Final Report

Are all lettuce created the same? *E. coli* O157:H7 and *Salmonella enterica* interaction with lettuce exudates

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Executive summary:

The consumption of leafy greens, such as Iceberg and Romaine lettuce has been on a rise in the last few decades. At the same time, there have been frequent foodborne outbreaks associated with these commodities. Pathogens such as *E. coli* O157:H7 and *Salmonella* Newport can contaminate leafy greens via different routes, such as soil, irrigation water, air, and animals' and birds' intrusion. Presence of sugars in plant metabolites can aid in both survival and population increase of foodborne pathogens, whereas certain fatty acids may be inhibitory to survival and proliferation of foodborne pathogens. This study was aimed to employ a metabolomics approach in conjunction with analysis of growth rates, chemotaxis and biofilm formation by *E. coli* O157:H7 and *Salmonella* Newport n exudates obtained from Romaine lettuce and Iceberg lettuce. Analysis of exudate composition indicated the presence of sugars but negligible amounts of fatty and amino acids. *E. coli* O157:H7 formed more EPS in presence of romaine lettuce exudates (both 8 and 12 week) than in presence of iceberg lettuce exudates. The results from the studies indicates that *E. coli* O157:H7 can form biofilms in presence of both romaine and iceberg lettuce exudates. *S.* Newport formed significantly higher biofilms in presence of both romaine and iceberg lettuce leaf exudate when obtained at the 8-week stage. No significant differences

was observed among the populations of *E. coli* O157:H7 indicating that the exudates did not differentially promote chemotaxis in *E. coli* O157:H7. The results of this study will help in better risk evaluation for produce growers.

Introduction:

Consumption of vegetables and leafy greens in the United States (US) has shown a considerable increase in the past two decades, with a notable rise in global distribution (USDA, 2011). In 2017, the US produced about 11.7 billion pounds of vegetables and leafy greens valued at 18.9 billion dollars. Head lettuce, and romaine lettuce also generated high production values at 1.6 billion, and 1.4 billion USD , respectively in 2017 (USDA, 2019). Leafy greens have been listed by the USDA among the top ten high-risk foods, with 606 outbreaks in the US between 1973 and 2012 (Herman et al. 2015; Heiman, 2015). On an average, with 20,003 foodborne illness cases, 1,030 hospitalizations, and 19 deaths, leafy vegetable associated outbreaks were larger than those attributed to other food types. Leafy greens ranked first in the proportion of illnesses associated with the outbreaks caused by food commodities from 1998-2018. Most of these outbreaks have been linked to Shiga toxigenic *Escherichia coli* (STEC), *Salmonella*, and Norovirus (8 consequently, several intervention techniques, for maintaining fresh produce safety, have been employed by the industry. Among the methods used by the fresh produce industry, the most common include, antimicrobial washes such as chlorine or surfactants in the processing water prior to packaging (Mandrell, 2009). However, chlorine efficacy is affected by organic matter.

The contamination of leafy greens in the pre-harvest environment could occur due to cross-contamination with contaminated soil, irrigation water, dust or soil amendments harboring foodborne pathogens. Row crops such as leafy greens are highly susceptible to contamination by *E. coli* O157:H7. Recent outbreaks associated with romaine lettuce grown in the Yuma region as

well as previous leafy green outbreaks have resulted due to contamination by *E. coli* O157:H7. Shigatoxigenic *E. coli* such as *E. coli* O157:H7 has been typically associated with cattle and can be introduced into the environment from cattle operation facilities. The presence of *E. coli* O157:H7 into soil or irrigation water can result in cross transfer to produce surface. Several studies have indicated that *E. coli* O157:H7 can persist on plants and even internalize sub-surface. Once present on the surface of leafy greens such as lettuce, several factors such as stage of development, presence of lesions, occurrence of spoilage organisms and plant metabolites could affect plant growth. Previous studies have indicated that the presence of sugars in plant metabolites could aid in both survival and population increase of foodborne pathogens while certain fatty acids might be inhibitory to survival and proliferation of foodborne pathogens.

While romaine lettuce has been associated with several *E. coli* O157: H7 outbreaks, iceberg lettuce has been less frequently associated with outbreaks caused by shigatoxigenic *E. coli*. Leaf placement, post-harvest processing and differences in metabolite availability could contribute to differences in pathogen harborage amongst these commodities. Several studies have indicated that metabolite variation such as the availability of sugars, carotenoids, cyanidin, polyunsaturated fatty acids, total phenolic contents (TPC), and antioxidant potential. could occur among different cultivars of lettuce. The presence/ absence and density of these compounds could vary among Iceberg and Romaine lettuce and could influence the survival and growth of *E. coli* O157:H7.

The objective of this project was to employ a metabolomics approach in conjunction with analysis of growth rates, chemotaxis and biofilm formation by *E. coli* O157:H7 in exudates obtained from Romaine lettuce and Iceberg lettuce. The results of this study will help in better risk evaluation for produce growers.

