CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Quantifying risk associated with changes in EHEC physiology during post-harvest pre-processing stages of leafy green production

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Objectives

1. *Determine the impact of pre-processing time delays on the physiological state of E. coli O157:H7 on Romaine lettuce.*
2. *Evaluate the impact of physiological state on detectability of E. coli O157:H7 on Romaine lettuce.*
3. *Quantify changes in E. coli O157:H7 risk over pre-processing cut-to-cool and refrigerated transport times.*

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FINAL REPORT

Abstract

If leafy greens are contaminated in the field, human pathogens such as *E. coli* O157:H7 are capable of surviving throughout the distribution chain. *E. coli* O157:H7 survival on lettuce in the field and during post-harvest washing has been quantified, but not during harvest and transport prior to processing. Leafy greens transported from the West Coast to the East Coast may be held for days at refrigeration temperature during transport prior to processing, likely impacting pathogen physiology. Physiological changes such as entering a dormant state can protect cells from environmental stress and decrease detectability. Recent studies have demonstrated that *E. coli* O157:H7 cells may enter a persister state, a type of dormancy characterized by resistance environmental stresses. Entering the viable but non culturable (VBNC) state impedes detection of *E. coli* O157:H7 by standard molecular detection methods, though cells remain virulent. While *E. coli* O157:H7 can enter the persister or VBNC state on lettuce, the extent to which this occurs during harvest, cooling, and refrigerated transport is unknown. Changes in the physiological state of *E. coli* O157:H7 have the potential to impact risk of illness associated with contaminated lettuce due to alterations in tolerance to sanitizers in wash water, virulence properties, and detectability. This study aimed to 1) characterize physiological changes in *E. coli* O157:H7 cells on Romaine lettuce during post-harvest pre-processing handling and refrigerated storage, 2) determine if changes in physiology impact detectability of *E. coli* O157:H7 on Romaine lettuce, and 3) evaluate how changes in *E. coli* O157:H7 physiology, virulence, and stress tolerance impact potential health risks through a quantitative microbial risk assessment (QMRA). Romaine lettuce plants were inoculated with *E. coli* O157:H7 in the laboratory and were held under conditions to simulate harvest and refrigerated storage. The proportion of *E. coli* O157:H7 cells that are culturable, persisters, or VBNC were quantified before and after harvest and every day for 5 days during refrigerated storage. Changes in acid and chlorine tolerance were assessed at each sampling point. Changes in virulence potential were determined by measuring relative pathogenicity of *E. coli* O157:H7 in *Galleria mellonella* larvae. Correlations between proportion of cells in the persister or VBNC state and changes in detectability with two standard molecular detection methods were determined. A QMRA model was developed to evaluate the risk of illness after consumption of contaminated lettuce using the expected prevalence and concentration of *E. coli* O157:H7 across the farm-to-fork pathway including processing, production, and supply chain stages. Experimental data was used to determine changes in risks corresponding to the physiological changes in *E. coli* O157:H7 as the result of in-field cut-to-cool temperatures and durations. A tool was developed to identify practices that are associated with reduced risk of *E. coli* O157:H7 illnesses transmitted via Romaine lettuce.

Background

In many of the recent outbreaks of *E. coli* O157:H7 linked to leafy greens, the majority of initial cases in the outbreak were from states on the East Coast or in the Midwest, as well as from the eastern provinces of Canada (Bottichio et al., 2020; Coulombe et al., 2020; Marshall et al., 2020). During these outbreaks, illnesses were often regional even though the implicated product was distributed nationwide. These observations suggest that product experiencing pre-processing time delays may be more likely to be associated with illness compared to leafy greens that are processed shortly after harvest. Prolonged storage at low temperature prior to processing, while not a lethal stress, impacts pathogen physiology. One strategy for bacterial survival under adverse conditions is to enter a state of dormancy, characterized by low metabolic activity with little or no growth (Ayrapetyan et al., 2018). The shift to a dormant state

includes a continuum of physiological states, from actively growing cells to stationary phase cells, to the formation of persister cells and viable but nonculturable (VBNC) cells. Studies have shown *E. coli* O157:H7 can form persister and VBNC cells under pre-harvest conditions on leafy greens and in water (Dinu & Bach, 2011; Munther et al., 2020; Thao et al., 2019). There is currently little information on how *E. coli* O157:H7 populations may shift to a dormant state during post-harvest, pre-processing handling. As observed in the referenced outbreaks, health risks associated with these changes in physiology and virulence may be increased.

Quantitative microbial risk assessment (QMRA) studies have previously focused on *E. coli* O157:H7 in leafy greens and lettuce in particular due to past outbreaks and to evaluate intervention strategies (irrigation water quality, temperature control, UV radiations, chlorine sanitizers, etc.) (Franz et al., 2010; Ottoson et al., 2011b; Pang et al., 2017; Tromp et al., 2010). However, these QMRA evaluations have almost always exclusively considered detectable, quantifiable vegetative bacteria; presumed homogenous growth and decay rates; and virulence for the microbial populations in the study. The previous QMRA models also address uncertainties in either pre-harvest contamination sources or post-harvest, processing handling conditions including cross contamination during washing and chopping, retail or home storage times and temperatures. Therefore, health risks associated with the times and temperatures during post-harvest, pre-processing handling and the induced changes in the pathogen have not been well established – a gap in our understanding of another potential critical control point. Our goal was to characterize physiological changes in the population of *E. coli* O157:H7 cells on leafy greens during pre-processing handling and refrigerated storage that may impede detectability and increase stress resistance and/or virulence. QMRA can be used to evaluate how these changes affect potential health risks so that critical control points within the farm-to-fork pathway can be identified and prioritized. A QMRA that specifically models the actual post-harvest practices including the environmental conditions and durations within each step will support the development of strategies to reduce health risks and potential outbreaks.

Research Methods and Results

Objective 1: Methods

Lettuce varieties and growth

Lactuca sativa var. Solid King and Parris Island were grown in the MSU Greenhouse Complex at 18°C to 24°C, with lights on for 14 h during the day cycle.

Harvest and cut-to-cool data and analysis

Cut-to-cool data was obtained from an industry partner for Romaine lettuce harvested in two production regions, Salinas Valley, CA, and Yuma, AZ. The measurements detailed in the dataset included harvest temperatures, produce temperatures at the start and end of cooling, harvest-to-cool time, harvesting start and end time and cooling start and end time. R programming language was used to generate summary statistics of the dataset. Median, 5^{th}, 25^{th}, 75^{th} and 95^{th} percentiles of the harvest temperature, cooling time, and cut-to-cool times were generated for use in subsequent experiments.

Strains

The *E. coli* O157:H7 strains used in this study are PNUSAE013458 (associated with the Yuma 2018 lettuce outbreak), PNUSAE019890 (associated with the Central Coast, CA 2018 outbreak), and PNUSAE044369 (associated with the Salinas 2019 lettuce outbreak). The Central Coast 2018 (also referred to as the Santa Maria 2018) and Salinas 2019 outbreak strains have recently been classified by the CDC as belonging to a reoccurring, emerging, or persistent subtype, REPEXH02 (Chen et al., 2023).

Inoculum growth and preparation
Bacterial strains were selected for rifampicin resistance through a sequential exposure to increasing concentrations of the antibiotic, starting at 4 ng/mL and reaching 80 ng/mL in 4 days. These strains were stored in 75% glycerol at -80°C, and for all experiments, a fresh streak was initiated from the freezer stock on each occasion. Bacteria from -80°C storage were streaked onto TSA with 80 µg/mL rifampicin and incubated at 37°C for 18 h. The next day, a single colony was transferred to 5 ml TSB with 80 µg/mL rifampicin, incubated at 37°C, 150 rpm for 18-20 h. On the following day, 100 µL from the previous culture was added to 100 mL TSB with 80 µg/ml rifampicin and incubated at 37°C, 150 rpm for 18 h. The culture was centrifuged at 8000 rpm for 10 min, the pellet suspended in PBS, and this suspension was used for inoculation.

Inoculation of Romaine lettuce
Romaine lettuce was inoculated with *E. coli* O157:H7 by spray inoculation with a Miles hand-held reagent sprayer in a biosafety cabinet. Each plant was kept in a container, closed with a plastic wrap, and bacteria was misted onto the surface of the Romaine plant through the slits made on the plastic wrap. Twenty plants were inoculated at the same time for one biological replicate of one strain. The target initial inoculum was ~ 5.5-6.0 log CFU/g.

Sample collection
After inoculation, Romaine lettuce plants were held at 17°C for 1 day in a CARON PLANT Growth chamber 730X-22-X. At the time of harvest, a sterilized knife was used to cut the plant at the base. Harvested plants were placed into a plastic lined bin and held at either 17°C or 9°C for 4 h and then moved to cold storage at 2°C for 5 days. At every time point, one whole head of harvested lettuce was chopped into strips of about 2”x5” with a sterilized knife on a disposable plastic cutting board in a biosafety cabinet. It was then thoroughly mixed in 16”x20” Whirl-Pak bags to ensure a uniform sample. Measurements of culturable cells, persister cells, and total viable cells were conducted at the time of inoculation, before and after harvest, and every day for 5 days of cold storage at 2°C. Acid tolerance and chlorine tolerance as well as virulence were assessed at the same time points.

Quantifying culturable cells
Ten grams of lettuce were homogenized in a 7.5”x12” filter Whirl-Pak stomacher bag with 90 mL PBS buffer for 1 minute. From this,100 µL was used for making dilutions in PBS (phosphate buffer saline) and culturable cells were quantified on TSA+ rifampicin plates.

Quantifying persister cells
From the same bag of homogenized lettuce used for culturable cells, a 1 mL sample underwent a 3-hour exposure to 10X MIC of ciprofloxacin at 150rpm, 37°C. The survivor cells were captured through a 0.22 um filter, were vortexed in 1 ml PBS for 3 minutes, and dilutions were plated to quantify persister cells.

Quantifying total viable cells and VBNC
From the same bag of homogenized lettuce used for culturable cells, a 10 mL sample was taken and centrifuged at 10000 rpm for 10 min at 8°C. The supernatant was discarded and 1000 µL of PBS was added to suspend the cell pellet, followed by 250 µL of PMA enhancer (Biotium, US) and PMA (Biotium) for a final concentration of 50 µm (3.125 µL of 20 mM). Subsequently, the samples were stored in the dark for 10 min, with frequent tube inversion. The next step involved exposing the samples to UV light for 30 min in the Biotium device, with intermittent vortexing at 2,000 rpm for 5 seconds every 10 min. The DNA extraction was carried out using the Powerlyzer kit from Qiagen. A working solution was prepared by mixing SYBR green (Qiagen),

qPCR grade water and Z3276 (forward- 5′-GCACTAAAAGCTTGGAGCAGTTC and reverse- 5′-AACAATGGGTCAGCGGTAAGGCTA) primers targeting ORF Z3276 gene (B. Li & Chen, 2012) at the ratio of 10:6:2. To 18 µL of the working solution, 2 µL of the sample was added and taken to the MSU Genomics core for reading in the QuantStudio 7 Flex qPCR (Thermo Fisher Scientific). The total viable counts of the samples were determined for the qPCR readings using a prepared standard curve (R^2>90%). The VBNC fraction was quantified by subtracting the total culturable fraction from the total viable cells.

Acid tolerance assay
Ten grams of lettuce were homogenized in a stomacher bag with 90 mL of filter-sterilized synthetic gastric fluid (SGF) (Beumer et al., 1992) at pH 2.5. The mixture was homogenized for 1 min at normal speed in a Seward STOMACHER 400. After appropriate dilutions, the homogenized solution was plated on rifampicin-supplemented TSA to quantify culturable cells pre-acid exposure. Bags were incubated at 37°C for 2 h. Subsequent dilutions were plated to quantify surviving culturable cells post 2-hour acid stress exposure.

