

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

PI: **Teresa M. Bergholz**, Ph.D., Michigan State University



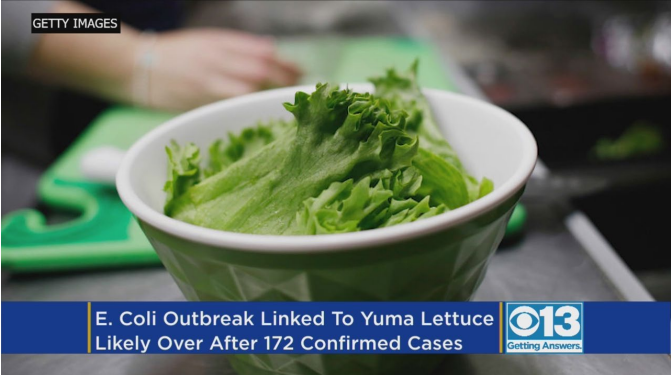
Research Team

- Jade Mitchell, Ph.D. – Co-PI
- Joshua Owade, Ph.D.
- Dimple Sharma
- Cleary Catur
- Shaney Rump
- Avery Evans
- Corrine Kamphuis



U.S. outbreaks of STEC O157 linked to leafy greens

Year	Food
2021	Packaged salads
2021	Baby spinach
2020	Leafy greens
2019	Romaine lettuce
2019	Salad mix
2018	Romaine lettuce
2018	Romaine lettuce



- Likely sources of contamination pre-harvest
 - Irrigation water
 - Runoff from cattle operations
 - Improperly treated manure

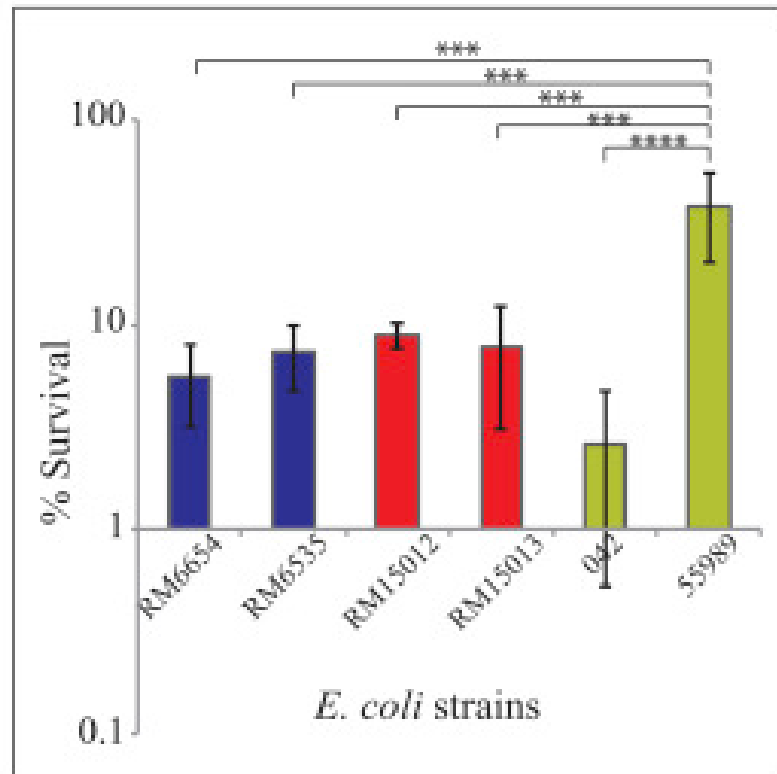
Project rationale

- Observations from 2018 and 2019 outbreaks
 - Initial cases in patients in eastern parts of U.S. and Canada
 - Product distributed nationally
- Difference in time before processing
 - Forward processing vs. immediate processing
- Potential for pathogen populations to shift to states that may impact stress tolerance, virulence, and detectability

Dormant states of bacteria

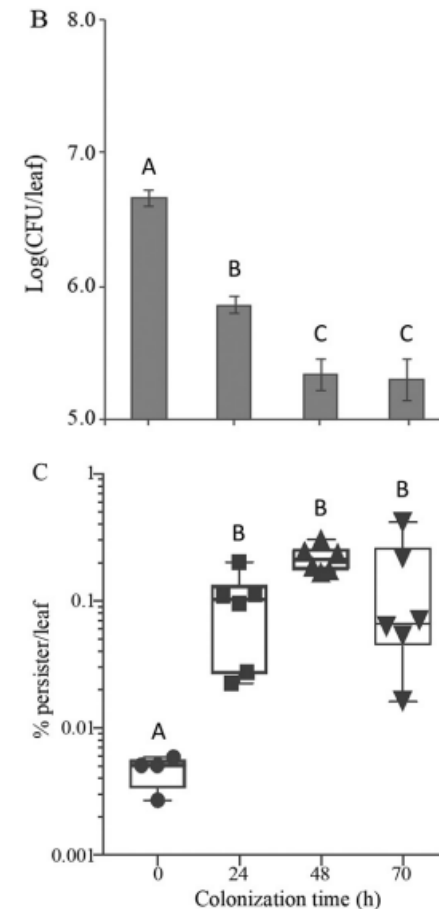
- Persister
 - Non-growing cells, resistant to antibiotics without specific resistance genes
 - Maintain metabolic activity
 - Can revert to actively growing state
- Viable but non-culturable (VBNC)
 - Cannot grow on/in culture media
 - Low level of metabolic activity
 - Maintain membrane integrity