Methods

Bacterial Culture

Escherichia coli O157:H7 H1730 and *Salmonella enterica* subspecies *enterica* serotype Newport stock was obtained from the culture collection of the Center for Food Safety at the University of Georgia. The frozen stock cultures were revived by culturing in Brain Heart Infusion Broth for 48h at 37°C. The *E. coli* O157:H7 culture was then streaked on Eosin Methylene Blue agar (EMB) and Chromogenic medium and the *S.* Newport culture was streaked on xylose lysine deoxycholate agar with tergitol supplmentation. Isolated colonies from the streak were confirmed as *E. coli* O157:H7 or *Salmonella* using serological agglutination test. Colonies with typical metallic sheen from EMB agar and black colonies on XLT4 were streaked on Tryptic Soy Agar. For experiments, several loopfuls of the culture was resuspended in sterile Phosphate Buffered Saline (PBS) and diluted to required cell densities for the experiments.

Lettuce

Iceberg lettuce heads and Romaine lettuce heads were harvested from the field at 4, 8, 12 weeks and at the harvest- ready stage using a clean blade. The harvested lettuce was placed inside a 2 gallon Ziploc bag. The bags should be placed on frozen icepacks and shipped to the University of Georgia, Center for Food Safety, Griffin through overnight shipping.

Collection of exudate

Each sample of lettuce was immersed in 1 L of autoclaved deionized water for a duration of 14h to collect the exudates from the lettuce. The water was then filter sterilized using a 0.2µm filter. The exudates from romaine lettuce at 8 weeks (RL8), 12 weeks (RL12) and harvest ready romaine lettuce (RLH) as well as Iceberg lettuce at 8 weeks (IL8), 12 weeks (IL12) and harvest ready iceberg lettuce (ILH) were frozen until further testing and stored at -20°C.

Glycosyl composition analysis

Glycosyl composition analysis was performed by combined gas chromatography-mass spectrometry (GC-MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the acid-hydrolyzed samples by acidic methanolysis as described previously by Santander et al. (2013). The samples were spiked with 20 µg of myo-inositol (internal standard) and heated in 1 M methanolic HCl in a sealed screw-top glass test tube for 18 h at 80 °C. After cooling and removal of the solvent under a stream of nitrogen, the samples were treated with a mixture of methanol, pyridine, and acetic anhydride for 30 min. The solvents were evaporated, and the samples were derivatized with Tri-Sil HTP (Pierce) at 80 °C for 30 min. GC-MS analysis of the resulting TMS methyl glycosides was performed on an Agilent 7890A GC interfaced to a 5975C MSD, using an Supelco Equity-1 fused silica capillary column (30 m ´ 0.25 mm ID).

Growth Curves

The growth of *E. coli* O157:H7 and *Salmonella enterica* in RL8, RL12, RLH, IL8, IL12, ILH was determined over a duration of 24h. An initial inoculum of 6 log CFU/ml was introduced into the growth medium. The populations of the *E. coli* O157:H7 *S. enterica* were measured through enumeration by spread plating with appropriate serial dilutions at 0, 4, 8 12, 16, 20 and 24h. The populations were plotted over time to obtain a growth curve.

Biofilm formation

The formation of EPS (exopolymeric substance) by *E. coli* O157:H7 in the exudates was determined by the crystal violet staining method (Dev Kumar et al., 2018). Briefly, 200µL of the exudates from romaine lettuce and iceberg lettuce at 8 weeks, 12 weeks and harvest were added to

the wells of a 96 well plate. These were inoculated with 5 log CFU/ml of *E. coli* O157:H7. The plate was incubated at 35℃ for 24H. following which, the exudate was removed. The wells were washed twice with sterile PBS to remove planktonic cells. The quantity of EPS was determined by adding 200 µL of 0.1% crystal violet. After staining for a duration of 5 minutes, the stain was aspirated, and the wells were washed 2 times with sterile PBS to remove unbound dye. Once dry, the wells were destained with 200 µL of 33% acetic acid. The quantity of unbound dye was determined by measuring the optical density at 570 nm (OD_{570}).

Bacterial cells in biofilm

The population of strongly bound bacterial cells in the biofilm matrix was also determined. Biofilms were formed as previously described. After aspiration of the growth medium and washing to remove the planktonic cells, the strongly bound cells were dislodged through sonication for 2 min using a Branson sonicator. The population of the cells was determined by enumeration though spread plating.

Chemotaxis

A chemotaxis assay was performed to determine if *E. coli* O157:H7 and *S.* Newport demonstrated a chemotactic response to lettuce exudates using a protocol adapted from Mazumder et al., 1999. Briefly 100µl of each exudate was loaded to 1ml tuberculin syringe with needle. A needle was attached to the syringe on top of the syringe. The needle was inserted into a pre-sterile 200 µl pipette tip containing 100 µl 8 log CFU/ml inoculum. After an incubation time of 60, 120 and 180 min at room temperature, the needle-syringe was removed from the bacterial suspension contained in the pipette tip. The population of bacteria that had moved from the bacterial suspension to the exudate was enumerated.