Chlorine tolerance assay
Forty grams of lettuce in 160 mL of KH2PO4 served as a negative control for culturable cell quantification, while another 40 grams were exposed to 20-25 ppm chlorine for 30 s in a 1L KH_2PO_4 solution with (XY-12). Free chlorine was measured using a Thermo SCIENTIFIC ORION AQUAfast AQ3700 device with the test kit-AC2071. To neutralize chlorine, lettuce was mixed with 160 ml of KH2PO4 and 90.4 µL of sodium thiosulfate in a Stomacher bag, then homogenized for 1 min. Dilutions were plated on rifampicin-supplemented TSA to quantify bacterial culturable cells post 30 s oxidative stress exposure.

Virulence assay using *G. mellonella*
The *G. mellonella* killing assay serves as a valuable tool in evaluating the virulence of bacterial strains (Cardenas-Alvarez et al., 2019; Ciesielczuk et al., 2015; Kuenne et al., 2013; Morgan et al., 2014). The protocol involves the transfer of strains from freezer stocks to LB agar plates, followed by overnight incubation at 37°C. Serial dilutions are then prepared, and cultures are plated to determine culturable cells. The bacterial cultures are centrifuged, resuspended, and injected into *G. mellonella* larvae at different concentrations using sterile Hamilton syringes and TRIDAK STEPPER to do baseline assays in triplicate for each strain. To identify the optimum inoculum, the dilution that kills 50% of larvae after 24 h at 37°C was chosen. The larvae are subsequently incubated for up to 5 days at 37°C in the dark, and survival is assessed every 24 h. For experiments with inoculated lettuce, at each timepoint 10 g of lettuce was added to 90 ml of PBS in a 7.5”x12” filter Whirl-Pak stomacher bag and homogenized at normal speed for 1 min. Out of this, a 1 mL sample was taken, and was also diluted 1/10. The larvae were injected with 20 µL of the sample and 20 µL of the 1/10 sample dilution. Larvae injected with PBS and only stabbed with no solution were used as negative controls. Killing assays were conducted for three biological replicates of each of the three strains at both harvest temperatures.

Data analyses:
Generating standard curve for qPCR
Stock cultures stored at -80°C were streaked onto TSA + rifampicin plates and incubated at 37°C for 24 h. A single colony from the streaked plates was transferred to 5 mL of LB + rifampicin broth and vortexed at 2,000 rpm for 5 s. The inoculated broth and a negative blank were incubated at 37°C with shaking at 150 rpm for 18-20 h. The negative control should have no growth. From the 5 mL broth, 100 µL was added to 100 mL of LB + rifampicin broth and vortexed. The inoculated broth and a negative control were incubated at 37°C with shaking at 150 rpm for 18 h. If the negative control had no growth, dilutions corresponding to microbial

loads of 10^8, 10^7, 10^6, 10^5 and 10^4 were added to stomacher bags with 10 g of lettuce. Samples were homogenized for 1 min in the stomacher and 10 mL collected in centrifuge tubes. Tubes were centrifuged at 10000 rpm for 10 min, supernatant was decanted, and the resulting cell pellet was mixed with 1 mL of PBS. To 1 mL of sample, 250 mL of PMA enhancer (Biotium) was added followed by PMAxx (Biotium) to achieve final concentrations of 75 µm (4.688 µL of 20 mM) and 50 µm (3.125 µL of 20 mM). The samples were exposed to the dark for 10 min with intermittent mixing followed by exposure to UV light for 30 min with intermittent vortexing at 2,000 rpm for 5 s every 10 min. The DNA was extracted using the Qiagen Powerlyzer kit and mixed with qPCR SYBR working solution prepared as described earlier. The samples were taken to the MSU Genomics core for reading in the QuantStudio 7 Flex qPCR (Thermo Fisher Scientific). Samples with 10^8, 10^6 and 10^4 loads were plated to verify concentrations. A standard curve of plate counts against qPCR readings was prepared, targeting R^2>90%. Standard curves were based on three biological replicates of each *E. coli* O157:H7 strain.

Statistical analysis

Analysis of the data was done in the R programming language (R Core Team, 2022), where the harvest temperature, strain, and cold storage time were identified as independent variables and log reduction values, microbial counts and fraction of the physiological states were the response variables. Summary statistics of the response variables grouped by single or combinations of independent variables were generated using the functions in the dplyr package (Wickham et al., 2023). A test for normality of the data was done using the Wilk's Shapiro test and the outliers in the data visualized using the box plots. For data that had normal distribution and limited or no outliers, correlation between the response variables and the cold storage time was tested using Pearson correlation, otherwise, Spearman rank correlation was used. The effect of the interaction of harvest temperature and strain was tested using analysis of covariance (ANCOVA) that had cold storage time, or in the case of chlorine tolerance the concentration of chlorine before treatment acted as the covariate. Means that were statistically ($p < 0.05$) different were separated using estimated marginal means in the emmeans package (Russell et al., 2024). Regression tree analysis was done to test the effect of all the independent variables on the response variable using the rpart package (Therneau et al., 2023). Wherever the assumptions of normality were violated, beta regression in case of proportions and generalized linear models were used.

Objective 1: Results

Selection of parameters for harvest temperature and time

The temperature data used in this study was collected using automated data loggers for a period of 3 years and 3 months (January 2016 to April 2019). Anonymized data was captured for the bin temperature of Romaine lettuce immediately after harvest and before and after cooling. A total of 5,615 and 4,623 entries were collected from the Salinas and Yuma regions, respectively, over the period. The average temperature of lettuce at harvest was 56.4 ± 8.1 °F whereas after cooling it was 35.1 ± 4.3 °F. A larger proportion of lettuce harvested in Salinas had significantly higher temperature than that harvested in Yuma ($p < 0.001$, Mode's Median test), while at the end of cooling, the produce had comparatively similar temperatures. The distribution of temperature of Romaine lettuce at harvest was a skewed distribution. The median cut-to-cool time was significantly shorter for Romaine lettuce harvested in Salinas (163 min) than in Yuma (232 min) ($p < 0.001$, Mode's Median test). The distribution of cut-to-cool times was a skewed distribution. Upon receipt at the cooling center, the difference in the median time taken to cool the lettuce in Salinas and Yuma was only 1 minute. Based on these data, we selected the 75th percentile for harvest to entry to cooling center, 240 min, as our harvest time period. We selected the values for the 25th percentile of harvest temperature from the Yuma

region, 48.2°F (9°C), and the 75th percentile of harvest temperature from the Salinas region, 62.6°F (17°C) for our two harvest temperatures to evaluate in these experiments.

Minimal changes in cell physiology during 4h at harvest temperature

To evaluate the effect of harvest temperature on *E. coli* O157:H7 physiology, culturable cells, persister population, and VBNC populations were quantified before and after incubating inoculated lettuce at each harvest temperature for 4 hours. Culturable cells did not significantly ($p > 0.05$) differ during the 4 h incubation for all strains between the two harvest temperatures (**Figure 1**). A slight increase in the percentage of the population in the persister state was found during harvest, with median values of 1.20–21.5% at time of harvest to 3.04–27.5% after 4 h at 9°C and 2.06–10.5% at the time of harvest to 4.51–14.0% after 4 h at 17°C, though the change was not significant (**Figure 2**). Regression analysis of the effect of harvest temperature on each strain showed no significant ($p > 0.05$) differences in the persister fraction at harvest. Similarly, the VBNC counts of strains on the Romaine lettuce did not significantly differ ($p > 0.05$) from the time of harvest to 4 h at harvest temperatures 9 and 17°C (**Figure 3**).

Survival of culturable cells on Romaine lettuce during cold storage

Correlation analysis of the log reduction of culturable cells during cold storage showed no significant trend ($p > 0.05$) for strains on Romaine lettuce harvested at either 9 or 17°C. The Central Coast 2018 outbreak strain had the highest average log reduction over 5 days of cold storage, 0.54 and 0.61 log for Romaine lettuce harvested at 9 and 17°C, respectively. This strain had comparatively higher reduction in culturable cells over the period of storage compared to the other two strains (**Figure 4**). The harvest temperature did not significantly influence the survival of culturable cells during cold storage. There was no significant ($p > 0.05$) difference in the reduction of culturable cells on Romaine lettuce during cold storage when it was harvested at 9 or 17°C. Regression tree analysis showed the Salinas 2019 strain averaged the lowest log reduction (-0.11) over 5 days of cold storage, whereas the outbreak strains Yuma 2018 and Central Coast 2018 averaged 0.43 logs (**Figure 5**). The Central Coast 2018 strain on lettuce stored for more than 12 h at 2°C averaged the highest log reduction (0.72).

Entry into dormant states on Romaine lettuce during cold storage

During cold storage, the percentage of cells in the persister state on Romaine lettuce harvested at 9°C had an average increase from 14.1 to 29.0% over 5 days of cold storage, which was higher than the average increase in persister cells on Romaine lettuce harvested at 17°C, with an average increase from 16.6 to 21.9% over the same period. Trend analysis of persister cell formation on lettuce during cold storage showed weak positive correlations for lettuce harvested at 9 ($r=0.12$) and 17°C ($r=0.14$). Over the 5-day cold storage period, all strains had comparatively higher median values of persister cell percentages for lettuce harvested at 9 than 17°C (**Figure 6**). Model fitting the effects of strain, cold storage time, and harvest temperature showed that harvest temperature, strain and cold storage individually affected the percentage of cells in the persister state (**Figure 7**), however, their interaction was not significant ($p > 0.05$). The percentage of cells in the persister state for the Yuma 2018 strain was less likely ($\beta=-0.48$, OR=0.6) to increase during cold storage compared to the Central Coast 2018 strain. Romaine lettuce harvested at 9°C had a significantly ($p < 0.001$) higher increase in percentage of cells in the persister state compared to lettuce harvested at 17°C. The percentage of cells in the persister state significantly ($p=0.008$) increased as cold storage time increased.

Notwithstanding the harvest temperature, VBNC formation for the Central Coast 2018 strain increased over the cold storage period, with an increase of 0.55–0.64 logs by the fifth day of cold storage. This strain consistently had a greater log increase compared to the Salinas 2019 and Yuma 2018 strains (**Figure 8**). For the Yuma 2018 strain, lettuce harvested at 9°C had a

higher VBNC accumulation than lettuce harvested at 17°C. The interaction of the harvest temperature and the strain significantly ($p < 0.001$, df=2) affected the change in the number of cells in the VBNC state during cold storage. The Central Coast 2018 strain had the highest increase in VBNC cells when Romaine lettuce was in cold storage for more than 12 hours (**Figure 9**). The lowest increase in VBNC cells occurred in the Salinas 2019 strain on lettuce in cold storage for less than 84 hours.

Acid and chlorine tolerance

Tests for correlation showed that cold storage time had a negative relationship ($r=0.25$, $p=0.023$) with log reduction during exposure to pH 2.5 of strains on Romaine lettuce harvested at 9°C, indicative of decreasing acid tolerance over cold storage. However, this was not the case for strains on lettuce harvested at 17°C ($r=-0.02$, $p=0.841$). Comparison of the effect of harvest temperature showed that the Salinas 2019 strain was less acid tolerant following cold storage for lettuce that had been harvested at 9°C, as it had higher log reduction values at 9°C than at 17°C. The effects of the interaction of all the factors were shown using regression trees (**Figure 10**). The lowest log reduction value (-0.06) was found for lettuce harvested at 17°C that was inoculated with the Salinas 2019 and Yuma 2018 strains and in cold storage for less than 36 hours. On the other hand, Romaine lettuce harvested at 9°C that was inoculated with the Salinas 2019 strain and stored for more than 36 hours had the highest reduction when exposed to pH 2.5. The study established that the effect of cold temperature storage on acid tolerance varied across strains, for instance the Salinas 2019 strain had higher acid tolerance on lettuce that was harvested at 17°C than at 9°C during the cold storage period. Strains on lettuce harvested at 9°C showed reduced acid tolerance with increasing cold storage time.

