Survival of *Listeria monocytogenes* on Lettuce With and Without Injury

John Grocholl

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In

Plant Science and Pest Management

Dr. Ryan Stewart - Plant and Environmental Sciences

Dr. Dixie Dalton

Dr. Chuanxue Hong

Dr. Josh Kardos

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ABSTRACT

Listeria monocytogenes (*Lm*) is a bacterium that causes listeriosis and outbreaks have been linked to the consumption of fresh produce. The purpose of this project was to establish if there is potential for *Lm* to persist on lettuce leaves. It also evaluated whether there is a difference in survival of this pathogen on injured and uninjured lettuce leaves. This study used *Lm* strain LS1061; a spontaneous mutation resulting in a rifampicin resistant strain. LS1016 is a serotype 4b isolate from the caramel-covered apple outbreak. Three conditions were evaluated: tear injury and 10µL *Lm* culture, no injury and 10µL *Lm* culture on the back of the leaf, no injured plus *Lm* on the midvein of the leaf. Three independent trials for Romaine lettuce and two independent trials for Iceberg lettuce were conducted. When plants were about half grown, lettuce leaves were injured by tearing a portion of the leaf, about an inch (2.5cm) from the tip to expose the inner tissues. Romaine trials showed that by day 4, positivity on all samples did not have major decline. Decline to ~50% or under was seen at Day 14. Injured Romaine had a high percentage (≥40%) of positive samples through Day 21. Like Romaine, Iceberg trials did not fall to 50% or lower positives until Day 14. Injured Iceberg and “cup” had positive samples through Day 21.

INTRODUCTION

Listeria monocytogenes (Lm) is a Gram-positive pathogen that causes invasive listeriosis, a rare, yet severe, human illness (Orsi and Wiedmann 2016). Ingestion of *Lm* can induce a benign and self-limiting gastroenteritis in immunocompetent individuals (Lecuit 2020). This illness consists of mild to severe symptoms that are induced when highly contaminated food (up to ~10^9 bacteria) is consumed (Radoshevich and Cossart 2018). However, in the case of neonates, elderly individuals, immunocompromised individuals, and pregnant women, even low levels of food contamination (~10^2–10^4 bacteria) can lead to severe illness, including bacterial sepsis, meningitis, and complications to pregnancy, such as premature birth, miscarriage, or stillbirth (Radoshevich and Cossart 2018). While the infection is rare, mortality, even with hospitalization, is 20-30%, making invasive listeriosis one of the leading causes of death due to foodborne illness (Behravesh, 2011). The cost associated with this illness is in the billions (Hoffmann et al. 2012), with hospitalizations due to *Lm* infections estimated at 94% (Scallan et al. 2011). Because of the major health implications related to *Lm*, identification of potential pathways for food contamination is imperative to protect public health.

Lm was first recognized as a foodborne pathogen after a 1981 outbreak in Canada that was linked to cabbage in coleslaw (Self et al. 2019). Outbreaks of listeriosis have historically been associated with ready-to-eat (RTE) deli meats and dairy products, but have more recently been associated with fresh produce, including sprouts, celery, cantaloupe, stone fruit, and caramel apples (Table 1) (Self et al. 2019). The presence of *Lm* in fresh produce, as well as other RTE foods, presents an increased risk that can lead to high hospitalization and death rates (Table 1).

Table 1. Produce-associated outbreaks in the U.S. for the last 15 years.

Year	Commodity	Cases	Hospitalizations	Deaths	Citation
2008	sprouts	20	16	0	Garner & Kathariou, 2016
2010	celery/chicken salad	10	10	5	Garner & Kathariou, 2016
2011	cantaloupe	147	143	33	Garner & Kathariou, 2016
2014	sprouts	5	5	2	Garner & Kathariou, 2016
2014	caramel-covered apples	32	31	6	Garner & Kathariou, 2016
2014	stone fruits	2	0	0	Garner & Kathariou, 2016
2016	packaged salads	19	19	1	Self et al., 2019
2016	frozen vegetables	9	9	3	CDC, 2016
2020	enoki mushrooms	36	31	4	CDC, 2020
2021	packaged salads	10	10	1	CDC, 2022
2022	packaged salads	18	16	3	CDC, 2022
2022	enoki mushrooms	5	5	0	CDC, 2023
2023	leafy greens	19	18	0	CDC, 2023

Despite a history of *Lm*-contaminated greens causing outbreaks, dating back to 1981, there is limited research on *Lm* persistence on field grown lettuce. Pre-harvest contamination is considered to account for many outbreaks, as it is extremely difficult to prevent (Harapas, Premier et al. 2010). If plants from a production field are contaminated with *Lm*, they could potentially cause finished product

contamination in three ways: 1) the contaminated produce itself, 2) carrying the bacterium into facilities and contaminating equipment, leading to contamination of other products, and 3) introduction of produce to other RTE products that are capable of supporting *Lm* growth.