Results and Discussion:

The study determined the composition of lettuce exudates from iceberg and romaine lettuce at 8 weeks, 12 weeks and harvest ready stages of growth as well as physiological responses of *E. coli* O157:H7 to the exudates. The physiological responses included growth, biofilm formation and chemotaxis in response to the presence of exudates. Foodborne pathogens such as *E. coli* O157:H7 have been known to persist in the produce production environment. Cross contamination due to contact with growth matrices such as soil, irrigation water, dust and soil amendments could result in the contamination of leafy greens such as lettuce.

Analysis of exudate composition indicated the presence of sugars but negligible amounts of fatty and amino acids. For all exudate samples, the main component is glucose. All samples also contained mannose and galactose, and with the exception of sample 3 (ILH) all samples contain arabinose. Sugar alcohols were detected in samples. Notoriously, all samples contained extra inositol peaks, in addition to the *myo*-inositol peak. Considering this had been observed in previous reports (CCRC code GK081619), and the fact that no additional inositol peaks were found in the standard runs (not shown), we believe the source of these peaks is not under derivatization, but possibly different inositol isomers present in the samples.

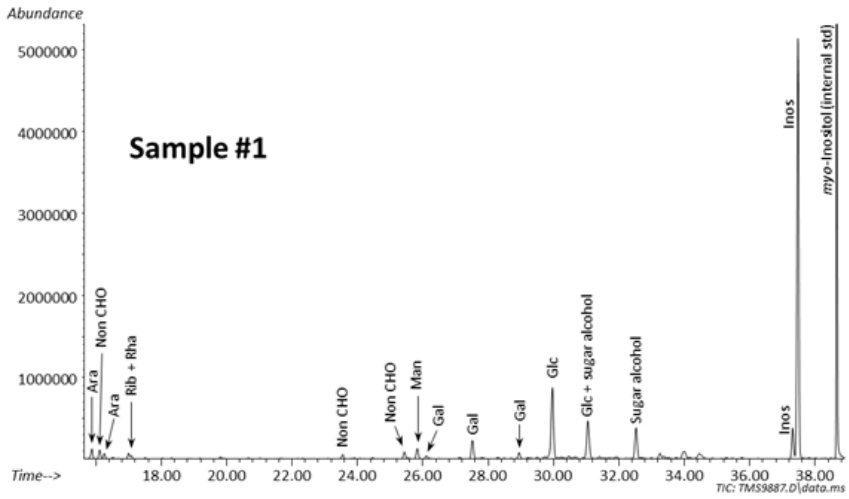


Figure 1: Sugars in Iceberg Lettuce at 8 weeks

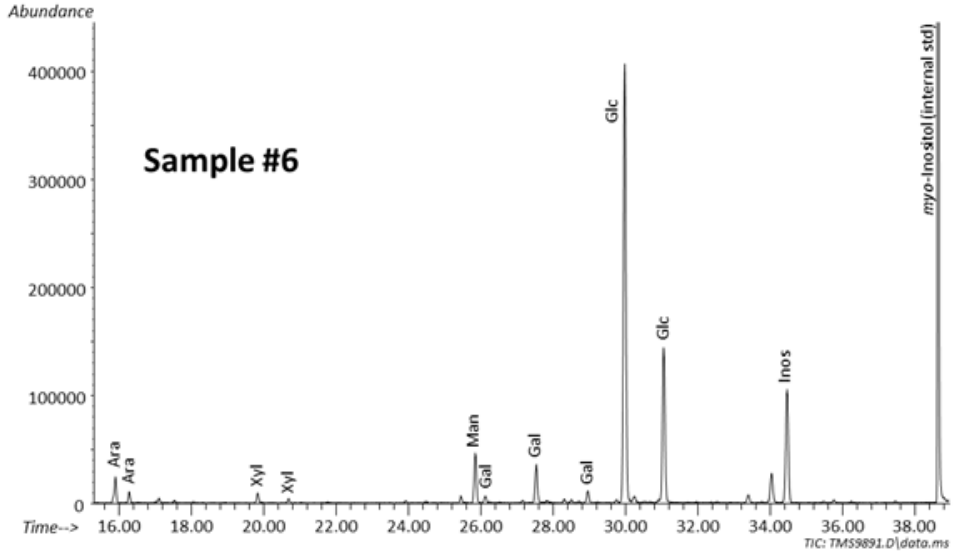


Figure 2: Sugars in Iceberg lettuce exudates at 12 weeks