There was no significant ($p > 0.05$) relationship between reduction due to chlorine treatment and cold storage time for strains on Romaine lettuce harvested at 9 ($r=0.06$) and 17°C ($r=0.19$). The average log reduction values due to chlorine exposure for the strains during the period of cold storage for lettuce harvested at 9 and 17°C was 0.53–0.73 and 0.58–0.88, respectively. ANCOVA analysis with chlorine concentration as a covariate showed the interaction between strain and harvest temperature significantly ($p=0.012$) affected the reduction due chlorine treatment of the lettuce. The Salinas 2019 strain had a higher log reduction due to chlorine exposure on lettuce harvested at 9°C than at 17°C. The interaction of the Salinas 2019 strain and chlorine concentration significantly ($p=0.028$) affected log reduction values on lettuce over the 5 days of cold storage (**Figure 11**). Higher reduction due to chlorine exposure was observed for the Salinas 2019 strain compared to the Central Coast 2018 strain. The reduction due to chlorine exposure varied across strains and harvest temperatures, though comparatively less chlorine tolerance was reported with increasing cold storage period.

Virulence

Strains inoculated onto Romaine lettuce that underwent harvest and cold storage were used to assess virulence in the *G. mellonella* model. Overall, the Central Coast 2018 strain was established as the most virulent out of the three strains, with lower survival of the inoculated *G. mellonella* larvae over a 4-day incubation period. Correlation tests revealed that after 4 days of incubation of larvae after inoculation, only the Central Coast 2018 strain had a positive correlation ($r=0$ 0.30) at harvest temperature 17°C. No significant ($p > 0.05$) correlations were reported at 9°C in any of the three strains. This is indicative of reduced virulence of the Central Coast 2018 strain over the duration of cold storage for the Romaine lettuce harvested at 17°C.

Objective 2: Methods

Strains used

The three outbreak strains described in Objective 1 were also used for these experiments.

Preparation of simulated agricultural water
Nutrient-limited agricultural water was prepared by adding ammonium ($2.67X10^{-4}$ M or Molar ammonium sulfate), phosphate ($2.6X10^{-5}$ or M potassium phosphate monobasic), sodium ($2.29X10^{-3}$ M sodium chloride), nitrate ($3.7X10^{-5}$M potassium nitrate), calcium ($9.2X10^{-4}$ M calcium chloride), and magnesium ($3.65X10^{-4}$ M magnesium chloride) (Avery et al., 2008). The water was sterilized by filtering through at 0.22 µm pore size filter. Sterilized agricultural water in 100 mL flasks was inoculated with three strains individually.

Growth of strains and inoculation into simulated agricultural water
Rifampicin resistant bacterial strains from -80°C stocks were streaked onto rifampicin supplemented LB plates and incubated at 37°C for 18-20 h. One colony was used to inoculate 5 mL of LB supplemented with rifampicin and grown at 37°C for 18 h at 150 rpm. A 1 mL sample was centrifuged at 4°C at 8000 rpm for 15 min, and the pellet was used for inoculation of agricultural water. Flasks with 100 mL of nutrient-limited agricultural water were inoculated with a target inoculum of 5.5–6.5 log CFU/mL and incubated at 15°C. This was done at different times, including 2,4,7,10,12, and 15 months before the detection experiments. One flask per strain was inoculated 2 days before the time of detection to serve as a control with a low level of persisters.

Monitoring persisters over time
Over time, cold stress and nutrient stress led to the formation of persister cells. The persister cell proportion differed in flasks mostly due to the length of time of incubation. From each flask, a 100 µL sample was diluted and plated on LB plates for quantification of culturable cells. For quantification of persister cells, 1 mL of sample was directly taken from the flasks and the procedure described in Objective 1 was followed.

Inoculation of lettuce for detection assays
Positive controls had freshly grown cultures added to lettuce samples. From –80°C freezer stock, bacteria were streaked onto rifampicin supplemented LB plates, and grown for 18-20 h at 37°C. Five mL of LB supplemented with rifampicin was inoculated with one colony and incubated at 37°C for 18 h at 150 rpm. Appropriate dilutions were made to inoculate 25 g of lettuce individually with approximately 2 MPN of each strain. To evaluate the impact of cells in the persister state on detection, samples were selected for inoculation of lettuce based on the percentage of cells in the persister state. A total of 21 flasks of inoculated agricultural water were set up, and 4-5 for each strain were used. Persister percentages were evaluated over time as the inoculated flasks were incubated at 15°C. Samples were selected based on persister percentages, to correspond with the following ranges: <1%, 1-4.9%, 5-9.9%, 10-19.9%, 20-40%, and 40-85%. A total of 27, 29, 14, 12, 12, and 6 samples had persister percentages of <1%,1-4.9%, 5-9.9%, 10-19.9%, 20-40%, and 40-85%, respectively. Twenty-five g of lettuce was placed into a Whirl-Pak bag and was targeted to be inoculated with 2 and 15 MPN of each strain at each level of persister percentages. They were then stored at 3°C for 21-22 h. Samples were sent to Eurofins (Battle Creek, MI) for BAX O157 testing; some samples were used for ANSR O157 testing, and some were used for calculation of MPN (most probable number).

Detection assays used
Two different detection assays were used: BAX for O157 and ANSR for O157. The BAX O157 testing was evaluated with two different enrichment times, 8 and 24 h, representing the range of potential enrichment times that can be used with this method. The ANSR for O157 enrichment time has only one option for leafy greens, which is 24 h. For BAX testing, samples were sent via local courier to the Eurofins laboratory (Battle Creek). An additional set of samples were

analyzed with Neogen's ANSR for *E. coli* O157:H7. For each 25 g of inoculated lettuce, 100 mL of freshly made ANSR *E. coli* O157:H7 enrichment media was added and samples were incubated at 42°C for 24 h. 50 µL of enriched sample and 450 µL of lysis reagent was added to each cluster tube. These were transferred to 37°C heat block for 10 min and then to 80°C heat block for 20 min. Out of this lysed sample, 50 µL was transferred to preheated lyophilized reagents at 56°C. After vortexing, tubes were placed in the ANSR reader and START command was given in the ANSR software. Reports were displayed as positive, negative, or invalid.

Determining MPNs

MPN was determined using the 5-tube MPN method scaled down for 96-well plates. 100 mL of TSB supplemented with 100 µg/mL rifampicin was added to inoculated lettuce (25 g), five aliquots of 1 mL were added to 96-well plates and were diluted twice in TSB supplemented with 80 µg/mL rifampicin. Plates were incubated at 37°C for 24 h and growth was noted for each well at each dilution. These results were used with the FDA MPN calculator to determine MPN for each sample (https://mpncalc.galaxytrakr.org/).

Objective 2: Results

Differing detection based on enrichment time

A set of control samples were evaluated for each strain. These consisted of each strain grown in LB (as described above), diluted in PBS and inoculated onto 25 g of Romaine lettuce at ~ 2 MPN. For the control samples, the Yuma 2018 strain was detected in 3/3 lettuce samples at both 8 and 24 h enrichment times. The Central Coast 2019 strain was detected in 0/3 lettuce samples with 8 h enrichment, but in 3/3 samples with 24 h enrichment. The Salinas 2019 strain was detected in 1/3 lettuce samples with 8 h enrichment, but in 3/3 samples with 24 h enrichment. These strain-based differences in detection by enrichment time were also seen in the larger experimental dataset when cultures were held in the simulated agricultural water to induce persister formation prior to inoculation onto lettuce.

A total of 208 spiked Romaine lettuce samples were evaluated using the BAX O157 method. 104 of the samples were incubated for 8 h prior to detection, while a duplicate set of 104 samples were incubated for 24 h prior to detection. For the samples incubated for 8 h, 78 (75%) were not detected, while 26 were presumptive positives (26/104, 25%). For the samples incubated for 24 h, 24 (23%) were not detected, while 80 were presumptive positives (80/104, 77%). No differences in detection were observed based on persister percentages (<1%–85%), but there were differences in detection by strain.

Of the 40 lettuce samples inoculated with the Yuma 2018 strain, 16 (40%) were positive after 8 h enrichment, while 32 (80%) were positive after 24 h enrichment. Of the 40 lettuce samples inoculated with the Central Coast 2019 strain, 9 (23%) were positive after 8 h enrichment, and 30 (75%) were positive after 24 h enrichment. Of the 24 samples inoculated with the Salinas 2019 strain, only 1 (4%) was positive after 8 h enrichment, while 18 (75%) were positive after 24 h enrichment.

Detection using different assays

Detection using the BAX for O157:H7 and the Neogen ANSR for *E. coli* O157:H7 were compared for a set of 12 samples with 24 h enrichment time. For Yuma 2018 and Central Coast 2019 strains, similar results were seen with both test types. Out of 12 inoculated lettuce samples for each strain, the Yuma 2018 strain was detected in 10 samples and the Central Coast 2019 strain was detected in 7 samples using both systems. For the Salinas 2019 strain, 9/10 samples were detected using the ANSR system and 11/12 samples were detected using the BAX system.

Objective 3: Methods

The following sub-objectives were completed in order to establish microbial kinetic data appropriate for completing a comprehensive risk assessment under various conditions across the value chain:

Systematic review of factors influencing growth and decay

A systematic review was done following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A string of search terms including "((("*Escherichia coli*" AND O157:H7) OR ("*E. coli*" AND O157:H7) OR (Ec.O157:H7) OR ((Shiga AND Toxin AND Producing) OR (enterohemorrhagic) OR (Verocytoxin AND Producing) AND *Escherichia* AND *coli*) OR EHEC OR STEC OR VTEC OR Pathogen OR Bacteria) AND (Growth OR Decay OR Kinetics OR Surviv* OR Fate OR Persistence) AND ((Leafy OR Green) AND Vegetables) OR "Salad Green" OR Lettuce OR Romaine OR "Lactuca AND sativa" OR Spinach OR "Spinacia oleracea" OR Iceberg" was used to extract review articles from Scopus and PubMed Central databases between April 1st and June 20th 2022. Only articles that focused on experimental trials, and if secondary data was used, details of the experimental conditions generating the data were provided were included in the study. Furthermore, the time series growth and inactivation data must document at least four time points. Articles that focused solely on substrates were excluded from the review. Analysis of the articles was done by extracting the bibliographic information, experimental design, treatments, and stage of the value chain of all the eligible and a summary prepared.

Data mining - Extracting datasets for modelling decay

From selected articles in the review, we extracted 530 datasets detailing the environmental conditions, storage, packaging, and processing conditions. Additionally, information on the reported sanitization process and biological characteristics such as type of produce, bacteriophage and inoculum size were collected. The extracted datasets had to have a minimum of four time points for microbial concentrations to be included in the study for ease of fitting two and three parameter nonlinear models as described in previous works (Dean & Mitchell, 2022).

Fitting of the inactivation models

To distinguish the inactivation from the growth data, a linear regression model with derived log reduction values (LRV), calculated by dividing the concentration at a time point by the initial concentration in each study, as the response variable and the y-intercept fixed at zero was fitted to the 530 datasets. Only datasets exhibiting a statistically significant ($p < 0.05$) negative trend were considered for inclusion in the subsequent inactivation modeling. A suite of 17 inactivation models (2 one-parameter, 10 two-parameter and 5 three-parameter models) was fitted to the microbial die-off datasets using MLE in a three-step optimization process. Model selection was done by calculating the Bayesian information criterion (BIC) and the goodness of fit evaluated using normalized root mean square error (nRMSE) and adjusted R-squared (R^2).

Factor analysis of conditions influencing inactivation

The survival metrics of 1–7 log reduction time (LRT), the time taken to achieve 1–7 log reduction in microbial concentration, was calculated for datasets that had at least one model selected as the best fit. For datasets that were best fitted by more than one model, a model averaging approach that assigns weighting to the models was used to generate a model averaged survival metric. A variety of factor analysis techniques including linear regression, GLM, random forest, quantile regression, regression trees and principal component regression were used in the evaluating conditions influencing decay. The performance and predictive

power of the factor analysis methods was used in selecting the technique that best described the effect of the conditions based on the fitting of the training and testing datasets, respectively, of the survival metrics.