STEC enters into dormant states under pre- and post-harvest conditions



<https://doi.org/10.1016/j.fm.2019.103241>

Thao et al., FM 2019

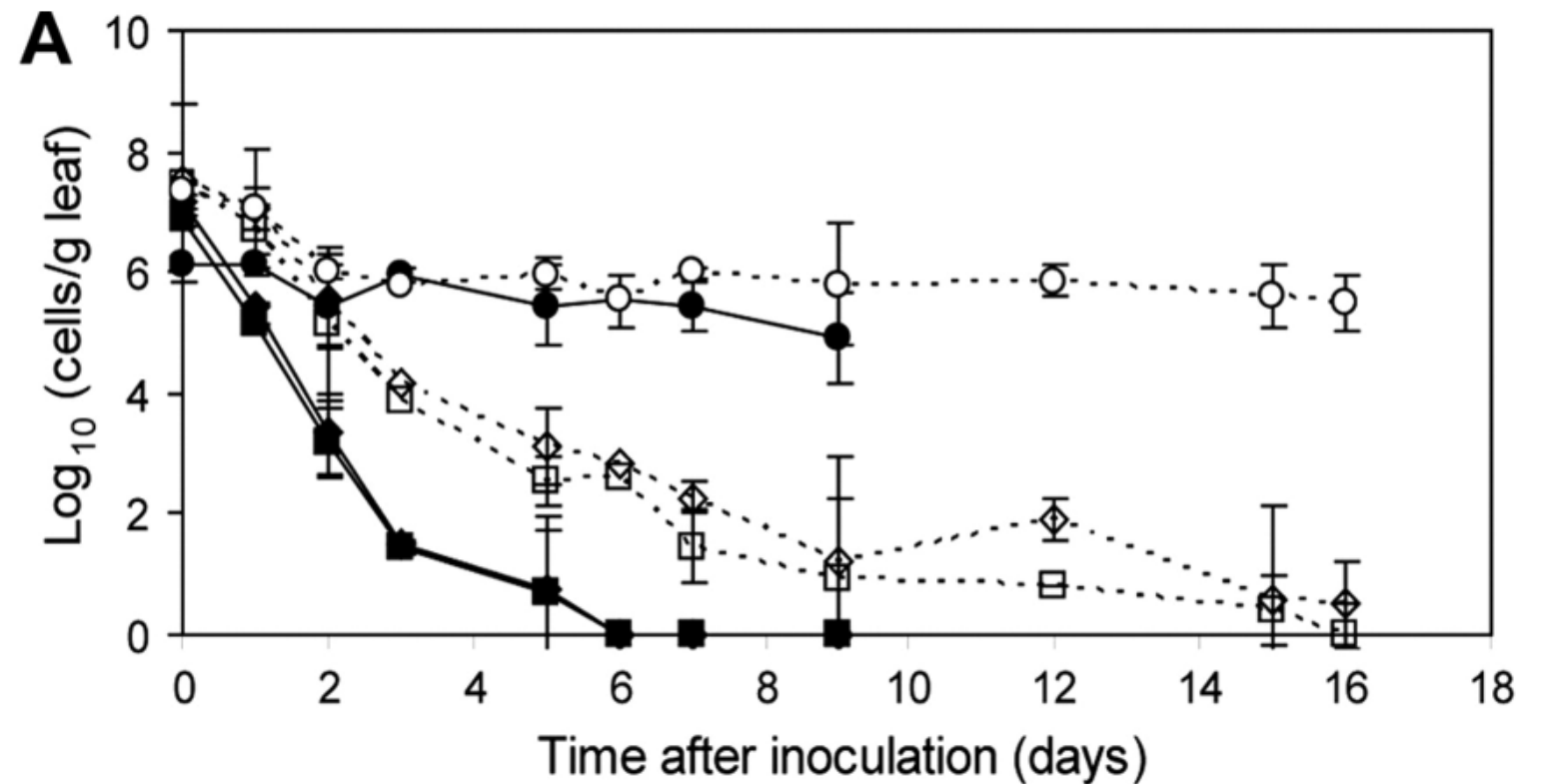


DOI: 10.1128/AEM.01602-19

Munther et al., AEM 2020

E. coli O157:H7 VBNC formation on lettuce leaves

- Culturable cells undetectable after 9 days on leaves at 8°C
- Shiga toxin detected from leaves at 16 days

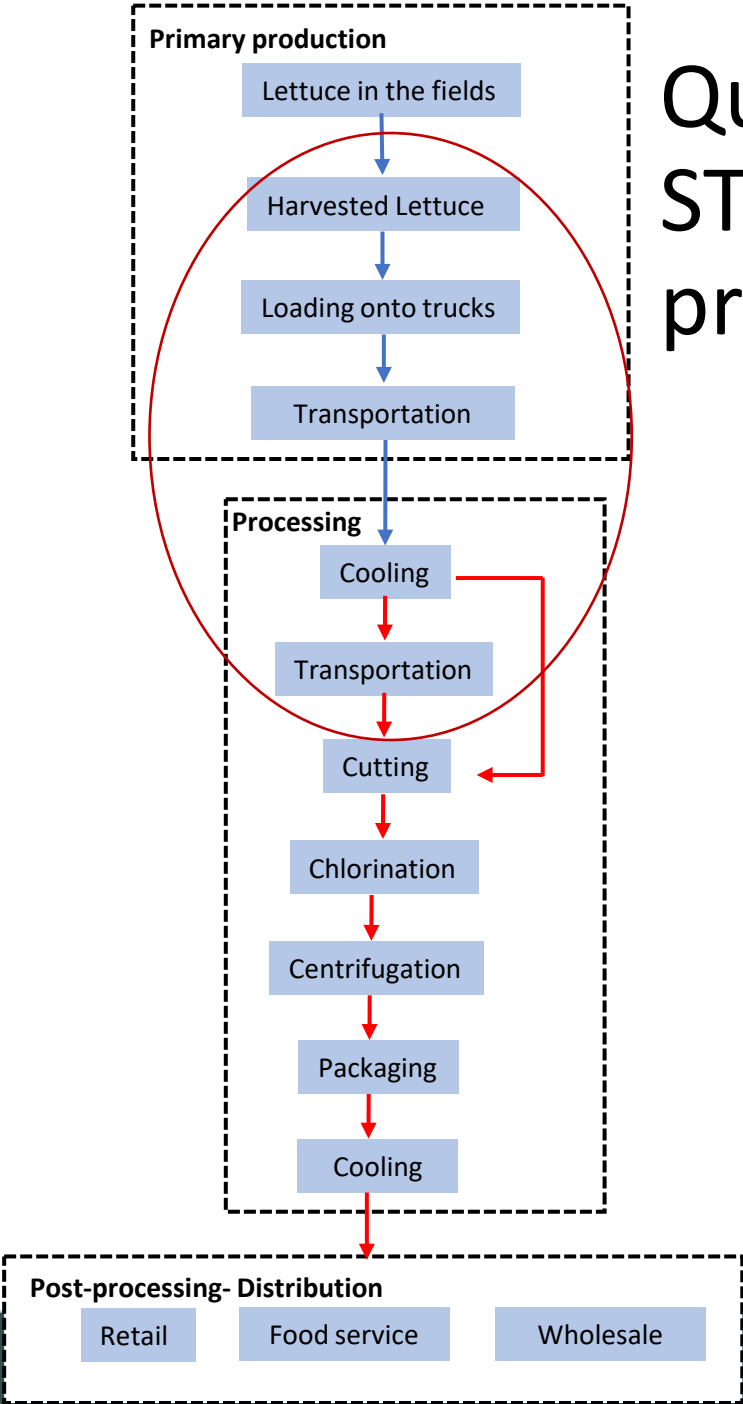


DOI: <https://doi.org/10.1128/AEM.05020-11>

Dinu and Bach, AEM 2011

Impact of persister and VBNC formation on STEC in the lettuce supply

- Bi-phasic decay on plants, remaining population likely to be in persister state (Brandl et al., 2022)
- Persister cells have enhanced survival to low pH, antimicrobial compounds and ROS (Gollan et al., 2019)
- VBNC cells form in the phyllosphere (Dinu et al., 2011) and in response to sanitizers used in lettuce processing (Truchado et al., 2023)
- Does this contribute to transmission and outbreaks? How?



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

- Effects of temperature and time on formation of persisters and VBNC cells on lettuce
- Changes in acid resistance and chlorine tolerance over time
- Outputs: QMRA

Research questions

- Does STEC O157:H7 enter into dormant states during cold storage?
 - If so, what are the implications for pathogen survival and human health risks?
 - How does this impact detectability?

Research objectives

- Quantify changes in *E. coli* O157:H7 physiology by lettuce harvest temperature and during refrigerated storage
- Quantify changes in *E. coli* O157:H7 risk over refrigerated transport times
- Evaluate the effect of *E. coli* O157:H7 in the persister state on detection on Romaine lettuce

Quantifying changes in STEC O157:H7 physiology on Romaine lettuce

- Identify ranges of temperatures at lettuce harvest
- Identify ranges of cut-to-cool times and temperatures
- Develop conditions for lab simulation of Romaine harvest and cooling

Temperature at harvest

- Salinas at harvest
 - Median = 57.9F
 - Mean = 57.8 ± 7.4 F
- Yuma at harvest
 - Median = 53.6F
 - Mean = 54.2 ± 8.2 F
- Temp after cooling
 - Salinas median = 34.5F
 - Yuma median = 34.7F