Pathogens can arrive in the field via animal manure used for fertilization and soil conditioning, contaminated irrigation water, wild and domestic animals, and floodwaters from a contaminated site, such as a cattle stock yard or sewage treatment facility (Harapas et al. 2010). Additionally, *Lm* is well-established as a soil microorganism, which may result in it being resident in the field itself or enable its persistence long after an introduction has occurred (Weis, 1975). A study evaluating strategies for risk management of pathogens, such as *Campylobacter* spp. and *Lm,* found their presence in fields can be influenced by the source of the irrigation water and the time interval between the last irrigation and harvest (Guevremont et al. 2017). In this study, *Lm* was detected in one lettuce sample (n=96) from one trial and none in the subsequent two trials (n=96). *Lm* was also recovered from one sample of aerated pond water in the first year of the trial, showing irrigation water may be a cause of contamination (Guevremont et al. 2017). Other research also showed a high prevalence of *Lm* in farm soils with highest prevalence during the winter and spring, as well as relatively high *Lm* prevalence in surface water (Ferguson, 2023). In agricultural regions, animal waste is often used to amend soils and there is known presence of *Lm* in farm animal feces. The use of these wastes without sufficient sanitation in farm fields can be a route of transmission to the soil and then cultivated plants (Vivant et al. 2013). Rules are in place to prevent produce affected by such events from entering market, but some events may go undetected. Current FDA guidelines recommend a pathogen die-off rate of 0.5 log per day to determine a time interval (in days), between last irrigation with untreated water and harvest with a maximum of four days (U.S. Food and Drug, 2015). Research on the possible sources of *Lm* contamination on produce will help prevent future outbreaks. Because listeriosis infections can be caused by consumption of leafy greens, research on potential pathways of *Lm* contamination during different stages of production and processing is crucial.

Lettuce has the potential to harbor *Lm* in leaf tissues. Research looking at internalization patterns of *Lm* among romaine lettuce plants 20 days post seed inoculation found the density of *Lm* was 3.9 cells/mm^3 of plant tissue. The author indicated that this was likely an underestimation as multiple bacterial cells could fit in the section (10µm) of plant tissue evaluated (Shenoy et al. 2017). *Lm* was found to be associated with every major tissue type, including the epidermis, cortex, pith, and vascular tissue (Shenoy et al. 2017). In a study looking at shoot injury and persistence of *Salmonella enterica* and *Listeria innocua* on lettuce, injury was inflicted to leaves, which were then inoculated using a spray bottle. Leaves were harvested for up to 21 days and *L. innocua* fell below detectable levels by Day 14 on injured lettuce versus Day 3 on uninjured leaves. Colony counts of *L. innocua* were greater on the injured versus uninjured shoots of lettuce as well (Harapas, Premier et al. 2015).

Experiments looking at pathways of *Lm* contamination have regularly used non-pathogenic members of the *Listeria* genus as surrogates, as seen in the aforementioned study. *L. innocua* has often been used as a surrogate for *Lm* but has limitations, as studies have shown differences in *Lm* and *L. innocua* stress response, as well as differences among the *Lm* subgroups (Milillo et al. 2012). Possibly the most significant genetic difference between the two organisms is the absence of *Listeria* virulence gene cluster (LIPI-1), the main pathogenicity island, in *L. innocua* (Milillo, Friedly et al. 2012). While the most notable difference is the absence of LIPI-1 from these surrogates, differences in the SigB regulon are likely to facilitate the survival and protection in harsh environmental conditions (Sibanda, 2022). It

was observed that *L. innocua* was significantly more sensitive to brine solutions than *Lm*. At 4°C, some *Lm* strains possessed better cold tolerance than other *Listeria* spp., including *L. innocua*. (Milillo, Friedly et al. 2012). Cold tolerance of *Lm* is key in its survival and its ability to outcompete other organisms. In food production, especially with produce, *Lm* can therefore have an advantage in refrigerated environments. Cold storage has already been presumed to lead to *Lm* outbreaks on produce. In an assessment of potential pathways for cantaloupe to be contaminated with *Lm*, it was proposed that a combination nutrients, water, and lack of pre-cooling before cold storage may have provided ideal conditions for *Lm* to grow and outcompete background microflora (U.S Food and Drug, 2011). Given the differences observed in response to stress, it is reasonable to presume survival differences.