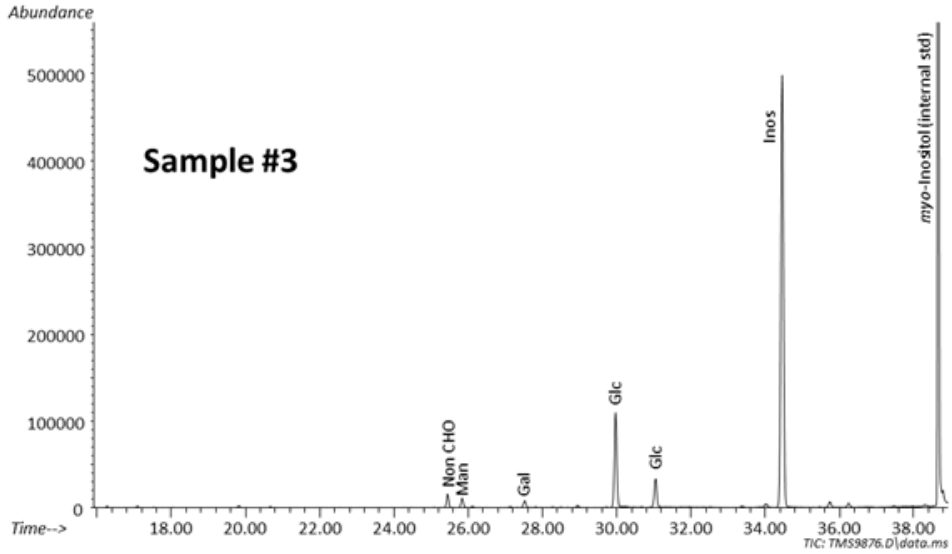


Figure 3: Sugars in Iceberg lettuce at harvest stage

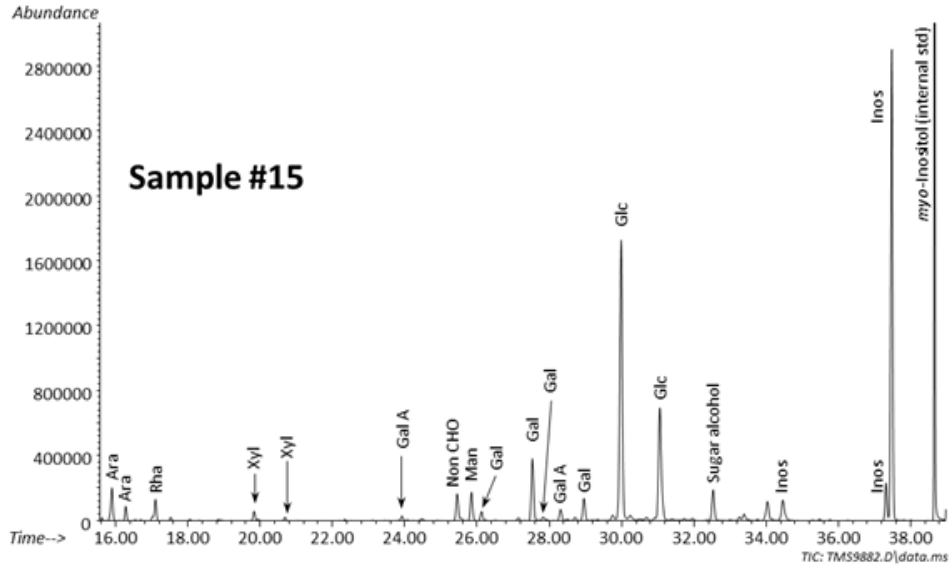


Figure 4: Sugars in Romaine Lettuce Exudate at 8 week stage

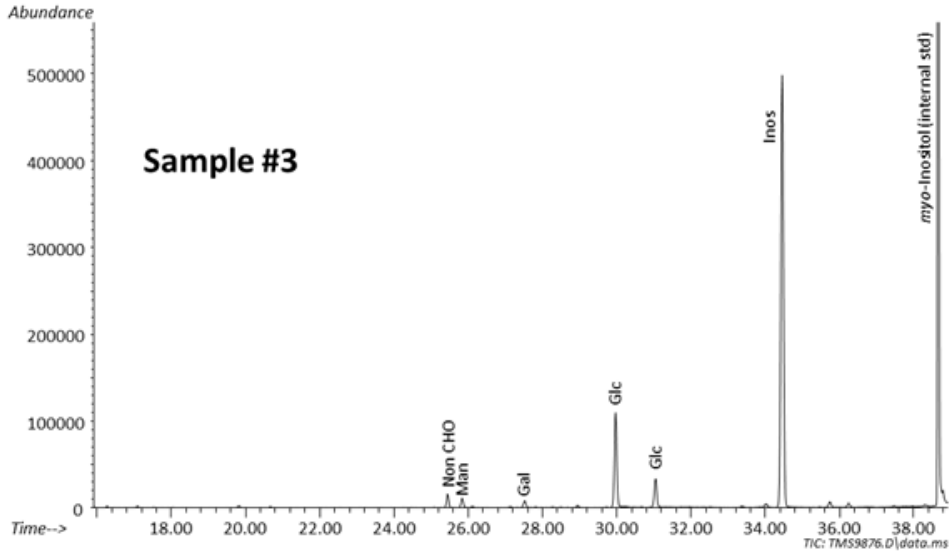


Figure 5: Sugars in Romaine Lettuce Exudate at Harvest stage

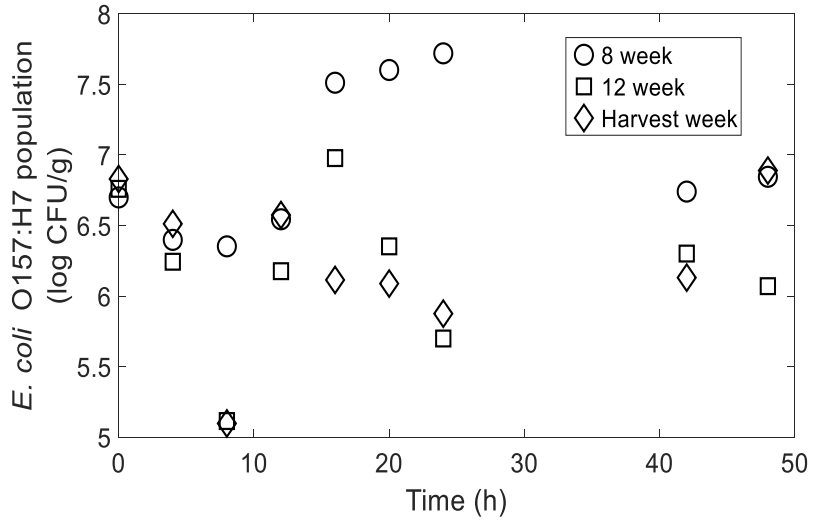


Figure 6: Growth of *E. coli* O157:H7 in exudates of Iceberg lettuce harvested at different time points

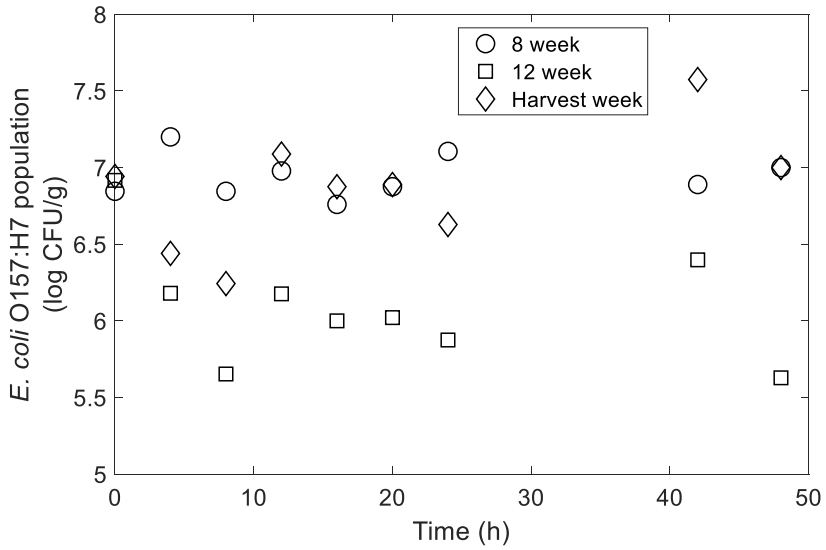


Figure 7: Growth of *E. coli* O157:H7 in exudates of Romaine lettuce harvested at different time points

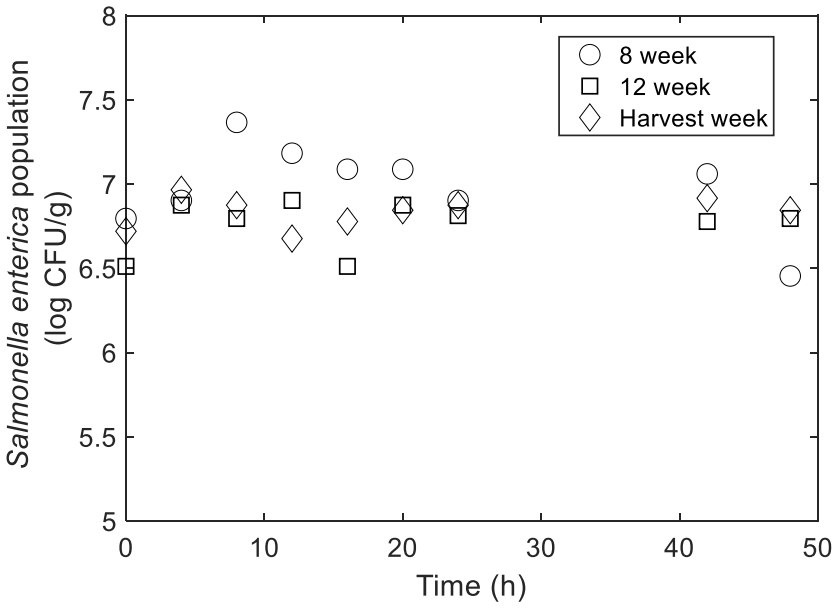


Figure 8: Growth of *Salmonella enterica* in exudates of Iceberg lettuce harvested at different time points