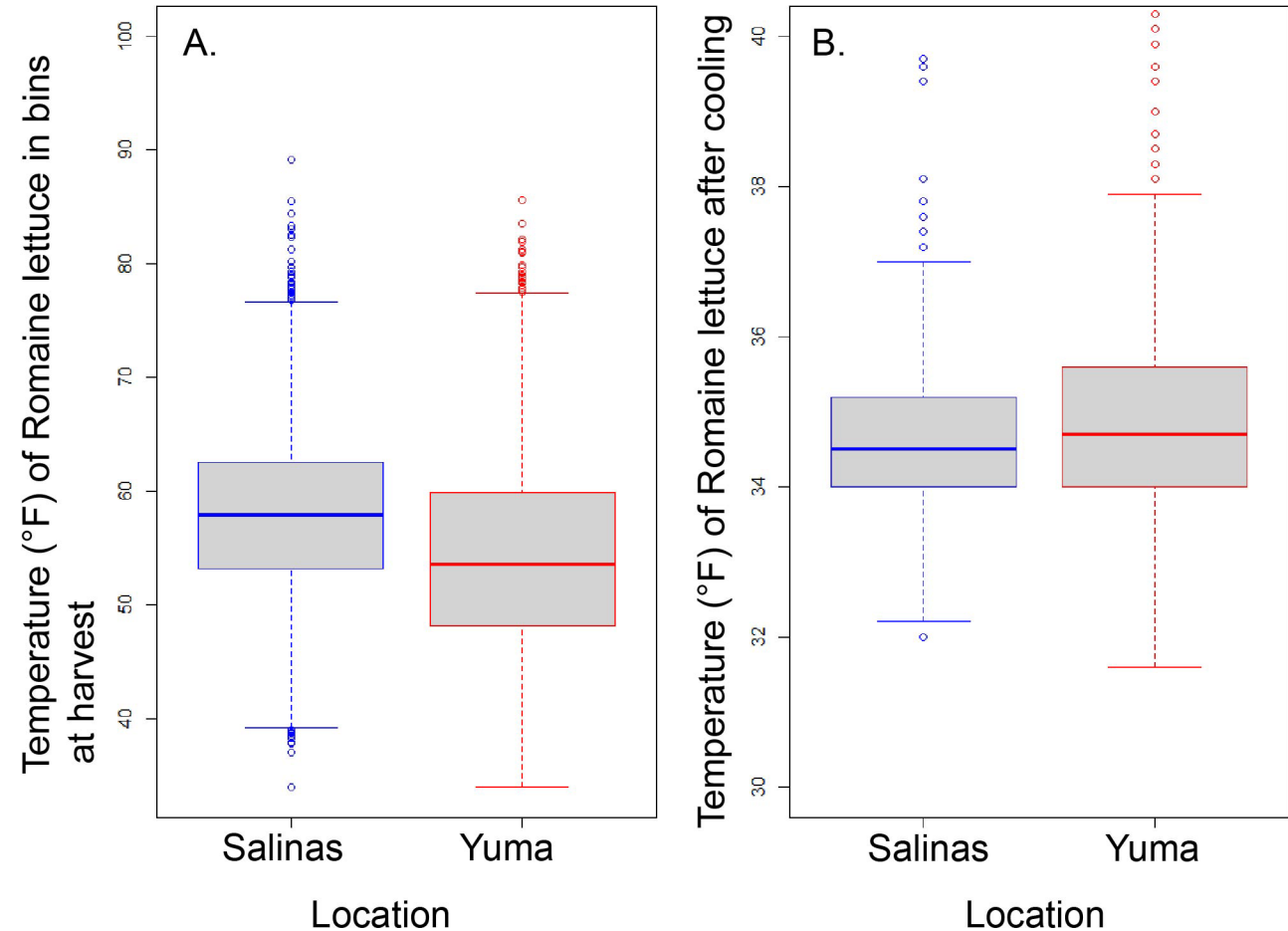


Figure 1. Temperature of Romaine lettuce in bins at harvest (A) and after cooling (B). Boxplots represent 5615 and 4623 datapoints collected from the Salinas and Yuma growing regions, respectively.

Cut to cool time

- Salinas = 163 min
- Yuma = 232 min
- Time to cool at cooling center
 - Salinas = 32 min
 - Yuma = 33 min

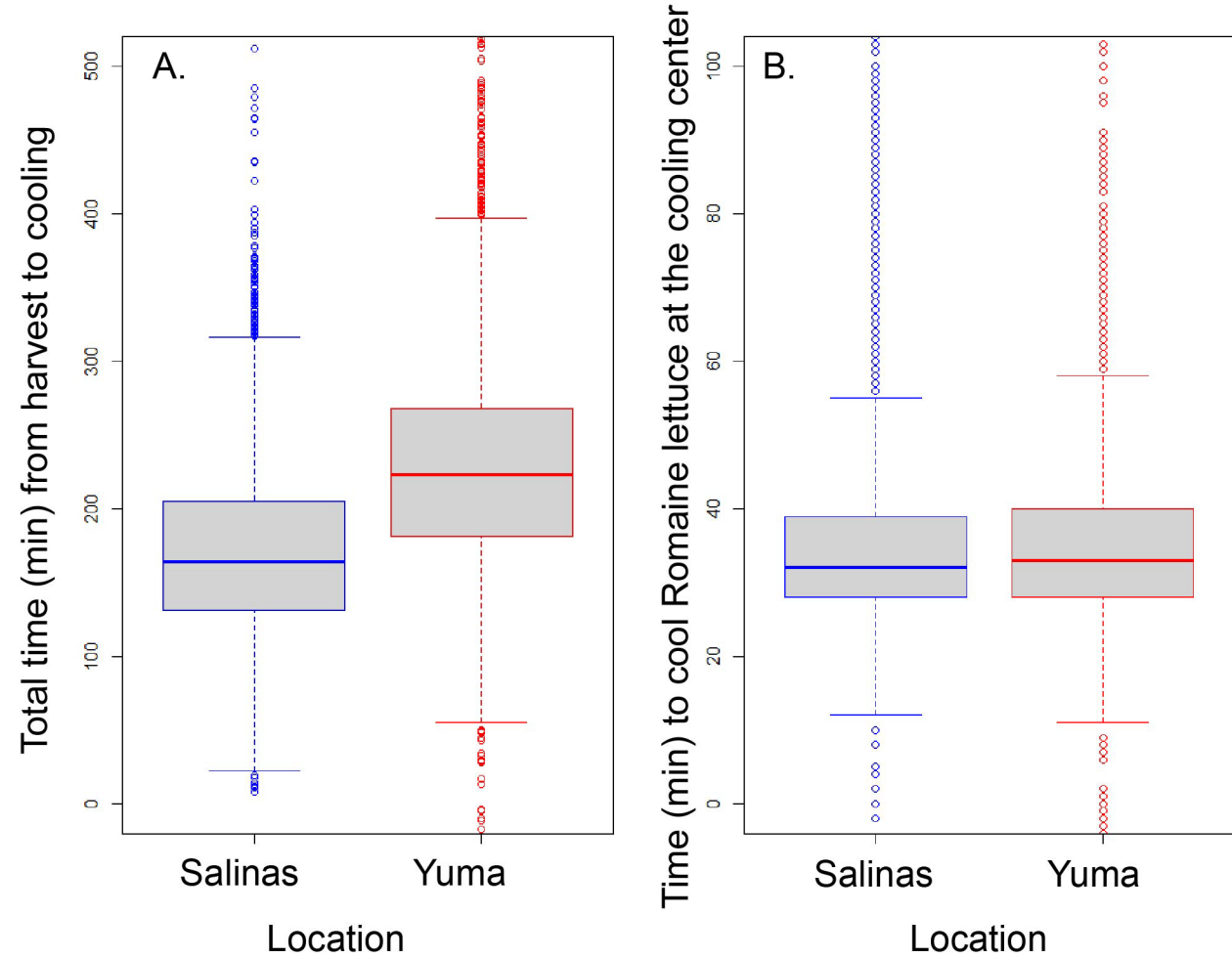


Figure 2. Cut to cool time for Romaine lettuce in bins (A) and time to cool Romaine lettuce at the cooling center (B). Boxplots represent 5615 and 4623 datapoints collected from the Salinas and Yuma growing regions, respectively.

