as O_2- and H_2O_2 in lettuce leaf lysate. They observed the up-regulation of a large set of EHEC O157 genes encoding proteins involved in oxidative stress. Two copper-zinc SODs were up-regulated after 30 minute exposure of the pathogen to lettuce leaf lysate. Therefore, in addition to the release of ROS as part of defense in plant cells and a result of mechanical injury, certain phytochemicals could also possess oxidizing activity towards microbial pathogens; however, this concept is yet to be tested.

Another class of enzymes called Methionine sulfoxide reductase (Msr) have also been found to play an important role in prevention of damage due to oxidative stress[73]. Oxidation of the amino acid methionine by ROS results in the formation of oxidized methionine sulfoxide (MetSo) leading to the loss of its biological activity. Two enzymes MsrA and MsrB are capable of reducing MetSo to methionine, thus restoring its biological activity. The role of these enzymes in protection against oxidative stress has been documented. For example, MsrA mutants were highly sensitive to hydrogen peroxide as well as nitric oxide and other free radicals [95, 96].

Genes encoding proteins involved in nutrient limitation and starvation response were observed in both strains of EHEC. In order to colonize the plant surface, a carbon source for energy, a nitrogen source and certain essential inorganic molecules such as inorganic phosphate must be present on leaves. Molecules leaching from the plants surface include a variety of amino acids, organic acids and sugars. Glucose, fructose and sucrose are the main sugars available on the leaf surface as carbon sources [23, 97]. However, the amount of nutrients available on the leaf surface is limited, rendering the lead surface as a hostile environment for bacterial colonizers. *psiF* and *phoE* are induced during phosphate-starvation. *phoE*, a member of General Bacterial Porin (GBP) family , is present in the outer

membrane of gram negative bacteria and facilitates efficient diffusion of phosphate and phosphorus-containing compounds across the outer membrane [64]. *phoU*, encodes an inner membrane protein that helps in phosphate uptake. Induction in these genes suggests the possible limitation of these nutrients on the lettuce leaf surface. Fink et al. (2010) also found that *psiF* was the most induced gene for phosphate starvation response in EHEC O157 on lettuce leaf surface. *malT*, encodes a protein that is involved in maltose metabolism and transport [98]. Maltose is the major product of starch degradation by chloroplasts [99]. *malE* was shown to be up-regulated in EHEC O157 when exposed to lettuce leaf lysate in a recent research study [31]. Thus, maltose might be the plant metabolite available as a carbon source on the leaf surface as depicted by up-regulation in *malT*.

Interestingly, multiple genes encoding proteins involved in biofilm formation were induced in both EHEC strains. Biofilms are an important survival strategy by bacterial pathogens allowing attachment to surfaces as well as formation of cell aggregates. EPS are also involved in biofilm formation and are major components of bacterial cell envelope playing an important role in interaction between the bacterial cell and the environment. Different types of EPS have been characterized in *E.coli* such as LPS, O-antigen, colanic acid, PGA. Colanic acid is a polymer of glucose, galactose, fructose and glucuronic acid. Production of this EPS is encoded by *cps/wca* operon. Colanic acid in EHEC O157 has been shown to be involved in attachment to alfalfa sprouts and to plastic surfaces in *E.coli* K-12 [49]. Therefore, EHEC strains used in this study could have utilized the production of colanic acid to attach to the leaf surface. *yjbE* has also been shown to encode a protein involved in production of an EPS different from colanic acid and PGA [67]. Thus, it is predicted that a new EPS could be produced by this gene in formation of biofilm and attachment to leaf surface.

Certain genes encoding proteins involved in flagellar formation and function were found to be up-regulated in the EHEC strains. Flagella are locomotory organelles and are also used in attachment to various surfaces including plants. *bssS*, encodes a protein involved in biofilm regulation, has been shown to be highly expressed in *E.coli* K-12 when exposed to cold temperature of 23°C[104]. *flhD* is the master regulator of flagella biogenesis and swarming motility [69, 101, 102]. One study have demonstrated the colonization of baby spinach and lettuce leaf surface by EHEC O157 via flagella [103]. Similarly, *S*. Thompson inoculated onto cilantro phyllosphere and observed through electron microscope revealed the

usage of flagella by the bacterial cells to anchor to the leaf surface. *ydeH*, encoding a dyguanylate cyclase, regulates the motility and biofilm formation in *E.coli* [105] and controls the transition from motile to biofilm lifestyle. EHEC inoculated on lettuce in our study could have utilized flagella in moving to protective niche as well as in formation of aggregates on the leaf surface.

ycfR has been shown to be turned on by a variety of stresses and encodes for a proteins involved in exhibiting resistance to multiple stresses including biofilm formation in *E.coli*. Fink et al. (2012) observed the up-regulation of *ycfR* in *E.coli* K-12 after the pathogen was inoculated on post-harvest lettuce. In another study by Wang et al. (2009), transcription of *ycfR* was upregulated by >10 fold under chlorine and hydrogen peroxide stress in EHEC Sakai and spinach strains, This suggests that *ycfR* is turned on under oxidative stress which suggests that EHEC spinach strain in our study could be exhibiting oxidative stress on lettuce. This could lead to enhanced survival under future decontamination treatment methods based on oxidative reagents.

ycfR has also been shown to encode proteins that are involved in attachment to produce surfaces. This gene was significantly induced in *S. enterica* upon exposure to chlorine treatment in a study by Salazar et al. (2013). To further characterize the role of *ycfR* in attachment in *S*. Typhimurium and *S*. Saintpaul in that study, deletion mutants were created for *ycfR*. Results demonstrated that deletion resulted in reduced bacterial attachment to fresh spinach and grape tomatoes. Deletion of *ycfR* in S. Typhimurium significantly reduced bacterial chlorine resistance. Thus, these evidences suggest that EHEC spinach strain inoculated on lettuce in our study could have utilized a protein encoded by *ycfR* in attachment to leaf surface, in addition to providing resistance to post-harvest chlorine decontamination.

Certain organelles in addition to flagella such as curile, fimbriae and Type three secretion system filaments (TTSS) are also involved in attachment or biofilm formation. Fimbriae are long proteinaceous organelles that protrude from bacterial cells and mediate various functions including adherence and biofilm formation. Pathogens could utilize these fimbriae to attach to lettuce leaf surface; however, if *yadM* encodes a fimbrial protein to play a role in adhering to lettuce phyllosphere has not been studied. The role of *espA*, a TTSS filamentous apparatus has been shown to be responsible for adherence in EHEC O157 as well as EHEC O26 in *Eruca vesicaria* leaves. The role of effector proteins (such as one encoded by (*espJ*) in facilitating attachment to leaf surface, however, has not been elucidated.

Taken together, all these data indicate that enteric pathogens experienced multiple stresses on pre-harvest lettuce and their physiology is affected by the pre-harvest environmental factors. This is indicated by the up-regulation of genes that encode proteins responsible for protection against cell envelope stress and oxidative stress. The induction of oxidative stress responses could lead to cross-protection of these pathogens against future stresses. In other words, the up-regulation of oxidative stress genes such as *sodC*, in EHEC O157 spinach strain could be responsible for encoding proteins that protect against chlorine decontamination washes on produce. Various genes encoding proteins involved in attachment, motility and biofilm formation were also up-regulated in both EHEC strains. Induction of such genes indicates that the pathogens could have formed biofilms or aggregates on the leaf surface to ensure effective colonization. Understanding the mechanisms of pathogens' physiological state on pre-harvest produce can provide insights into development of effective post-harvest decontamination treatments.

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