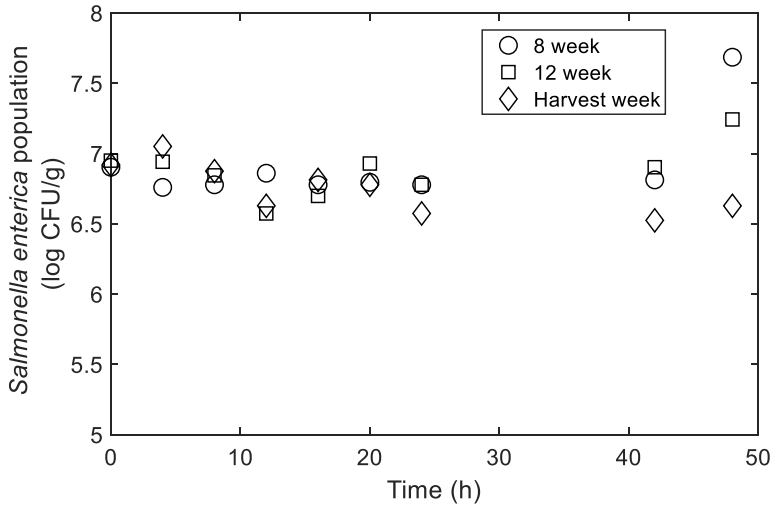


Figure 9: Growth of *Salmonella enterica* in exudates of Romaine lettuce harvested at different time points

The populations of *E. coli* O157:H7 and *Salmonella enterica* were slightly higher in the 8-week and harvest week Iceberg and Romaine lettuce samples, as compared with the 12-week samples (Figure 6-9). These results indicate that the metabolomes at 8-weeks may support the growth of foodborne pathogens, as compared with the leafy greens harvested at the later stages. Further studies are needed to corroborate these trends. These results may be useful in developing recommendations for identifying the optimum time period for harvesting leafy greens.

Biofilm formation is a well-known strategy for environmental persistence by foodborne pathogens. Biofilms consist of bacteria encased in an exopolymeric matrix. A comparison of EPS production by *E. coli* O157:H7 indicated that exposure to exudates from romaine lettuce ready for harvest resulted in the highest amount of EPS. *E. coli* O157:H7 formed more EPS in presence of romaine lettuce exudates (both 8 and 12 week) than in presence of iceberg lettuce exudates. The formation of EPS by *E. coli* O157:H7 helps in protection from environmental and chemical stressors such as

heat, starvation and sanitizers such as chlorine and peracetic acid. Increased resistance to these stressors could contribute to better survival and even higher cross contamination during washing.

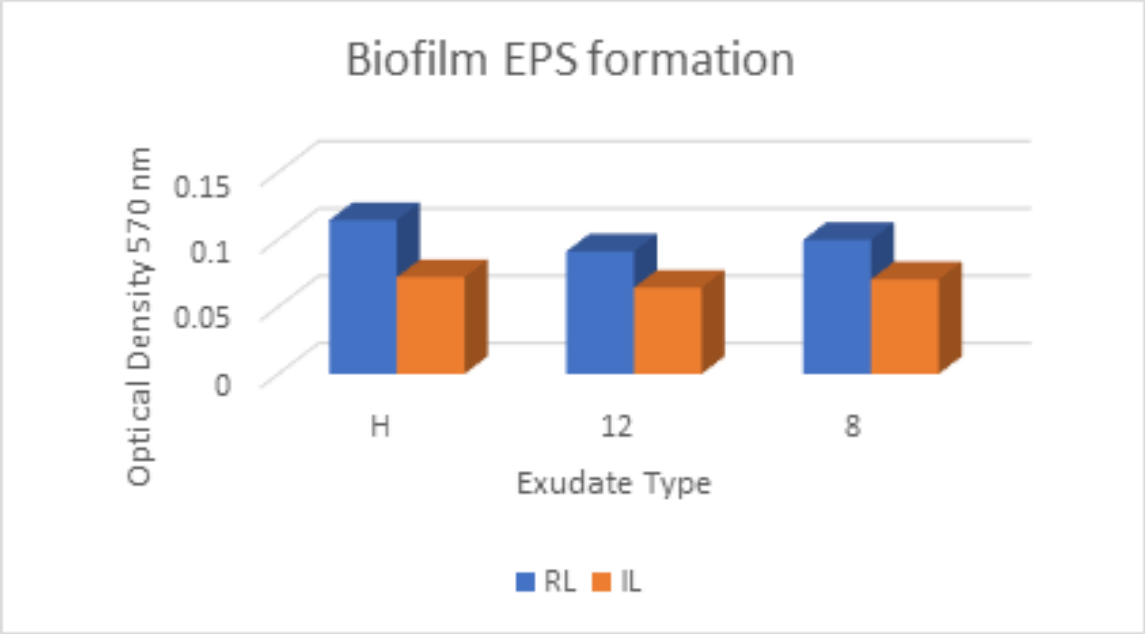


Figure 10: Biofilm EPS formation in Iceberg and Romaine lettuce exudates harvested at different time points