EHEC O157:H7 strain set

- Obtained from patient isolates associated with Romaine outbreaks
 - Yuma 2018
 - Central coast, CA 2018
 - Salinas 2019
- All strains selected for rifampicin resistance

Quantifying physiological changes during cold storage

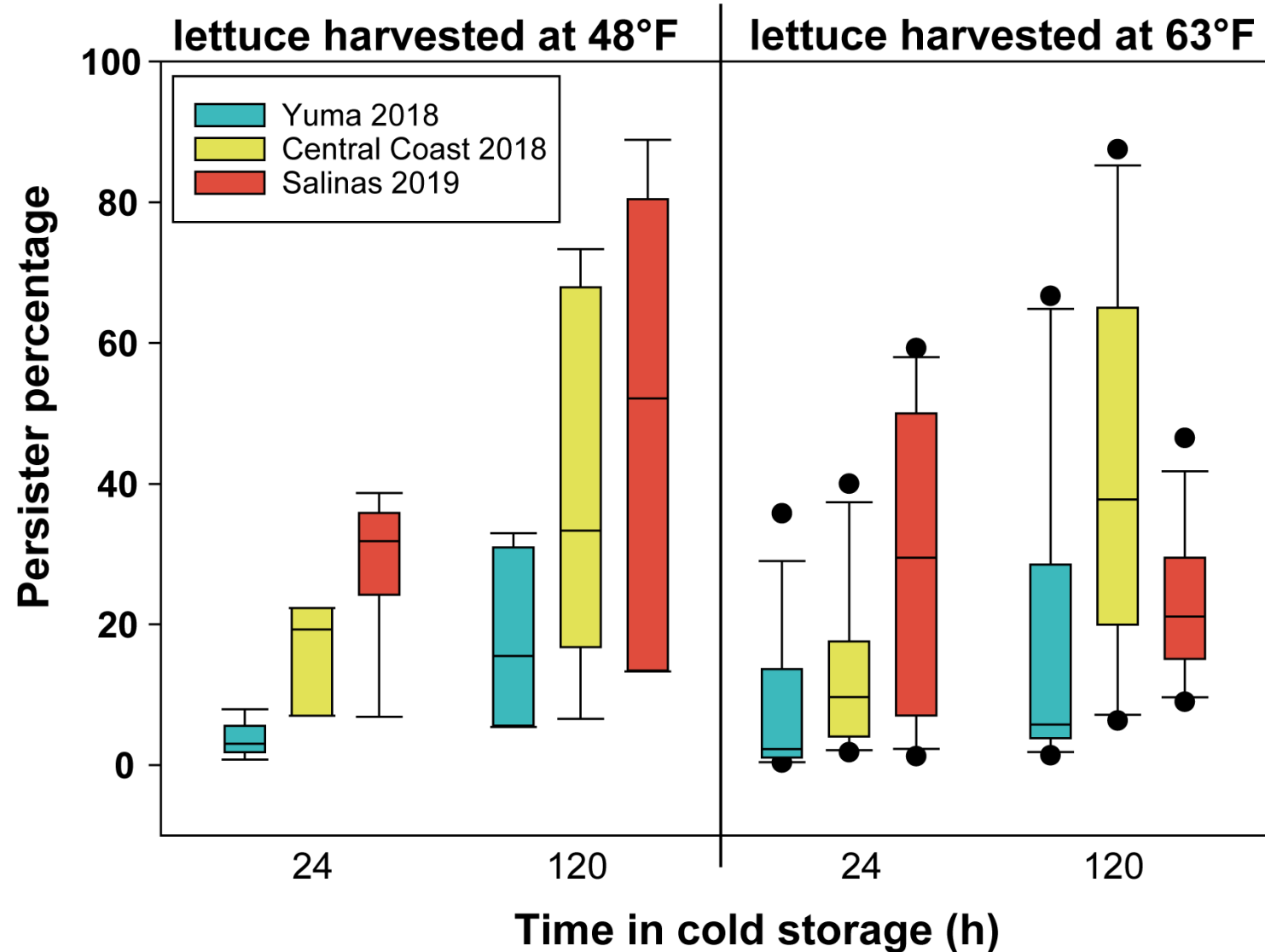
Lab simulation of Romaine harvest and cooling

- Greenhouse grown, Parris Island and Solid King varieties
- Spray inoculation of plants with 3 different *E. coli* O157:H7
- Harvest temperatures determined from industry supplied data
- Monitored culturable cells, persisters, VBNC, acid tolerance and chlorine tolerance over 5 days of cold storage



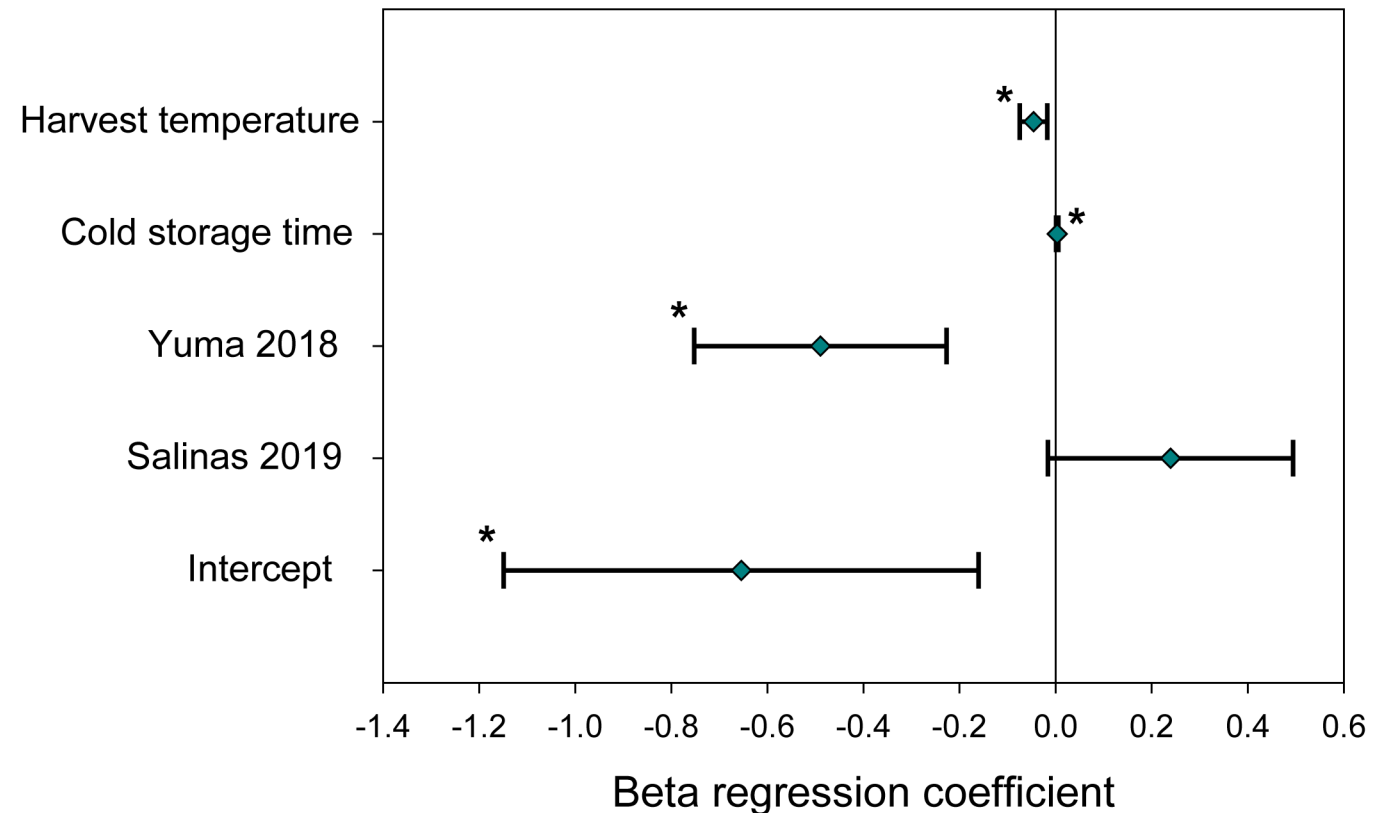
Minimal change in culturable cells on lettuce, increase in dormant cells

- Minimal change in *E. coli* O157:H7 culturable cells during cold storage
 - ~ 0.25 log reduction over 5 d
- Increase in persister cells and VBNC during cold storage
 - Dependent on strain and harvest temp