The purpose of this project is to establish if there is potential for *Lm* to persist on lettuce leaves and whether that persistence varies based on location or injury. Lettuce can be damaged by animals and field workers during production that can expose the internal tissues to pathogens like *Lm*. Factors that could cause a contamination event including splashing due to irrigation or rain and contact with animal feces. This research will look at the possibility for lettuce to support *Lm* survival and determine if there is a difference in survival of this pathogen on injured and uninjured lettuce leaves.

METHODS

Plant Cultivation. Research took place the Applied Research Plant Growth Facility (ARPGF) at FDA MOD-1 in Laurel, MD. Plant growth made use of two environmentally controlled areas. Seeds were incubated in CONVIRON GEN 1000 (Winnipeg, Manitoba, Canada) growth chambers, which control light intensity, temperature, and humidity, until ready for transplantation. Lights were set to run for 12 hr and would gradually increase intensity over a 4 hr period until they were at full strength, after which they would gradually decline. Temperature was set to range from 18°C-22°C with the lowest temperature occurring during evening and ramping up to 22°C when lighting was most intense. Humidity followed a similar trend and was set to 65% relative humidity during evening and would decrease to 55% during the day. After transplantation, lettuce plants were grown in grow rooms which allowed control of temperature, light duration, and humidity up to 40%. The grow rooms had a single set temperature of 18°C, except in trial 3 the temperature was increased to 22°C, and 30-40% humidity. Plant grow lights were set for 12 hrs day/night cycles, with no changes in intensity. These rooms were used to provide adequate room for growth of the plants. Seeds were started in Miracle-Gro® Moisture Control Potting Mix in Viagrow Standard Flat Insert compartmental tray and transplanted to Scotts Premium Topsoil in Greenhouse Megastore 300 Series 0.7-gallon, injection-molded polyethylene containers.

Bacterial strain. This study used LS1061, a spontaneously generated rifampicin-resistant strain derived from LS1016. LS1016 is a serotype 4b isolate from the caramel-covered apple outbreak and was isolated from an apple by the FDA Denver District Laboratory. LS1061 was routinely plated on brain heart infusion agar (BHIA) without rifampicin (Sigma Life Science, St. Louis, MO) (100 μg/mL) and incubated for 16 hrs at 37°C. On day 0, a 4-6 hr BHI broth culture was used to prepare an inoculum, adjusted to 10^6 CFU/mL in 0.85% sterile saline, based on the absorbance (600nm). The suspension was plated on BHIA, containing rifampicin, to determine the actual density.

Lettuce leaf trials. Three independent trials were conducted with romaine lettuce (*Lactuca sativa* var. longifolia) and two trials using iceberg lettuce (*Lactuca sativa* var. capitata). Three conditions were evaluated: injury and *Lm* inoculum, no injury and *Lm* inoculum on back of leaf, no injury plus *Lm*

inoculum to the midvein of the leaf. When plants were about half grown, lettuce leaves were injured by tearing a portion of the leaf about an inch from the tip (Figures 1&2), to expose the inner tissues (n=5-6 leaves per plant). Controls using saline were conducted concurrently with the experimental groups. The prepared *Lm* suspension or a saline control was spotted in a 10 µL aliquot to the leaf as indicated by the trial condition. Each condition was represented by three to six plants with one leaf harvested from each plant during each successive timepoint. Leaves were then harvested at Day 0, 3 or 4, 7, 14, and 21. Samples harvested on Day 0 were weighed and sterile saline was added to obtain a 1:10 dilution and manually massaged to homogenize the sample prior to enumeration. On subsequent days, the saline was replaced with Buffered *Listeria* Enrichment Broth (BLEB), but all other sampling steps were performed as on Day 0. Samples were enumerated via serial dilution and plating on BHIA with rifampicin. No enrichment was performed for samples collected on Day 0. Leaves harvested at the subsequent time points were processed for enrichment. All samples were incubated for 24-72 hrs at 37°C, with rifampicin antibiotics added after 4 hrs of incubation. After 48 hrs, a 10µL aliquot of the enrichment was struck out on RAPID'L.mono agar (Bio-Rad, Hercules, CA). Samples positive in enrichment but with no countable colonies from direct plating were assigned value of 75 cfu. Estimates based on colony counts below the level of significance were assigned Arabic numerals.

Figure 1. Romaine lettuce after injury, indicated by the yellow arrow.

Figure 2. Iceberg lettuce after injury, indicated by the yellow arrow.