Quantitative microbial risk assessment of *E. coli* O157:H7 in leafy greens

To facilitate quantification of changes in *E. coli* O157:H7 risk over pre-processing cut-to-cool and refrigerated transport times, a peer-reviewed QMRA focusing on the farm-to-fork supply chain of Romaine lettuce, with the steps documented broadly as primary production, processing, transportation, storage and consumption, was selected to serve the basis for model development. The previously described QMRA for lettuce from farm-to-fork by Pang et al. (2017) was determined to be comprehensive and reproducible but did not consider the effect of change in physiological states due to exposure to stress factors which were studied herein and will be used to expand the model. The farm-to-fork QMRA framework considered on-field production of lettuce, with the eventual outcome as probability of illness upon consumption of the lettuce (Ottoson et al., 2011a). Mathematical parameters for the model were sourced from literature or calculated as outlined in the following steps.

Exposure assessment

1. On-field harvested produce contamination

The contamination routes for shredded-packaged lettuce included cross-contamination from soil and harvesting equipment and irrigation water, whereas the Romaine hearts included only the latter. The *E. coli* O157:H7 concentration in harvested lettuce was determined by calculating contamination resulting from irrigation water and cross-contamination from soil via harvesting tools (Pang et al., 2017). Contamination of lettuce arising from irrigation was determined based on the assumption that all pathogens in water remaining on the leaves after irrigation will attach to it (Pang et al., 2017). A uniform distribution (min=1, max=235) was used to describe the concentration of *E. coli* O157:H7 and the amount of water retained on the leaves determined based on a normal distribution (μ=0.108 ml/g, σ=0.019 ml/g) (Hamilton et al., 2006; Pang et al., 2017). The in-field inactivation of the cells was a described over the holding time post-irrigation, also referred to as preharvest holding time, that was described using a triangle distribution, min=2, max=8, mode=4 (Pang et al., 2017). The transfer of pathogens from the soil to the leafy greens was described using microbial concentrations and transfer coefficients utilized in a previous QMRA (Pang et al., 2017).

2. Cooling

Produce harvested from the fields was transported to the cooling plants for temperature reduction before being taken to processing or retail. The logger data from the industry partner was used to describe the distributions of temperature and time during transport and cooling. Distributions were fitted to the logger data using the fitdistr function (Fitdistrplus package) in R programming language, and the generated distributions evaluated using Kolmonogov-Smirnov (KS) test. Post-cooling storage and transportation data were both described using uniform distribution (min=0, max=48).

3. Processing

Whereas the shredded-packaged lettuce underwent processing that included the washing step, the Romaine hearts did not. The processing steps detailed in this QMRA included washing and shredding (Mokhtari et al., 2022). Washing potentially removes pathogen from romaine lettuce. The concentration of *E. coli* O157:H7 in washed produce was determined using triangular distribution of values sourced from literature, min=0.6 mode=1 and max=1.4 log (Pang et al., 2017). Cross-contamination of produce in processing was due to contact with processing surfaces such as flume tank, shredder, conveyor belts, shaker and centrifuge. A triangular

distribution was used to describe the final microbial concentrations in the produce due to cross-contamination using transfer rates from previous studies (Pérez Rodríguez et al., 2011). Inactivation during the chlorine washing was modelled using the parameters generated from our meta-analysis.

4. Storage and transportation

The transportation of produce occurs just after harvest, post-cooling, post-processing and retail-home. All transportation steps were considered in detailing microbial kinetics. As previously stated in a study, the critical temperature of 5°C was selected to indicate minimum growth conditions of *E. coli* O157:H7 in leafy greens in storage and transportation. To model real-world scenarios, a time-temperature profile obtained from an industry partner was used in this study, instances where there were gaps data was sourced from literature to describe distributions. Temperature-dependent microbial inactivation parameters were obtained from our meta-analysis study to show survival both in transportation and storage. Data for the retail-end to home storage temperature was generated using normal distribution, (μ=8.3858°C, σ=3.8314°C), truncated at 0°C and 20°C (Pang et al., 2017).

Dose response assessment

Beta-poison model focusing on the risk of illness due to exposure to foodborne *E. coli* O157:H7 that was used in earlier studies was used to derive the risk of illness per serving (Pang et al., 2017). Consumption data from the USDA Food and Nutrient Database for Dietary Studies 2019-2020 showed that serving sizes of lettuce consumed raw especially in salads is 70 g (USDA-ARS, 2022).

$$P = 1 - (1 + \frac{D}{\beta})^{\alpha}$$

Where P is the probability of illness due to ingested dose, D is the number of organisms per serving and α and β are parameters model parameters.

Risk characterization

The probability of illness due to consumption of contaminated lettuce was calculated by multiplying the prevalence by the probability of illness per serving (dose), with annual number of cases in the United States computed by multiplying probability of illness per serving with the number of servings consumed annually (Pang et al., 2017). Monte Carlo simulations were set at 10,000 iterations to describe the distributions. The end point of our model was the annual risk of illness that was calculated using the cumulative risk equation (Hamilton et al., 2006).

$$P_{A.ill} = 1 - (1 - P_{ill})^{np}$$

Where $P_{A.ill}$ is the annual risk/ person, P_{ill} is the risk of illness in per serving per person per day and np are the number of servings in a year per person. Sensitivity analysis using Spearman correlation test was done to determine input variables that are most associated with risk of illness.

Evaluation of different scenarios

In modelling different scenarios, we considered the harvesting temperature, median values generated from objective 1 and switch rates to VBNC fraction that was determined in objective 2. The VBNC switch rates were found to be determined by the harvest temperatures and cold storage time. The cold storage times considered in our scenarios were from 0 to 5 days.

Objective 3: Results

Identifying factors influencing microbial growth and decay

Using the search criteria and the inclusion and exclusion criteria, 65 articles were selected for the review. The supply chain of the leafy greens was documented as primary production (23), processing (20), transportation (4) and consumer and retail storage (34). It was evident that the least studied step in the supply chain was transportation. Descriptive analysis of the articles focused in identifying factors documented in various studies to affect growth or inactivation of E. coli O157:H7 in leafy greens in the farm-to-fork supply chain (**Figure 12**). The broad classification of factors identified by the review to influence the fate of *E. coli* in leafy greens included environmental, chemical and biological factors, and handling practices.

In primary production, environmental factors were identified as temperature (Ottoson et al., 2011b), relative humidity (Moyne et al., 2020), water quality especially during irrigation (Markland et al., 2013) and soil conditions (Ibekwe et al., 2007). The biological factors included type (Işık et al., 2020) and maturity of vegetables (Moyne et al., 2020) and physiological state and inoculum size of the cells (Bezanson et al., 2012; Moyne et al., 2013). Of the ten studies that studied temperature and other atmospheric conditions, five investigated the effect of atmospheric/environmental conditions outdoors. Other than affect microbial growth and inactivation, environmental factors such as temperature influenced the transition to non-culturable state (Dinu & Bach, 2011).

Processing steps were documented as cutting or shredding (Gleeson & O'Beirne, 2005) and washing and sanitization processes (Davidson et al., 2017; Gleeson & O'Beirne, 2005). The factors documented in processing steps included environmental such as temperature (Y. Li et al., 2001), chemical including use of sanitizers and chlorine washing (Abnavi et al., 2021), biological including size of inoculum and use of bacteriophages and handling practices such as shredding (Gleeson & O'Beirne, 2005). The most studied antimicrobial treatment was chlorine washing (12 articles). The concentration of chlorine solution in the studies was 12–50 ppm of free chlorine over treatment time of 30–90 seconds (Abnavi et al., 2021; Doering et al., 2009). Other antimicrobial agents studied included chlorine dioxide gas (Mahmoud & Linton, 2008), organic acids (Davidson et al., 2017) and non-conventional techniques of irradiation Neal et al., 2008), cold plasma nitrogen (Cui et al., 2018) and electrolyzed water Electrolyzed water (Posada-Izquierdo et al., 2014).

In storage, the identified factors included temperature (Uzeh & Adepoju, 2013), relative humidity (Wang et al., 2012) and packaging (Oliveira et al., 2010), the chemical factors detailed the effect of sanitizers in storage (Doering et al., 2009; Mahmoud & Linton, 2008) and the biological factor was size of the inoculum (Lee & Baek, 2008). The transportation stage had a single environmental factor identified, temperature (Koseki & Isobe, 2005).

Microbial inactivation models describing the die-off of *E. coli* O157:H7 in leafy greens

Of the 530 extracted datasets, 270 showed an inactivation trend ($r<-0.5$, $p < 0.05$). Of the inactivation datasets, 266 were successfully converged by at least of model in the suite. A preliminary study of these datasets detailed natural die-off which indicated inactivation due to natural conditions such as environmental factors and antimicrobial-induced die-off which was due to sanitization processes such as washing in chlorine. The datasets that showed natural die-off were 195, whereas 71 had antimicrobial-induced die-off. The majority (50.0%) of the datasets were best-fitted by the two-parameter log-logistic Juneja and Marks model (jm2). The one-parameter exponential model was selected for only 19.5% of the datasets. Across the stages in the supply chain, the two-parameter models best fitted higher proportions of the datasets than the one-parameter exponential model.

The log reduction time was generated utilizing the best fitted models to compare the performance of the model in describing pathogen survival (**Figure 13**). Whereas the exponential model generated higher median values for the 1–3 log reduction times (LRTs), the model averaged medians were higher for >4 LRTs (**Figure 13**). This depicts the tendency of *E. coli* O157:H7 to have tailing effect in its survival in leafy greens.

Factor analysis of conditions influencing microbial inactivation

Various factor analysis methods were used to evaluate the conditions describing the survival of *E. coli* O157:H7 in leafy greens. Random forest technique consistently had the best performance and predictive power on the training and testing datasets, respectively, thus was chosen for further evaluation. Lettuce (156), spinach (32), and cabbage (4) were the leafy greens that were studied in this meta-analysis for the natural die-off. Relative humidity, temperature and inoculum sizes were isolated as the most important factors that described pathogen survival in natural die-off. Interactions between relative humidity and temperature and size of the inoculum were significant (H statistics >0.1) in explaining the survival of the pathogen in natural die-off.

Of the datasets describing survival of the pathogen in antimicrobial-induced die-off, 60 focused on lettuce whereas 11 focused on spinach. The average concentration of chlorine washing solution was 18.5 (0.5–50) ppm free chlorine, and that of chlorine dioxide gas 7.66 (0.12–50) ppm. Organic acids were also documented as an antimicrobial treatment by six studies with an average concentration of 40.1 ppm (0.5–50) ppm. Random forest technique identified size of the inoculum, stage in the value chain and temperature as the most important factors in describing the inactivation of *E. coli* O157:H7 in leafy greens in antimicrobial-induced die-off. There were no significant interactions of the factors in affecting survival of the pathogen (H statistic <0.1).

QMRA tool for risk assessment

The risk of illness from consuming Romaine hearts, median = 4.61×10^{-8} $(95\% CI = 3.34 \times 10^{-6} - 8.22 \times 10^{-10})$, was higher than for the shredded-packaged lettuce, 9.37×10^{-10} $(95\% CI = 4.45 \times 10^{-5} - 3.54 \times 10^{-13})$ (**Figure 14**). Inactivation due to cold storage and transportation had a nonlinear pattern over time. Sensitivity analysis established that the post-processing (chlorine washing) including retail temperature, retail display time and home storage temperature are the most important model inputs impacting the predicted risk for shredded-packaged lettuce. On the other hand, field exposure factors such as amount of *E. coli* O157:H7 in the irrigation water and distribution factors such as the home storage temperature were the most important in impacting the risk in Romaine hearts. The effect of cold storage on VBNC switch rates resulted in minimal increase in the risk of illness for Romaine hearts harvested at warm (17°C), however no change in risk was reported for shredded-packaged lettuce.