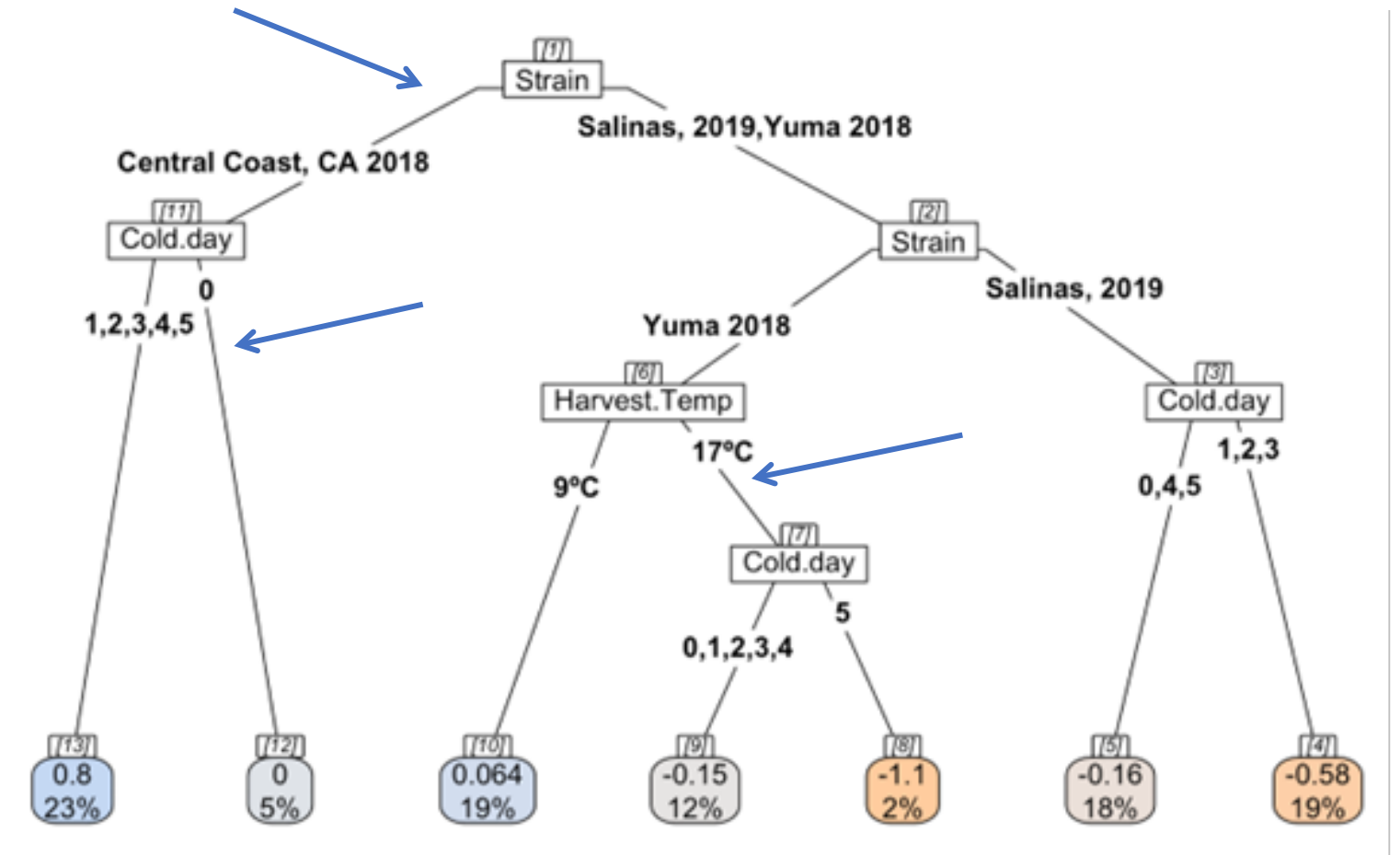
Factors contributing to persister formation

- Persister percentage increases as harvest temperature decreases
- Persister percentage increases as cold storage time increases
- Central Coast and Salinas strains had significantly higher persisters compared to Yuma 2018



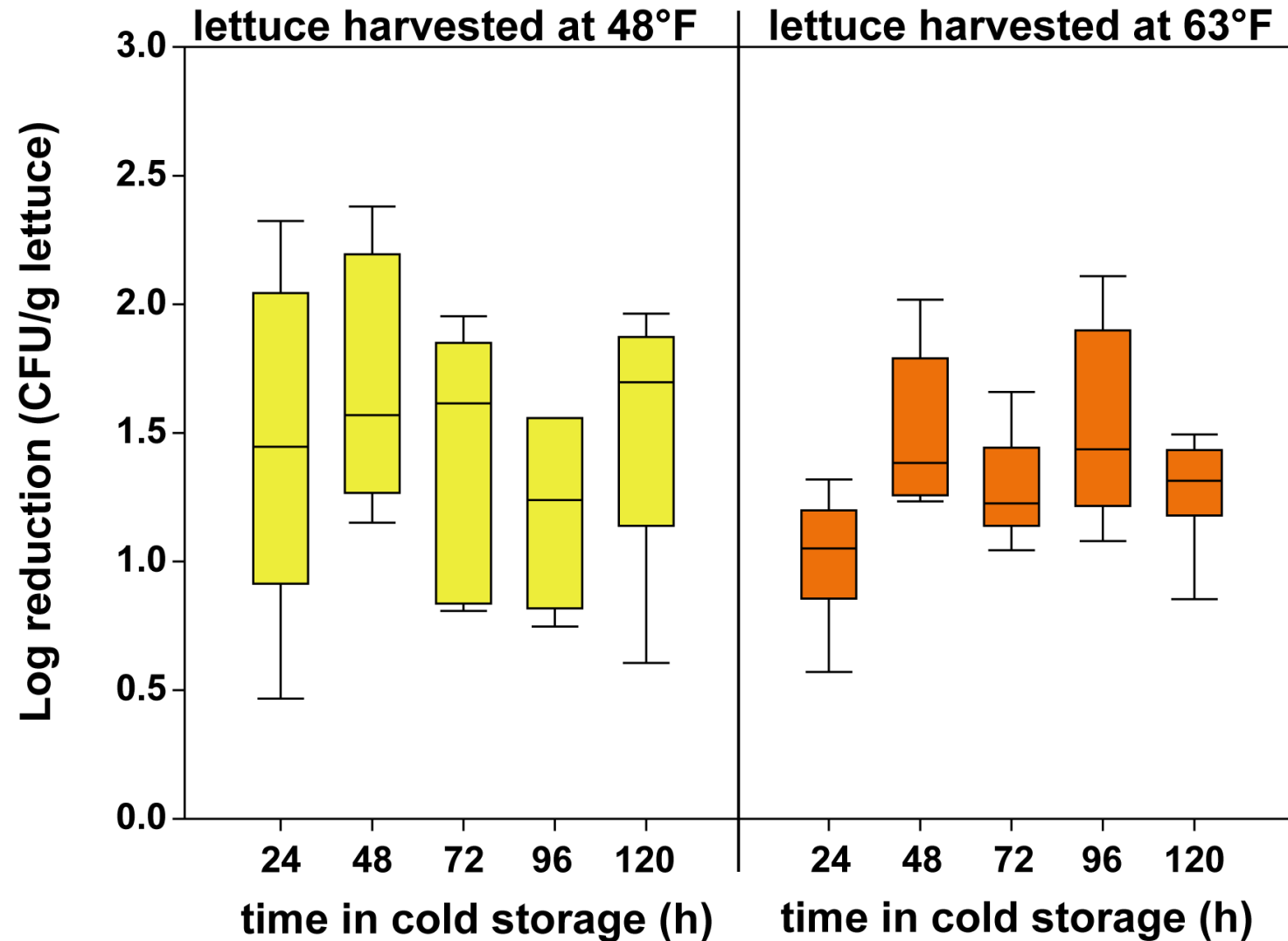
Factors contributing to VBNC formation

- Main difference among strains
 - Central Coast higher VBNC formation
- Greater VBNC formation when lettuce harvested at 48F for Yuma 2018