The population of *E. coli* O157:H7 in the biofilm matrix was also determined. The lowest density of *E. coli* O157:H7 cells, 4.63 log CFU/ml, was found in biofilms formed in presence of harvest ready iceberg lettuce exudate and the highest density of *E. coli* O157:H7 cells, 6.17 log CFU/ml, was found in biofilms formed in presence of 8-week iceberg lettuce exudate. The average population of cells in biofilms formed by *E. coli* O157:H7 in romaine lettuce exudates was 5.47 log CFU/ml.

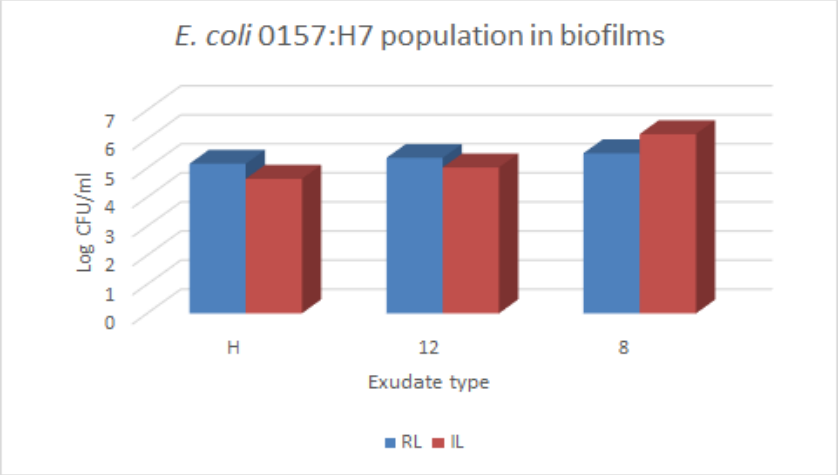
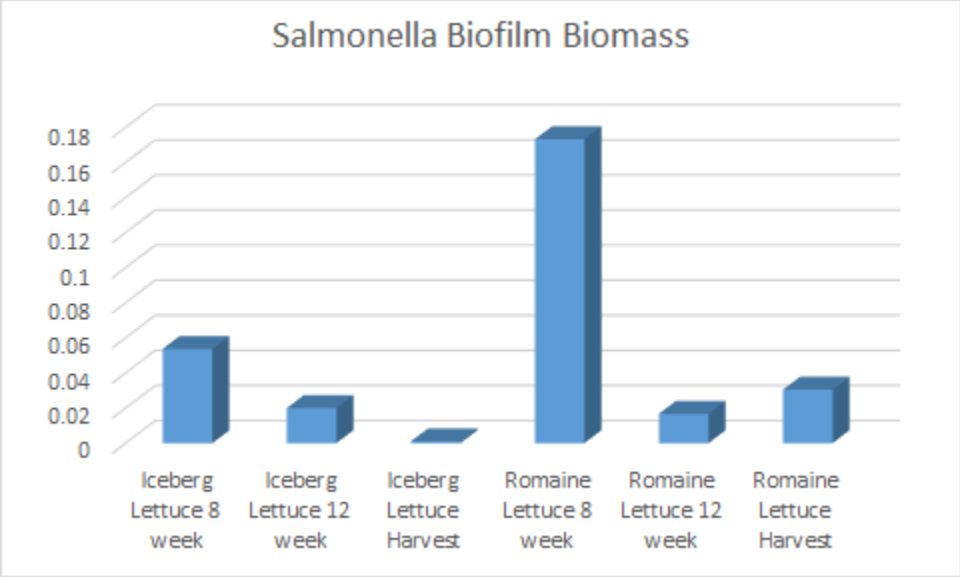


Figure 11: *E. coli* O157:H7 in biofilms formed in Iceberg and Romaine lettuce exudates harvested at different time points