Outcomes and Accomplishments

1. Impact of harvest temperature and length of cold storage on *E. coli* O157:H7 physiology and response to subsequent stress:
    - Harvest temperature (9 or 17°C) did not significantly change *E. coli* O157:H7 physiology, in terms of changes in culturable cells, percentage of the population in the persister state, or the amount of cells in the VBNC state.
    - During cold storage, culturable cells on Romaine lettuce decline in a strain-dependent manner, but the overall change is ~0.22 log CFU/g over the 5 days of cold storage.
    - During cold storage, the percentage of the population in the persister state varied by strain and by harvest temperature. The general trend is an increase in the percentage of persister cells on Romaine lettuce over time of cold storage, though this occurred to the greatest extent for the Central Coast 2018 strain on lettuce that was harvested at 17°C.
    - During cold storage, the amount of cells in the VBNC state also varied by strain and harvest temperature. The Central Coast 2018 strain, which also had greater percentage of cells in the persister state, also had higher levels of cells that were VBNC, also on lettuce that was harvested at 17°C.
    - While there were differences among the strains, the general trend was for acid tolerance and chlorine tolerance to decrease as the length of time in cold storage increased. This means that populations became more sensitive to acid stress (pH 2.5) and chlorine stress (free chlorine ~ 20 ppm) the longer the lettuce was held at 2°C prior to exposure to low pH or chlorine.

2. Effect of persister cell formation on detection of *E. coli* O157:H7 on Romaine lettuce:
    - Two different detection methods were used, and for one of the methods with a range of enrichment times, we assessed the shortest and longest enrichment times.
    - Detection of *E. coli* O157:H7 on Romaine lettuce was impacted by enrichment time. Detection was not influenced by the proportion of the population in the persister state but was impacted by strain. Strain based impacts on detection were notable for the shorter enrichment time (8 h) but were resolved with the longer enrichment time of 24 h.

3. Quantifying changes in *E. coli* O157:H7 risk over pre-processing cut-to-cool and refrigerated transport times:
    - Temperature had a multi-stage effect on the survival of microbial counts on the produce along the supply chain. However, limited studies documented microbial survival during transportation, making the stage the least studied.
    - Two-parameter rather than the one-parameter exponential models better fit the survival of *E. coli* O157:H7 in leafy greens. Therefore, the first order 1 LRT (log reduction time) is not adequate as a measure of the inactivation of pathogens especially in processes aimed at reduction of the microbial loads.
    - Whereas in natural die-off the interactions between relative humidity and either size of the inoculum or temperature affected microbial inactivation, such interactions were not reported for antimicrobial-induced die-offs. Overall, temperature was ranked the most important factor influencing microbial reduction both in natural and antimicrobial-induced die-offs.

- While the risk of illness due to consumption of shredded-packaged lettuce is low due to the inclusion of additional hurdles in processing, such as chlorine washing to prevent cross-contamination, Romaine hearts rely largely on temperature controls, and abuse of such present higher risks. Formation of VBNC cells due to cold stress during storage of Romaine hearts resulted in minimal increase in risk of illness, however, this had no effect on the risk associated with shredded-packaged lettuce. Among the factors that influenced predicted risks, the postprocessing temperature and time distributions were the most important for the shredded-packaged lettuce whereas for Romaine hearts in-field factors were also important.

Summary of Findings and Recommendations

- The longer Romaine lettuce was held in cold storage, the proportion of cells in dormant states (persister and VBNC) increased. This did not lead to overall greater stress tolerance of *E. coli* O157:H7 on lettuce, as both acid tolerance and chlorine tolerance decreased the longer the lettuce was in cold storage.
- Pre-processing time delays, indicated in our experiments by the length of time in cold storage prior to washing with 20 ppm free chlorine, did not result in greater tolerance of *E. coli* O157:H7 to chlorine.
- Improved detectability was observed for *E. coli* O157:H7 on Romaine lettuce when longer enrichment times were used.
- Whereas previous models developed for quantifying the risk of illness assumed a linear relationship for the inactivation of cells, our models incorporate temperature-dependent parameters to detail a nonlinear pattern.

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APPENDICES

Publications and Presentations

Publications

J. O. Owade, T. M. Bergholz and J. Mitchell. A meta-analysis of factors influencing the inactivation of Shiga toxin-producing *Escherichia coli* O157:H7 in leafy greens. [Under revision, Comprehensive Reviews in Food Science and Food Safety]

J. O. Owade, T. M. Bergholz and J. Mitchell. A review of conditions influencing fate of Shiga toxin-producing *Escherichia coli* O157:H7 on leafy greens. [Under review, Comprehensive Reviews in Food Science and Food Safety]

Presentations

D. Sharma, J. O. Owade, C. Kamphuis, A. Evans, J. Mitchell, and T. M. Bergholz. "Dynamics of change in physiological state of *E. coli* O157:H7 during cold storage of Romaine lettuce". International Association for Food Protection annual meeting, July 15, 2024. *Dimple was selected as a finalist for the developing scientist competition.

J. O. Owade, T. M. Bergholz and J. Mitchell. "A meta-analysis of conditions affecting decay and growth of *Escherichia coli* O157:H7 on leafy greens". Society for Risk Analysis Annual meeting, December 2023.

T. M. Bergholz. "Quantifying risk associated with changes in EHEC physiology during post-harvest pre-processing stages of leafy green production", CPS Symposium, Atlanta, Georgia. June 20, 2023.

T. M. Bergholz. "Quantifying risk associated with changes in EHEC physiology during post-harvest pre-processing stages of leafy green production", International Association for Food Protection Produce Safety Professional Development Group Webinar. March 21, 2023.

T. M. Bergholz. "Resilience of foodborne pathogens in the food supply", New Zealand Microbiology Society Conference, Wellington, New Zealand. November 22, 2022.

Poster Presentations

Owade, J. O., J. Mitchell, and T. M. Bergholz. Identifying the Best Fit Models Describing the Persistence of *Escherichia coli* O157:H7 in Fresh Vegetables Consumed in Salads. International Association for Food Protection Annual Meeting, Toronto, Canada. 2023.

Sharma, D., C. Catur, and T. M. Bergholz**.** Quantifying Physiological Profiles of Shiga Toxin-Producing *E. coli* O157:H7 during Post-Harvest Pre-Processing Stages of Romaine Lettuce Production. International Association for Food Protection Annual Meeting, Toronto, Canada. 2023.

T. M. Bergholz. "Quantifying risk associated with changes in EHEC physiology during post-harvest pre-processing stages of leafy green production", CPS Symposium, La Jolla, California. June 2022.

Budget Summary

The total amount of research funds awarded to this project was $328,442. As of this report, we have spent $308,628. Remaining funds will be used to support the research team's travel to the CPS symposium.

Figures 1–14

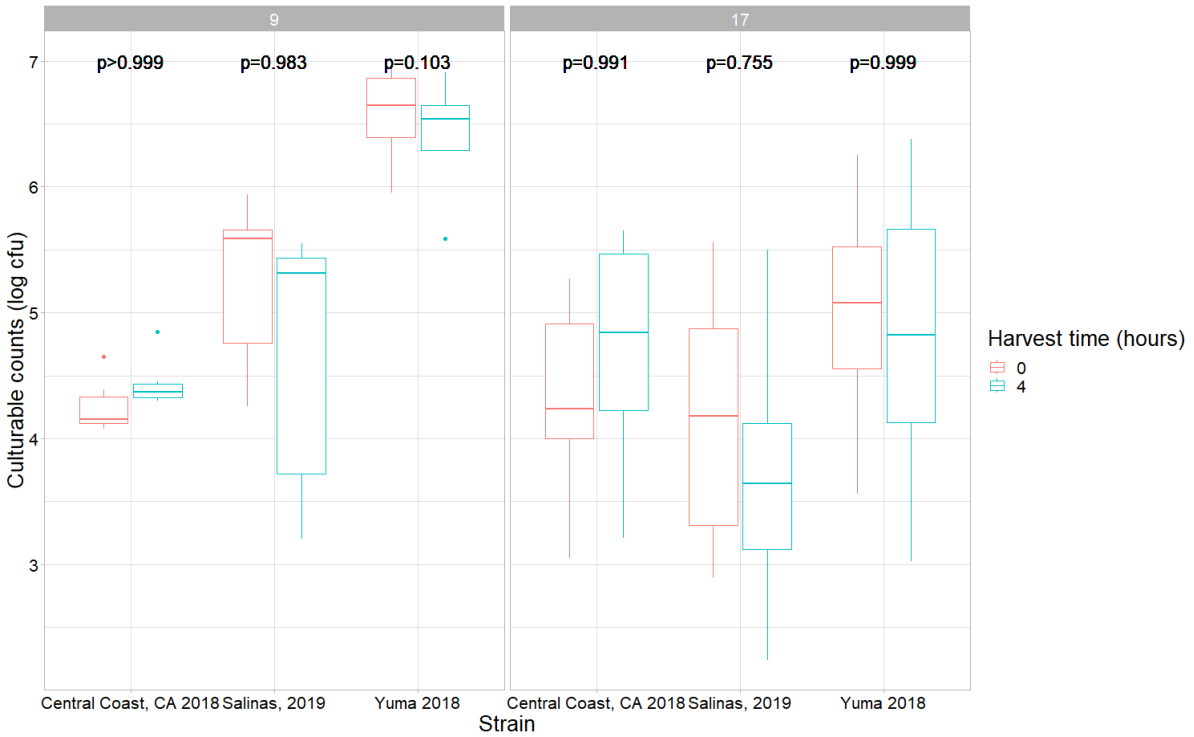

Figure 1: Effect of harvest temperature on the culturable cells of each *E. coli* O157:H7 strain on Romaine lettuce. Each boxplot shows the median value and the first and third quartile of the dataset. Points in the data indicated outside the range of the whisker are outliers in the dataset. The p-value indicated for each pair of boxplots depicts the test of statistical differences done using repeated measures analysis of variance and the means separated using Tukey's HSD.

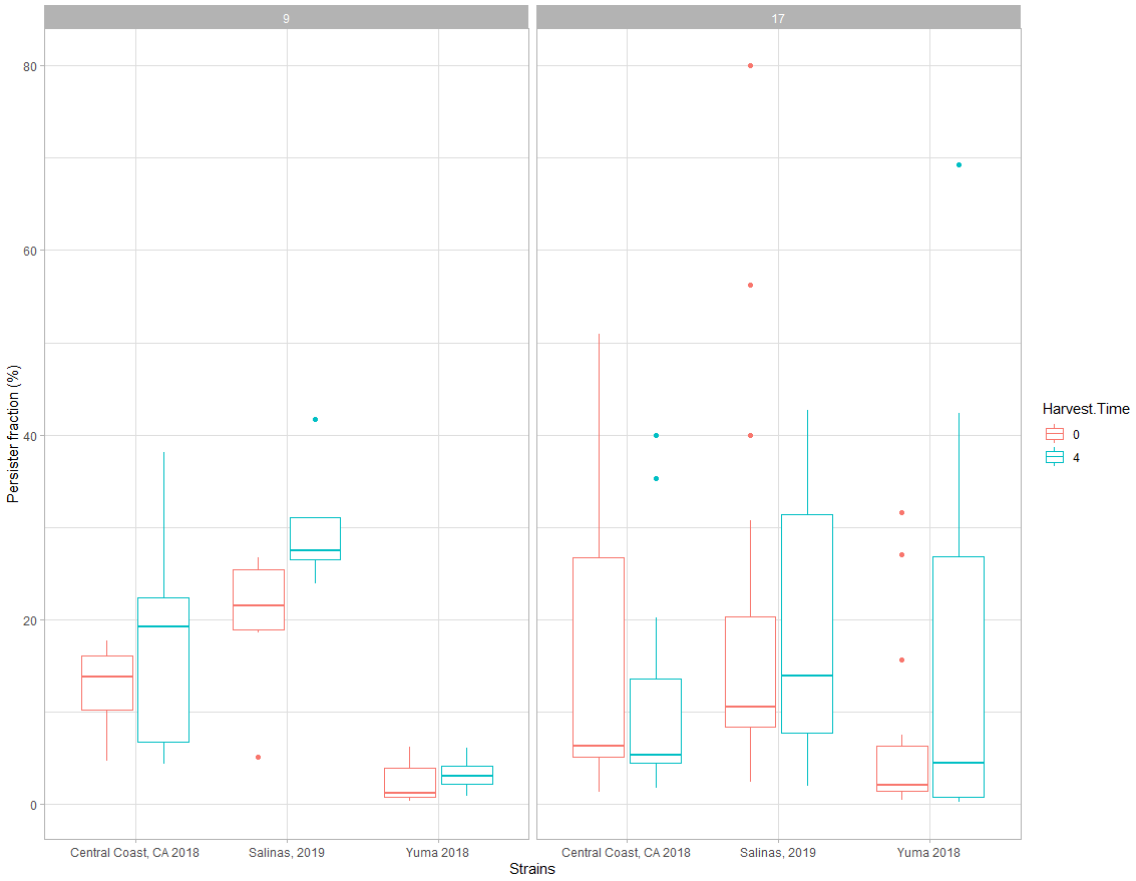

Figure 2: Box plots of the percentage of cells in the persister state for each *E coli* O157:H7 inoculated onto Romaine lettuce and harvested at 9 and 17°C.
The boxplots indicate the median values, first and third quartile of the data. The points beyond the whiskers are outliers in the data.