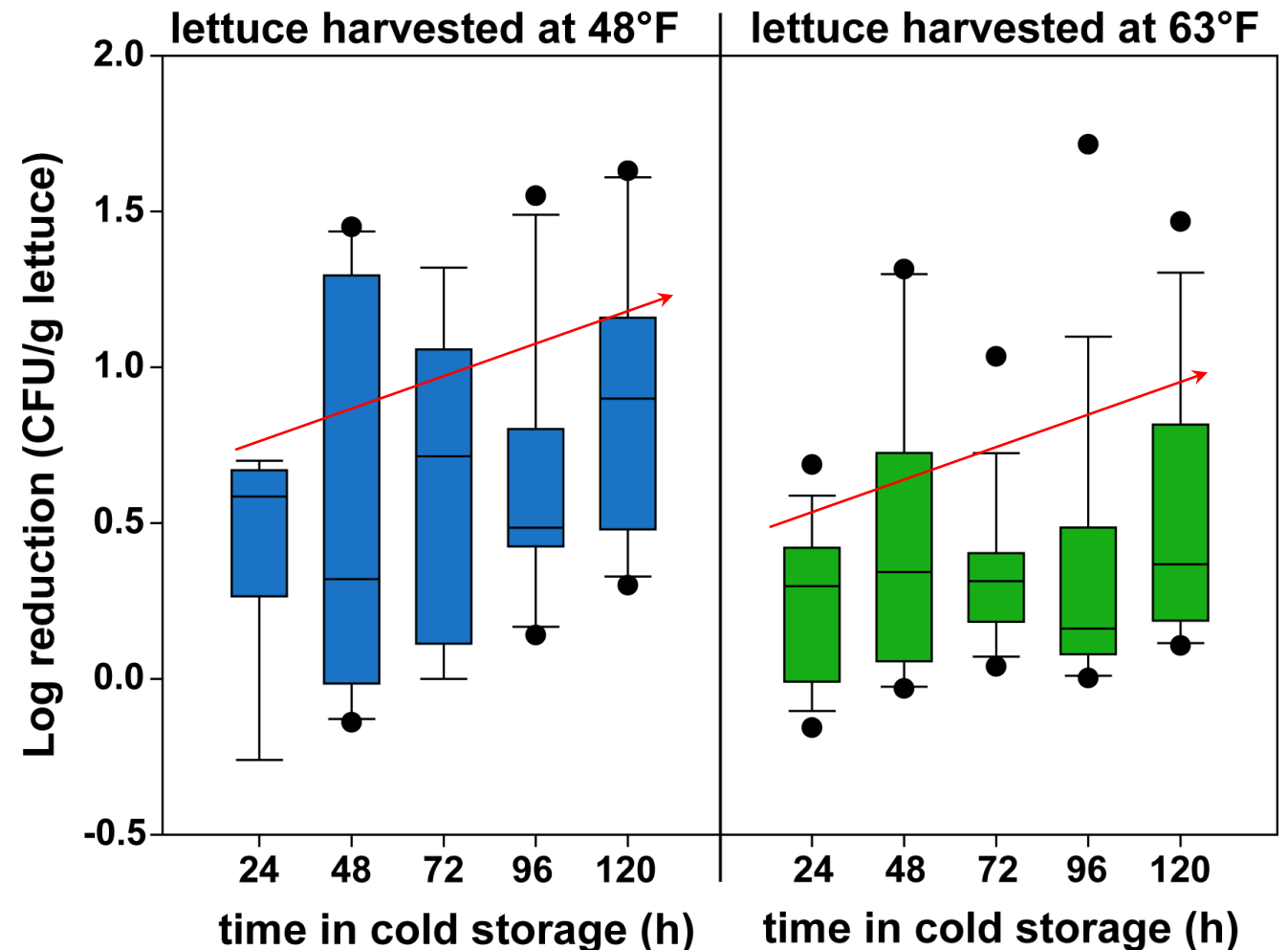
Chlorine tolerance not impacted by length of time in cold storage

- Chlorine tolerance assay
 - 30s exposure to 18-20ppm free chlorine



STEC O157:H7 decreased acid tolerance as cold storage increased

- Greater reductions during exposure to pH 2.5 occurred as length of time in cold storage increased
- Greater reductions for lettuce harvested at lower temperature



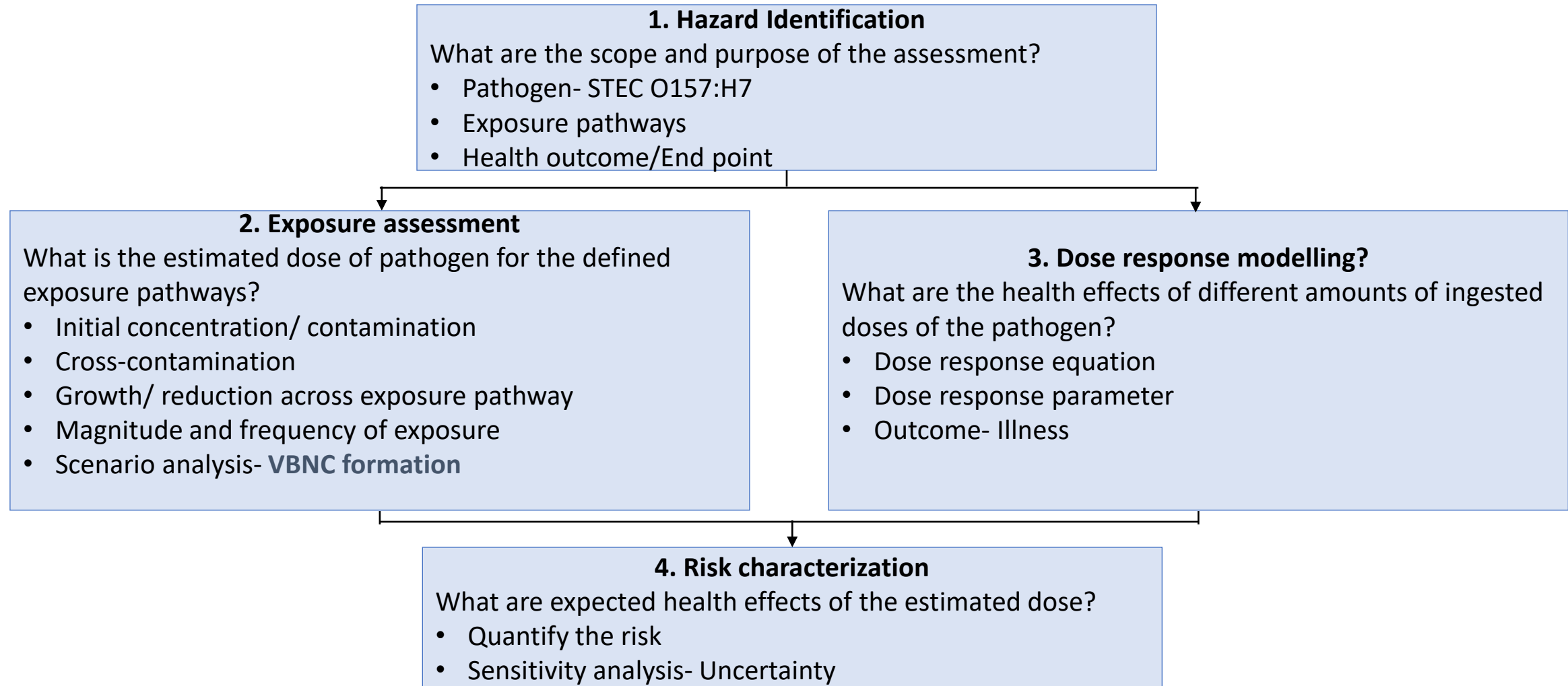
Impacts of cold storage on STEC O157:H7 physiology

- Does STEC O157:H7 enter into dormant states on lettuce during cold storage?
 - Yes
- Increase in proportion of cells in dormant states as cold storage time increases
- No increase in chlorine tolerance
- Decrease in acid tolerance as cold storage time increases