The biofilm biomass and the population of attached cells was estimated for *Salmonella* Newport biofilms formed in presence of the exudates. The population of *S.* Newport cells, 6.5 log CFU/ml and 6.4 log CFU/ml, was significantly higher in biofilms formed in presence of harvest ready iceberg lettuce and iceberg lettuce exudate after 12 weeks of growth respectively, than that of *E. coli* O157: H7. The population of *Salmonella* cells in iceberg lettuce exudate obtained from crop after 8 weeks of growth was 6.48 log CFU/ml. In presence of romaine lettuce exudates, S. Newport cell populations found in biofilms formed in presence of romaine lettuce exudates at harvest, 12 weeks and 8 weeks was 6.49 log CFU/ml, 6.09 log CFU/ml and 6.21 log CFU/ml respectively and was higher that *E. coli* O157:H7. S. Newport form the highest biofilm biomass in presence of Iceberg lettuce exudate formed during the 8-week stage. S. Newport formed the highest amount of biofilm biomass in presence of romaine lettuce exudate obtained at 8 weeks of growth (O.D 570- 0.17). Iceberg lettuce exudate from the 8 weeks for growth also resulted in increased biofilm biomass formation by *S.* Newport (O.D 570- 0.05).. *S.* Newport formed

significantly higher biofilm in presence of romaine lettuce exudate than in comparison to iceberg lettuce exudate obtained at the harvest stage.



The results from the studies indicates that *E. coli* O157:H7 can form biofilms in presence of both romaine and iceberg lettuce exudates. The high amount of EPS formed in presence of romaine lettuce exudate could indicate the possibility of better protection from stressors during survival on produce surfaces and during post-harvest handling. Formation of biofilms in presence of lettuce exudates could indicate that the recycling of wash water during hydrocooling and washing should be avoided as leachates might promote biofilm formation by *E. coli* O157:H7 on produce contact surfaces.

Chemotaxis is defined as the movement of bacteria towards a stimuli that might be benificial such as an energy source. No significant differences was observed among the stimuli migrated populations of *E. coli* O157:H7 after different durations of exposure to the exudate. This indicated that the exudates did not differentially promote chemotaxis in *E. coli* O157:H7. The

population of *E. coli* O157:H7 in RLH and ILH was 6.01 and 5.96 log CFU/ml respectively. In RL12 and IL12 the population of *E. coli* O157:H7 was 6.13 and 6.06 log CFU/ml respectively. For RL8 and IL8 the population of *E. coli* O157:H7 was 6.03 and 6.09 log CFU/ml respectively.

Iceberg lettuce exudates did not promote chemotaxis of *S.* Newport and the population of *S.* Newport did not increase significantly over time. The population of S. Newport was approximately 6.3 log CFU/ml over all durations tested. In presence of iceberg lettuce exudate obtained at 8 and 12 weeks of growth a population increase of approximately 1 log CFU/ml was observed between 60 and 120 min, indicating the presence of chemotaxis.

Conclusions: The results of this study indicate that certain differences might occur between the metabolites of Iceberg and Romaine lettuce such as the presence of sugars, phenolics and sugar alcohols. The concentration of these compounds might change during growth and development and could lead to growth of the pathogen as well as stimulate persistence mechanisms such as biofilms. There was more EPS formation in Iceberg lettuce as compared with the Romaine lettuce, which indicates that biofilms might be formed by *E. coli* O157:H7 in presence of lettuce metabolites. The formation of biofilms in processing and wash water contact surfaces by *E. coli* O157:H7 can serve as a source of recurrent contamination. In addition, the results indicate that Iceberg lettuce harvested at 8-weeks may better harbor the growth of pathogens, compared with the lettuce harvested at 12-weeks and harvest week.

Salmonella Newport demonstrated chemotaxis towards romaine lettuce metabolites at the 8 and 12 week stage of growth. This could indicate that the exudates could contain nutrients that could result in *Salmonella* translocation to the leaf surface. The increased biofilm biomass in presence of romaine and iceberg lettuce metabolites obtained at the 8-week stage of development and

might indicate that persistent contamination of both iceberg and romaine lettuce by foodborne pathogens could occur during the early stages of plant development.

References:

- Herman KM, Hall AJ, Gould LH. 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973–2012. Epidemiol Infect 143:3011–20. https://doi.org/10.1017/S0950268815000047.
- U.S. Department of Agriculture. 2011. Economics, statistics and market information system. U.S. Department of Agriculture, Washington, DC. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID_1576.
- Heiman KE, Mody RK, Johnson SD, Griffin PM, Gould LH. 2015. *Escherichia coli* O157 outbreaks in the United States, 2003–2012. Emerg Infect Dis 21:1293–1301. https://doi.org/10.3201/eid2108.141364.
- Mandrell RE. 2009. Enteric human pathogens associated with fresh produce: sources, transport, and ecology, p 5–41. In Fan X, Niemira BA, Doona CJ, Feeherry FE, Gravani RB (ed), Microbial safety of fresh produce. Wiley-Blackwell, Ames, IA.
- U.S. Department of Agriculture. 2019. USDA economic research service – food availability (per capita) data system. Retrieved from http://www.ers.usda.gov/dataproducts/food-availability-(per-capita)-data-system/.aspx, Accessed date: 3 October 2019.