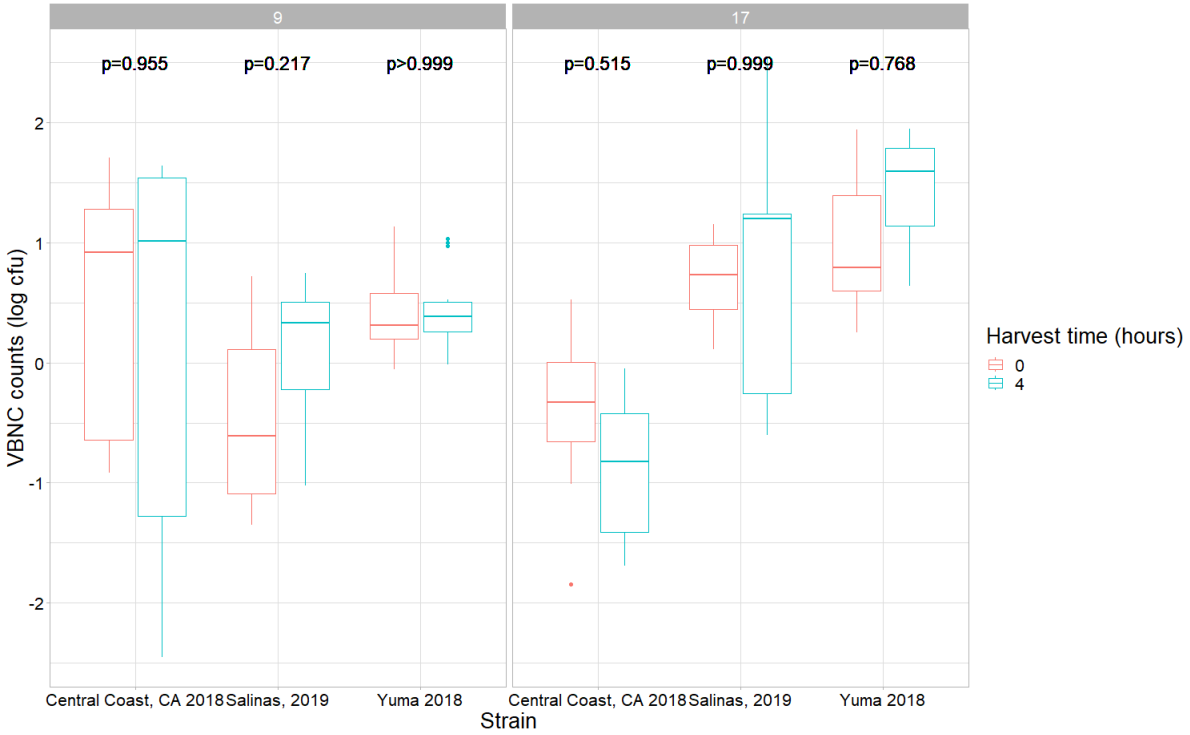

Figure 3: Effect of harvest temperature on the VBNC counts of *E. coli* O157:H7 strains on Romaine lettuce during the harvest period. The boxplots show the range of values for specific strain harvested at 9 and 17°C, with the harvesting done after 4 hours of being held at the 2 different harvest temperatures. The p-values indicate statistical difference between before and after harvesting. The p-value was generated using repeated measures analysis of variance, and the means separated using Tukey's Honest significant difference.

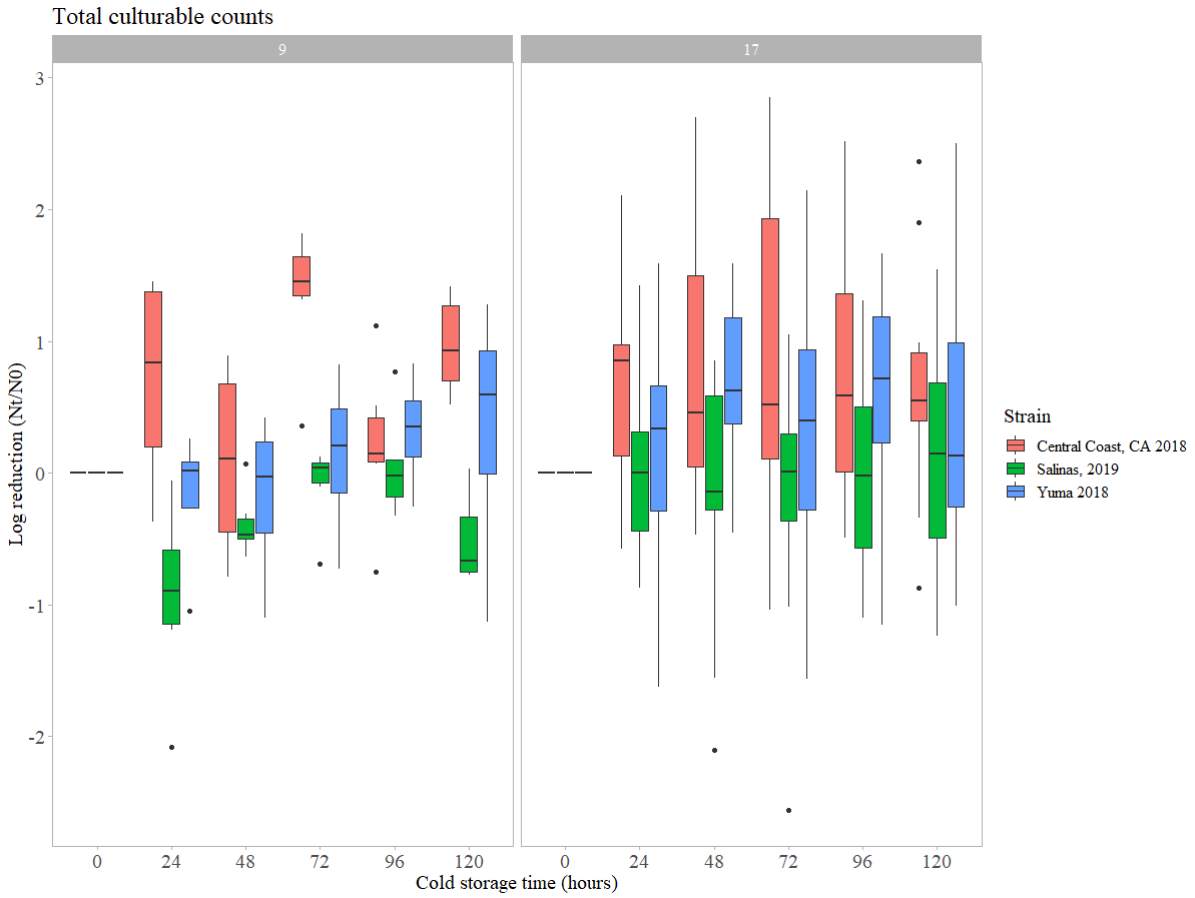

Figure 4: Boxplots of log reduction values for culturable cells of different strains of *E. coli* O157:H7 on Romaine lettuce that were harvested at different temperatures and stored at 2°C for 5 days.

Log reduction values were derived by calculating log (N_t/N_0) where N_t is concentration of microbial cells at time t and N_0 is the initial concentration at time zero of cold storage.

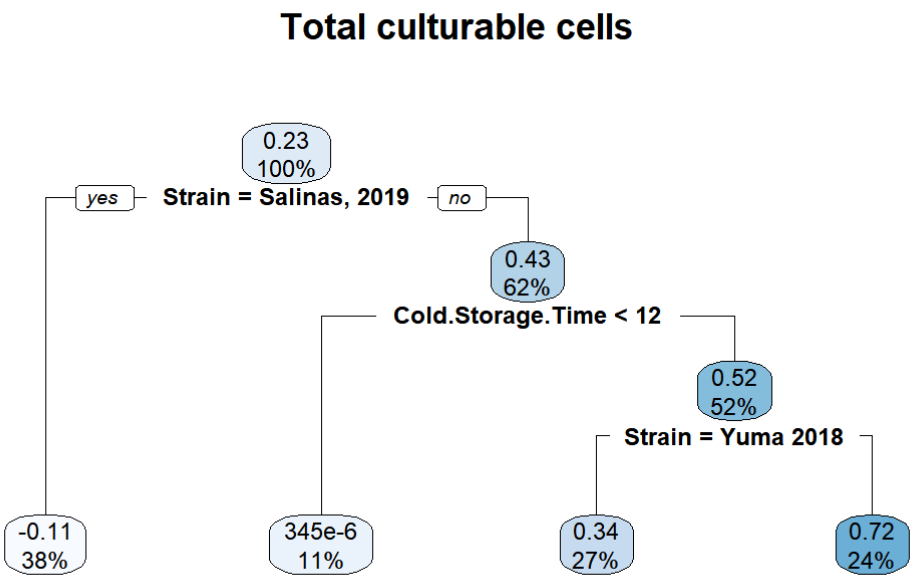

Figure 5: Regression tree of the effect of harvest temperature, strain and cold storage time on the log reduction of the culturable fraction. The log reductions were derived as Nt/N0, with Nt as culturable cells at time t and N0 as the initial population. The first node was split by the decision criteria strain, and the outbreak strains Central Coast and Yuma reported a higher reduction in the culturable fraction, than the outbreak strain Salinas 2019.