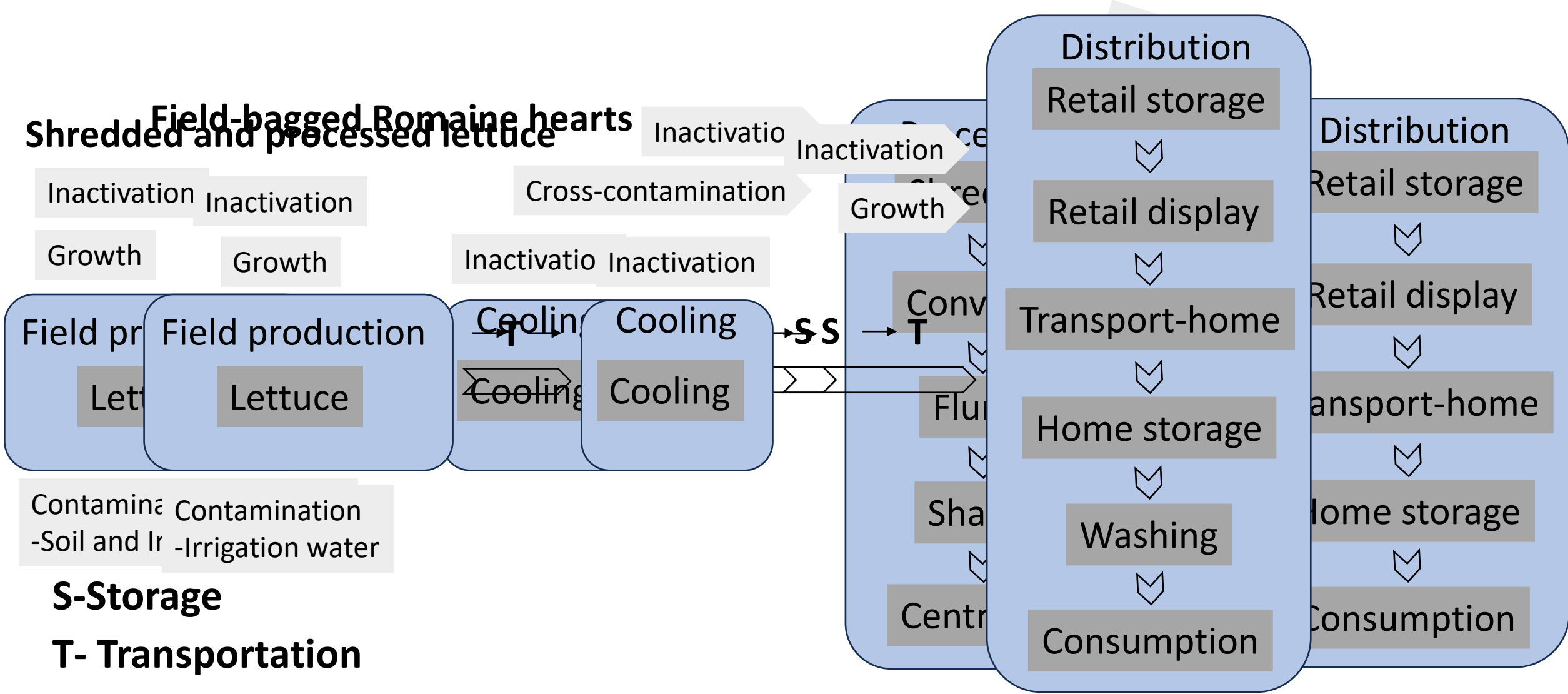
Research questions

- Does STEC O157:H7 enter into dormant states during cold storage?
 - Yes
- Does entry into dormant states impact human health risks?