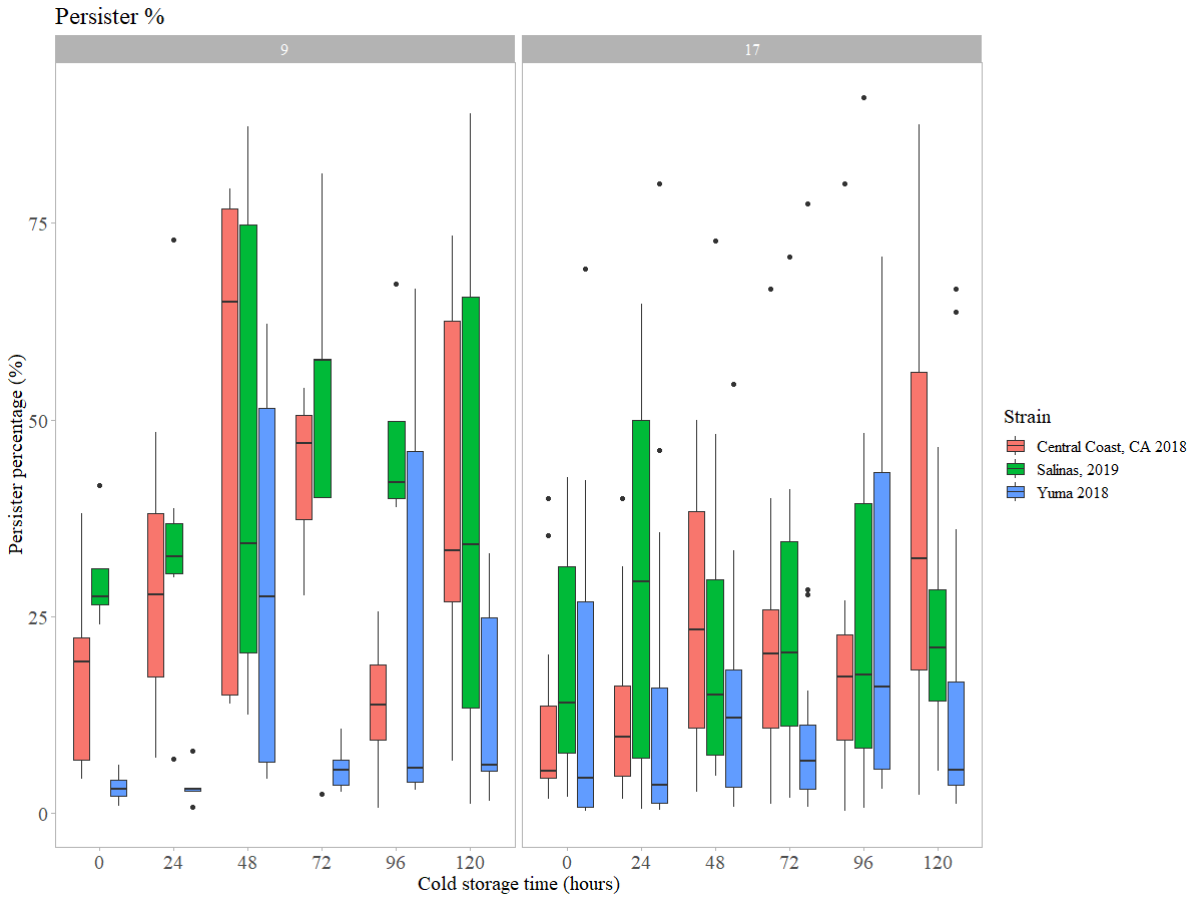

Figure 6: Boxplots of persister percentage of romaine lettuce harvested at different storage temperatures and stored in cold over 5 days. The middle line in the boxplot is the median value and the lower and upper lines of the box are the first and third quartile. The points outside the boxplots are outliers in the data.

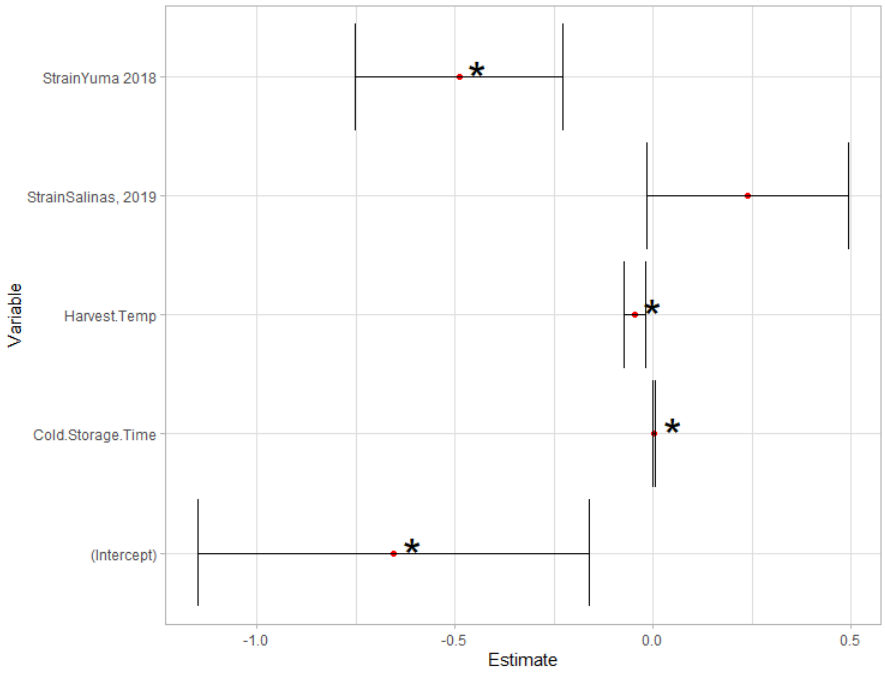

Figure 7: Effect of strain, harvest temperature and cold storage period on the persister fraction on Romaine lettuce. The beta coefficients were generated from beta regression models of transformed persister fractions to range between 0 and 1. Values with an asterisk (*) indicate significant effect at $p < 0.05$.

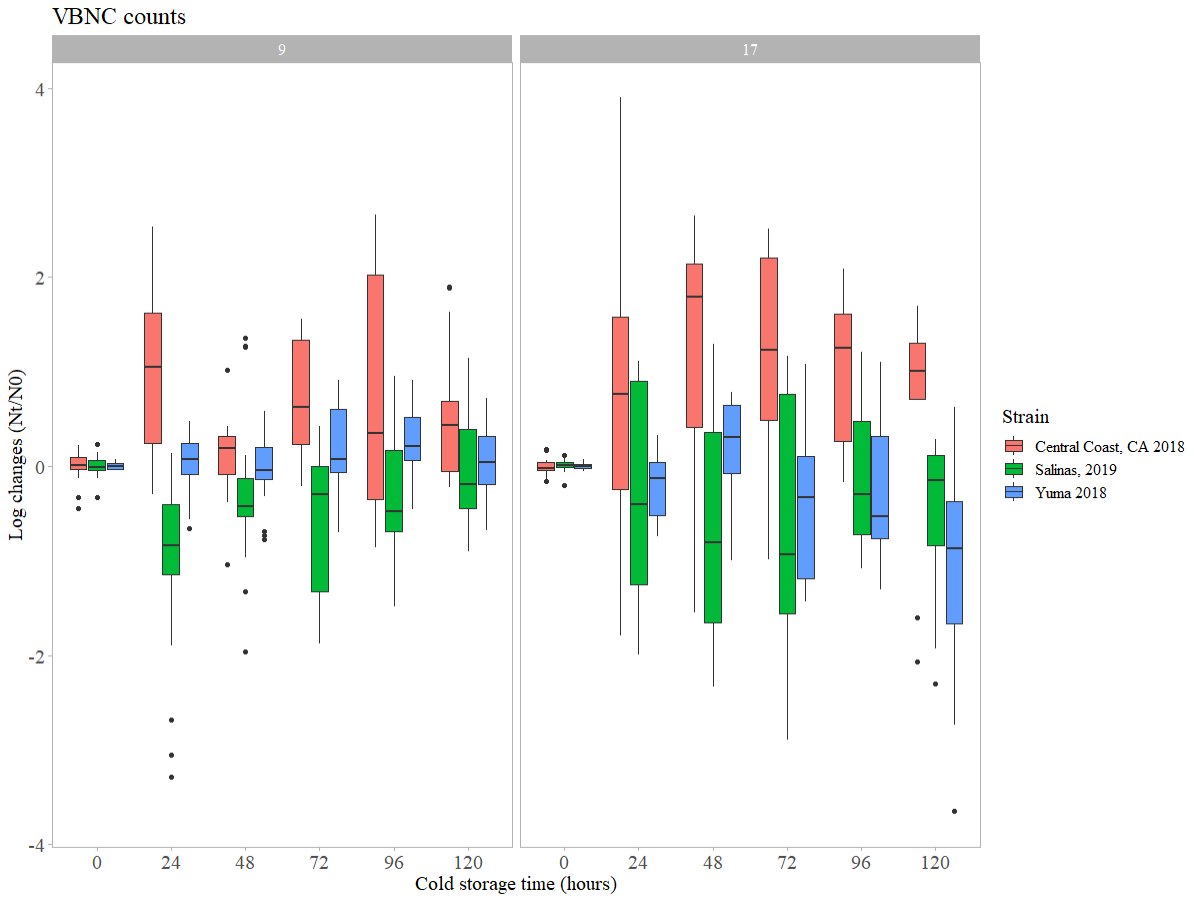

Figure 8: Boxplots of VBNC counts of romaine lettuce harvested at different storage temperatures and stored in cold over 5 days. The middle line in the boxplot is the median value and the lower and upper lines of the box are the first and third quartile. The points outside the boxplots are outliers in the data.

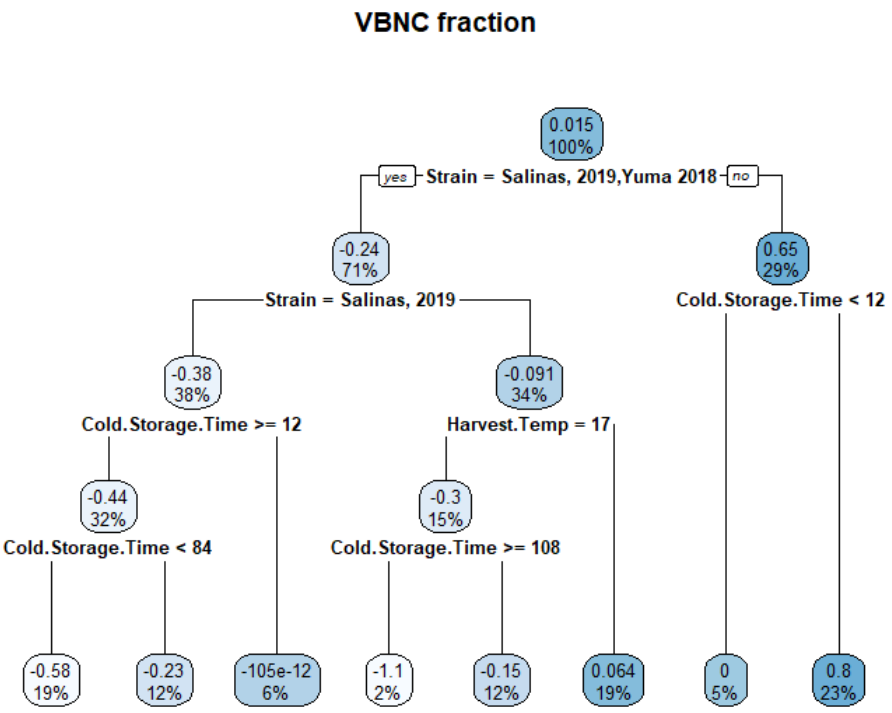

Figure 9: Regression tree analysis of the effect of harvest temperature, strain and cold storage time on VBNC fraction. The increase in VBNC fraction was derived as log (Nt/N0), where Nt is the VBNC counts at time t and N0 is the initial VBNC counts. The first node was split using the strain, with outbreak strains Salinas 2019 and Yuma 2018 showed lower VBNC increase during cold storage than the outbreak strain Central Coast 2018.

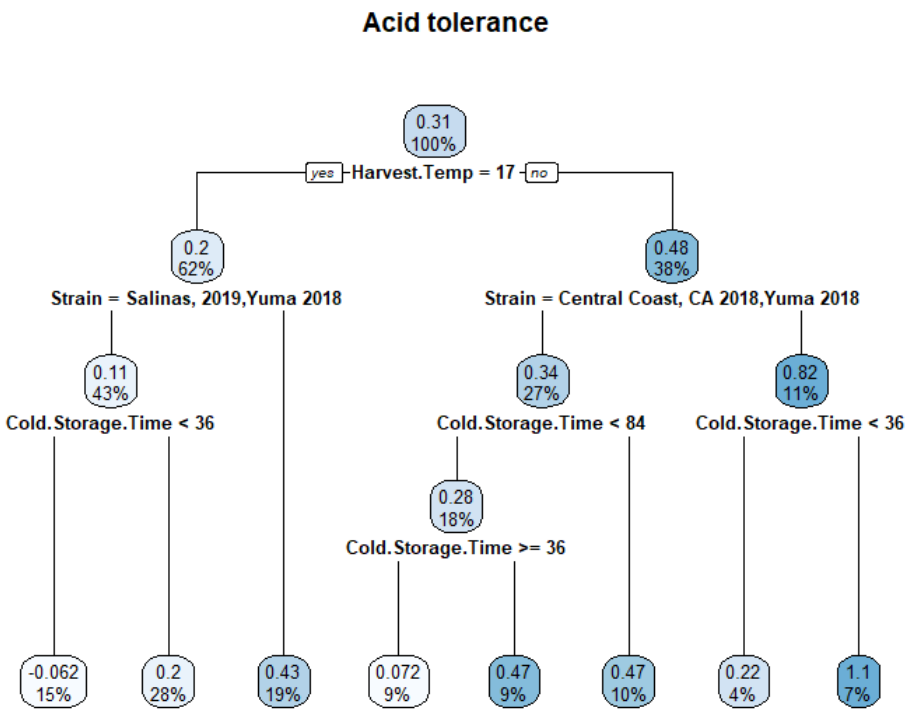

Figure 10: Regression tree analysis of the effect of harvest temperature, strain and cold storage of acid tolerance. The first decision node was split by harvest temperature, with romaine lettuce harvested at 9°C having lower log reduction values than those harvested at 17°C.