Overview of the QMRA framework

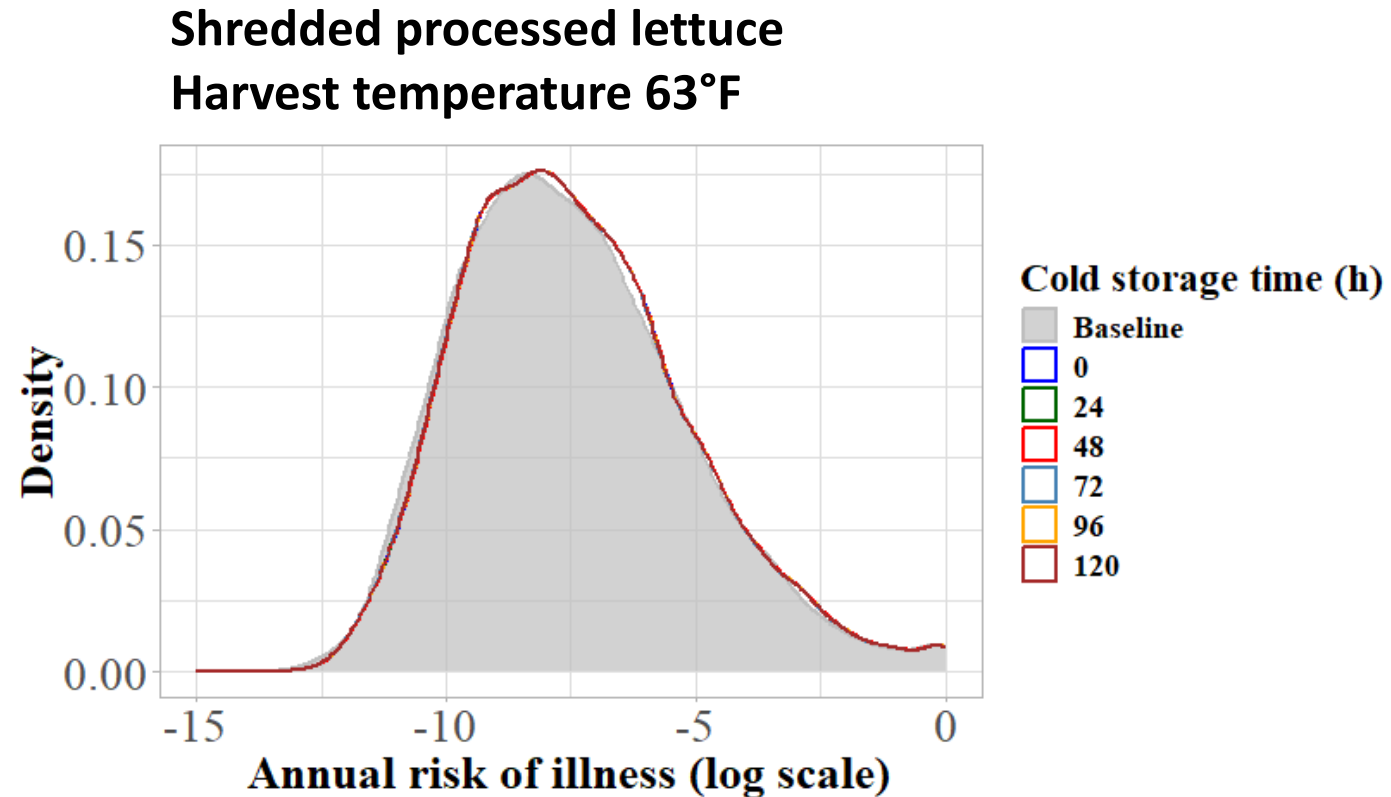


Overview of QMRA model for lettuce

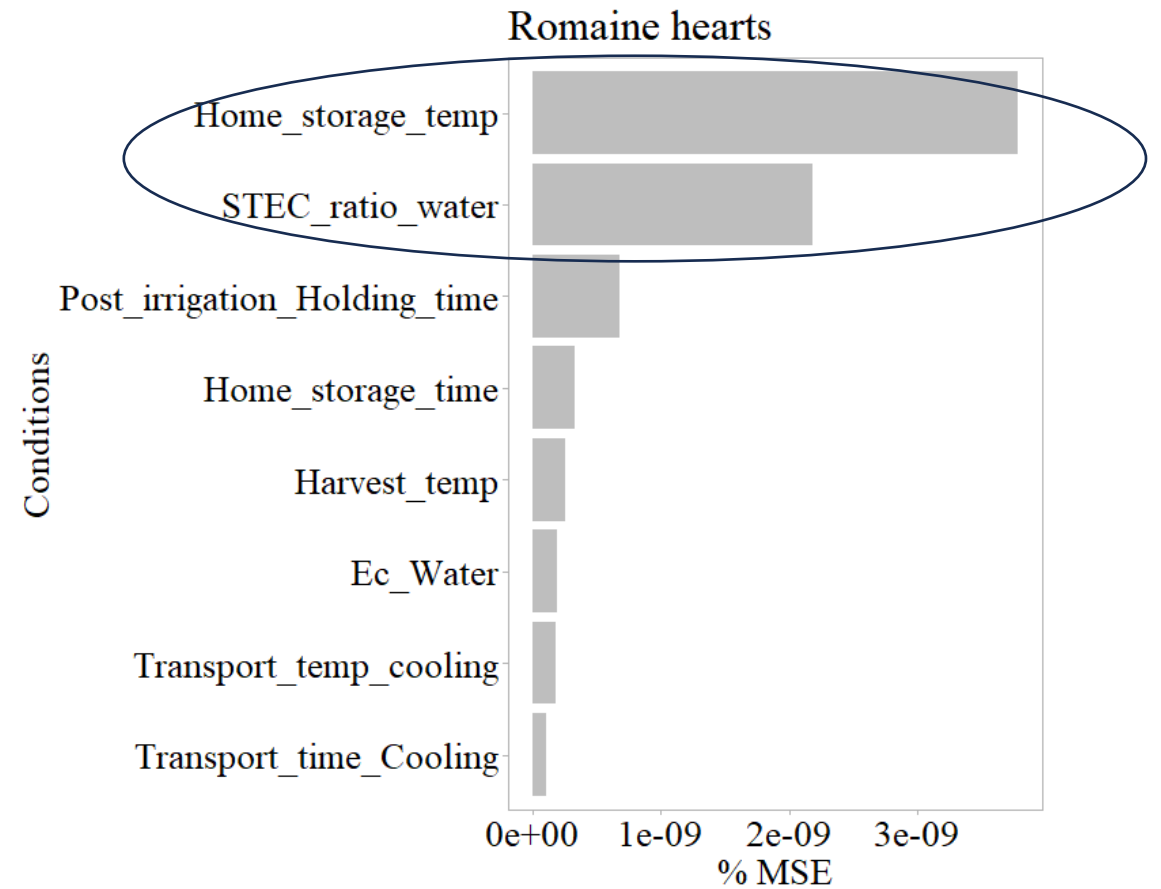
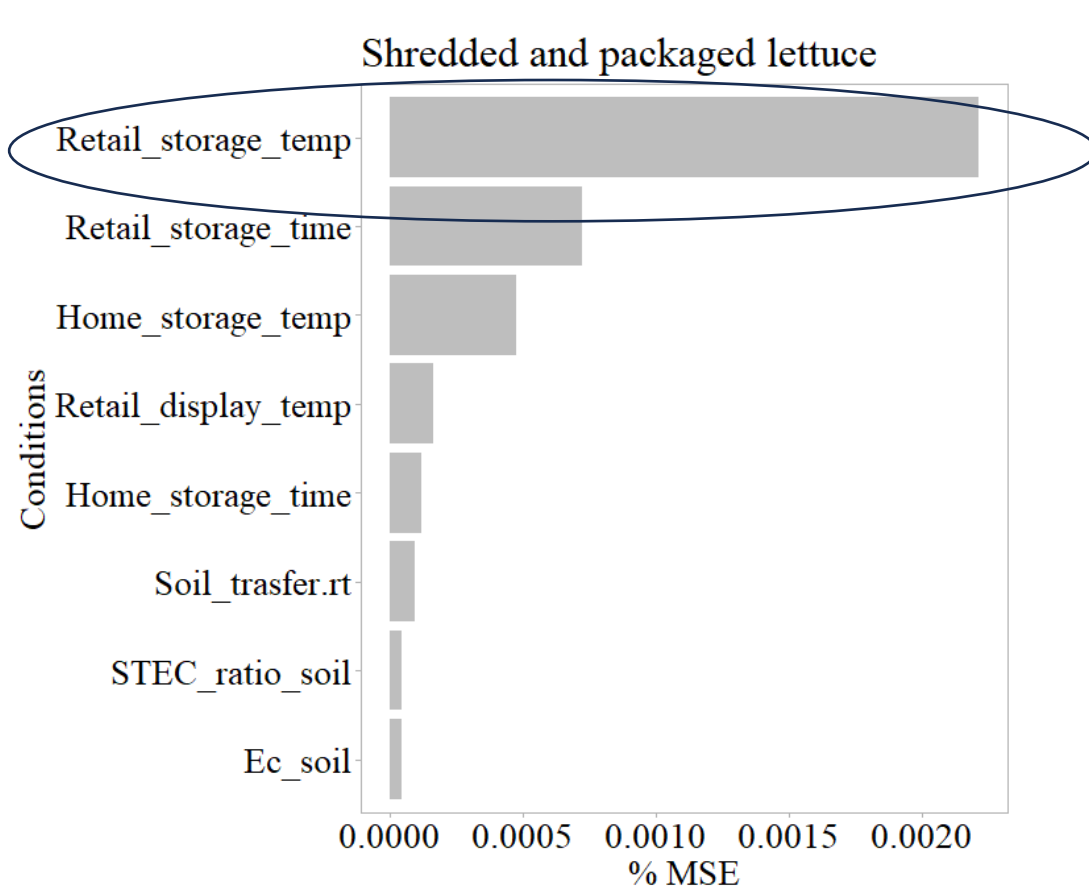


Length of time in cold storage did not impact risk of illness

- The annual risk of illness per person
 - Romaine hearts (median 4.61×10^{-8})
 - Processed lettuce (median = 9.37×10^{-10})
 - Risk distributions did not change with length of cold storage or VBNC state



Important factors influencing risk of illness



Impact of cold storage time and O157:H7 physiology on risk of human illness

- Does entry into dormant states impact human health risks?
 - No
- Maintaining cold chain during transport and storage most important for reducing risk of transmission



Research questions

- Does STEC O157:H7 enter into dormant states during cold storage?
 - Yes
- Does STEC O157:H7 in the persister state impact detectability on Romaine lettuce?

Do cells in the persister state impact detection?

Induction of persister state and detection

- 3 different *E. coli* O157:H7 inoculated into water
 - Yuma 2018, Central Coast 2018, Salinas 2019 outbreak strains
 - Induced persister cell formation
- *E. coli* O157:H7 with varying persister percentages spiked onto 25g samples of Romaine lettuce (2 and 15 MPN)
- Collaborated with third party testing company to use 8h and 24h enrichment for BAX O157 testing

Ranges of persister percentages inoculated onto Romaine lettuce	Number of samples
< 1%	27
1 – 4.9%	29
5 – 9.9%	14
10 – 19.9%	12
20 – 39.9%	12
40 – 85%	6

Proportion of cells in the persister state did not influence detectability

Ranges of persister percentages inoculated onto Romaine lettuce	Number of samples	Positive after 8h enrichment	Positive after 24h enrichment
< 1%	27	6 (22%)	25 (93%)
1 – 4.9%	29	0 (0%)	19 (66%)
5 – 9.9%	14	5 (36%)	8 (57%)
10 – 19.9%	12	4 (33%)	10 (83%)
20 – 39.9%	12	4 (33%)	11 (92%)
40 – 85%	6	3 (50%)	6 (100%)

Detection varied with enrichment time and by strain

<i>E. coli</i> O157:H7 strain	Positive after 8h enrichment	Positive after 24h enrichment
Yuma 2018	40% (16/40)	80% (32/40)
Central Coast 2018	23% (9/40)	75% (30/40)
Salinas 2019	4% (1/24)	75% (18/24)

Detection varied with enrichment time and by strain

- Does STEC O157:H7 in the persister state impact detectability on Romaine lettuce?
 - No
- To be determined
 - Extremes of enrichment time range evaluated
 - Which times lead to greatest recovery and detection?
 - Optimize recovery from stressed states



Fuel the change

- While the proportion of cells in dormant states increased the longer Romaine lettuce was held in cold storage, this did not lead to increased subsequent stress tolerance of *E. coli* O157:H7 on lettuce
 - Pre-processing time delays did not result in greater tolerance of *E. coli* O157:H7 to chlorine
- Maintaining cold chain during transport and storage most important for reducing risk of transmission
- Improved detectability for *E. coli* O157:H7 on Romaine lettuce with longer enrichment times
 - Understand what enrichment time(s) are used for your sample detection

Acknowledgements

Project team

- Dr. Jade Mitchell
- Dr. Joshua Owade
- Dimple Sharma
- Cleary Catur
- Corrine Kamphuis
- Avery Evans
- Shaney Rump
- Diane Luong

Industry partners

- data sharing, discussion and feedback