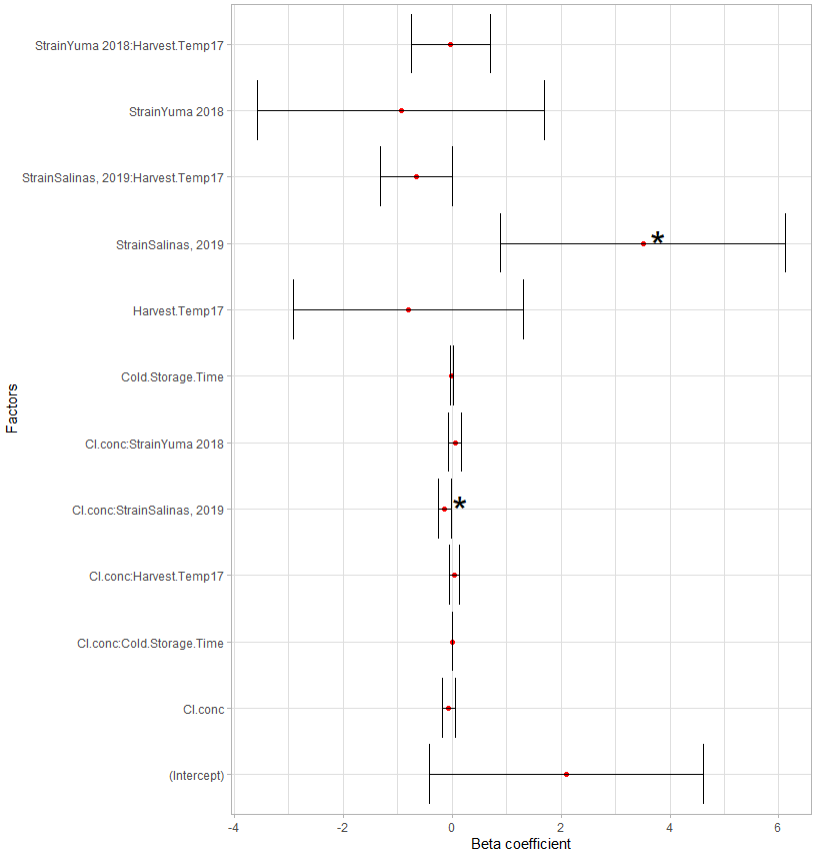

Figure 11: Effect of strain, harvest temperature and cold storage period on the chlorine tolerance of bacterial cells on romaine lettuce. A generalized linear model was used in fitting the effect of chlorine concentration, cold storage time, harvest temperature and strain on the log reduction of *E. coli* O157:H7 on romaine lettuce. Data points indicated with an asterisk (*) significantly affect log reduction values at $p < 0.05$.

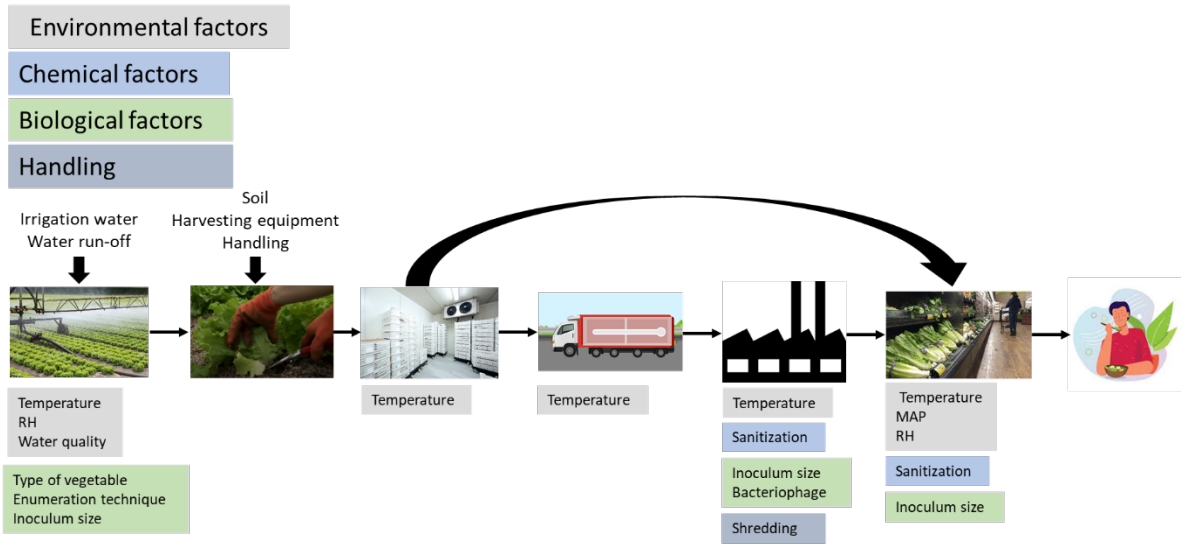

Figure 12: Descriptive identification of factors influencing growth and decay of *E. coli* from farm-to-storage.

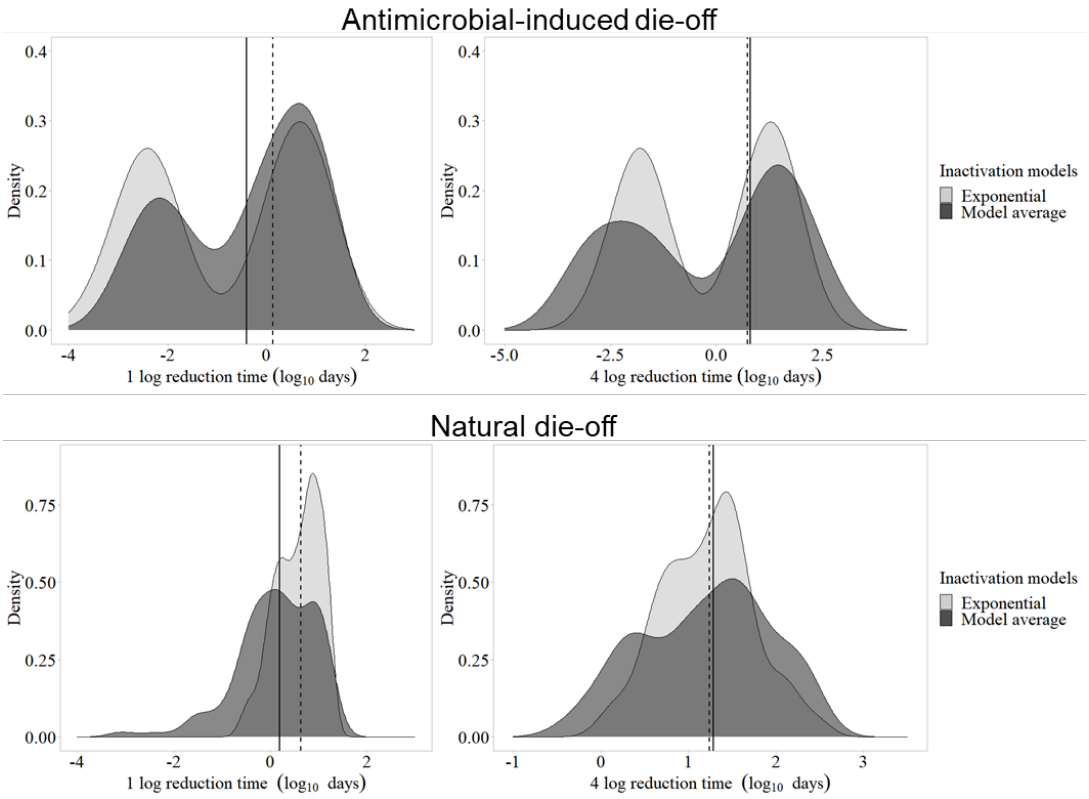

Figure 13: Distribution of the time taken to achieve 1 and 4 log reduction (log reduction times) of *E. coli* O157:H7 in leafy greens. Natural die-off describes inactivation due to natural causes such as environmental factors whereas antimicrobial-induced die-off refers to inactivation due to antimicrobial treatments such as chlorine washing.

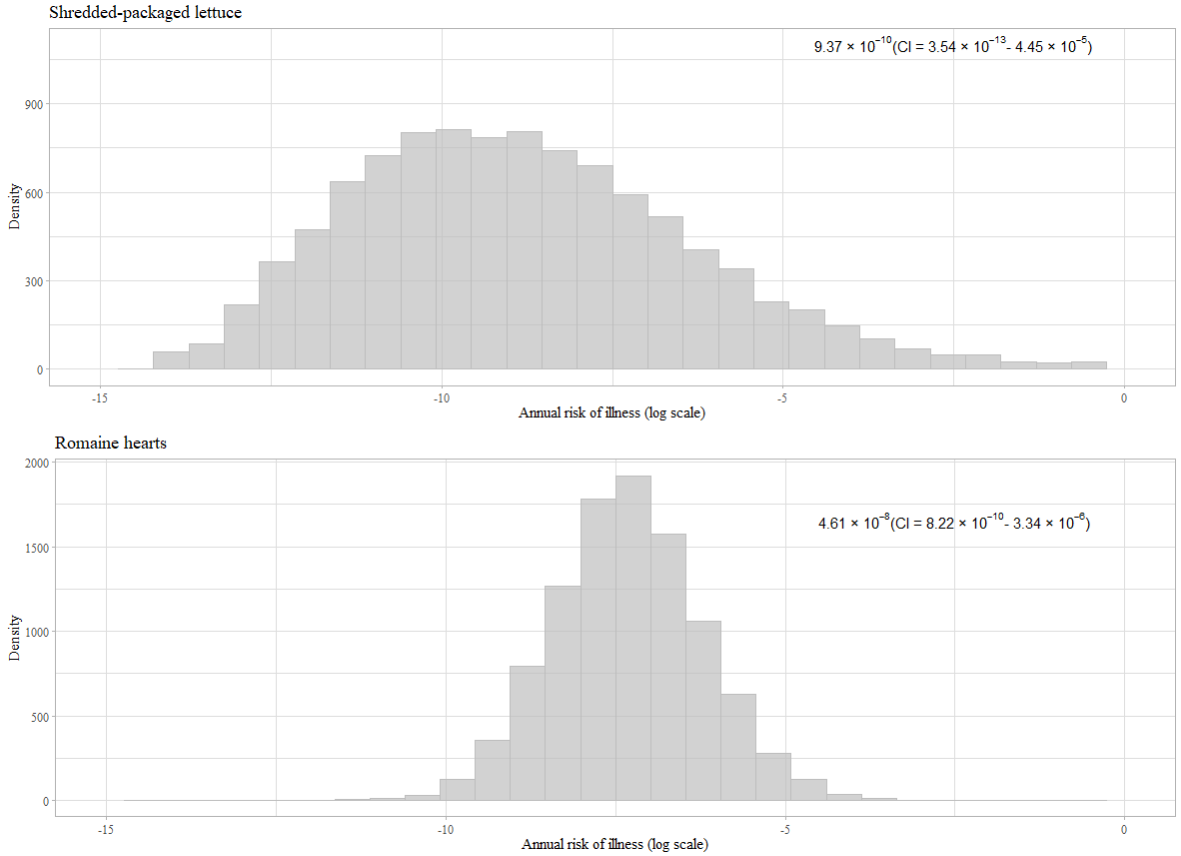

Figure 13: Annual risk of illness (per person) from the consumption of packaged lettuce and Romaine hearts contaminated with *E. coli* O157:H7.
Confidence interval (CI) describes the range of distribution of 95% of the values.