RESULTS

Survival of *Lm* on Romaine lettuce. Results of the trials can be evaluated both by the levels of *Lm* recovered at each timepoint, as well as by looking at the percent positivity as a measure of persistence. Evaluation of enumeration results on Day 0 indicated a consistent inoculum (~$5x10^4$cfu/mL) across the samples. By the second sampling, romaine lettuce leaves showed a reduction in the level of *Lm* on injured leaves by no more than 1-log in the first two trials and about 1.5-log in the third (Figure 3). Conversely, uninjured leaves had a greater reduction by about 2 and 3-log in the first two trials and less than 1-log in the third (Figure 4). By Days 14 and 21, colonies were only sporadically observed during enumeration. The third trial evaluating romaine lettuce, as above though temperatures are 22°C, have found similar results. This trial includes a third experimental group with *Lm* inoculum being added to the front side of the leaf (Figure 5).

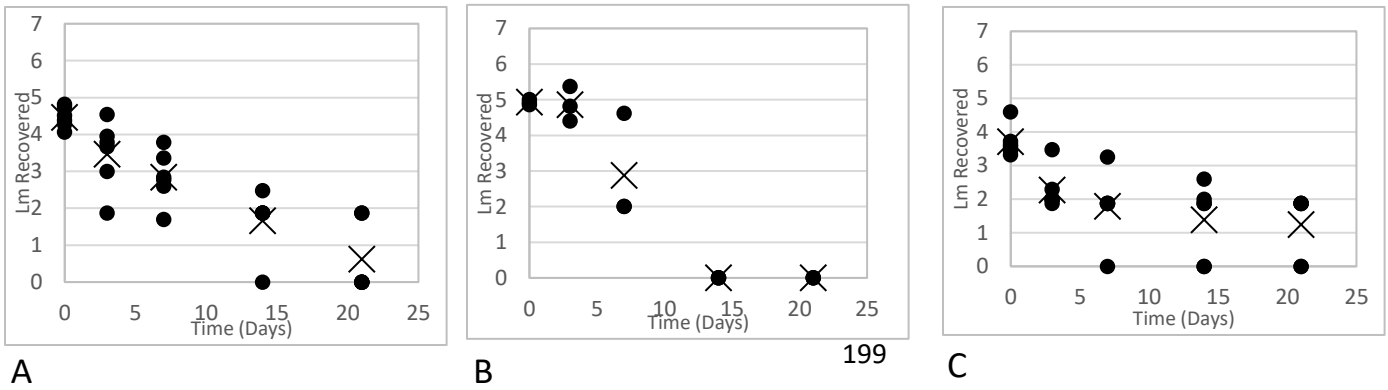

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Figure 3. Levels of *Lm* recovered from romaine leaves during the first (A), second (B), and third (C) trials. Circles represent the individual values for each leaf tested, while the "X" indicates the average of those values. Values below Log_{10} 2 are estimates.

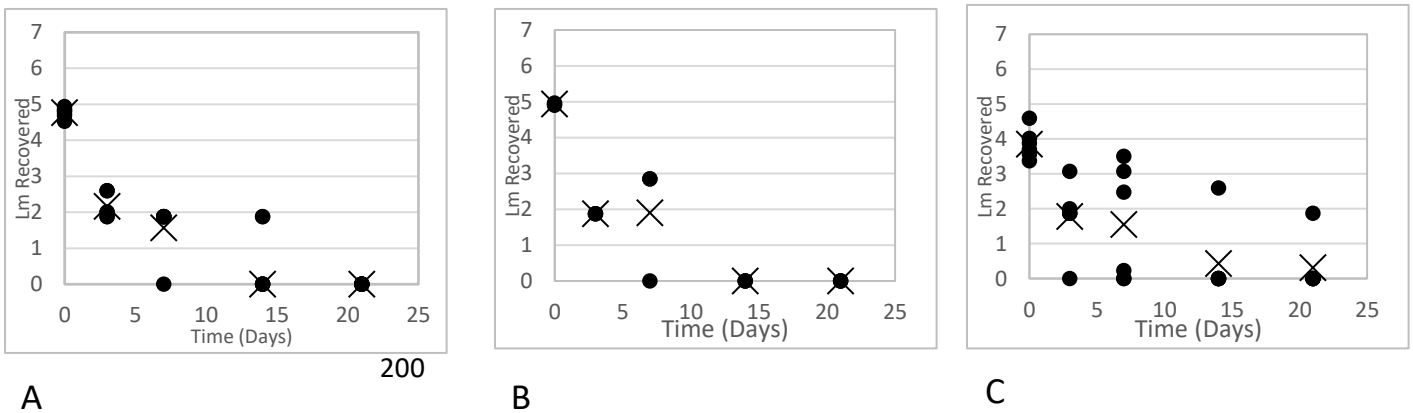

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Figure 4. Levels of *Lm* recovered from the back of romaine leaves during the first (A), second (B), and third (C) trials. Circles represent the individual values for each leaf tested, while the "X" indicates the average of those values. Values below Log_{10} 2 are estimates.

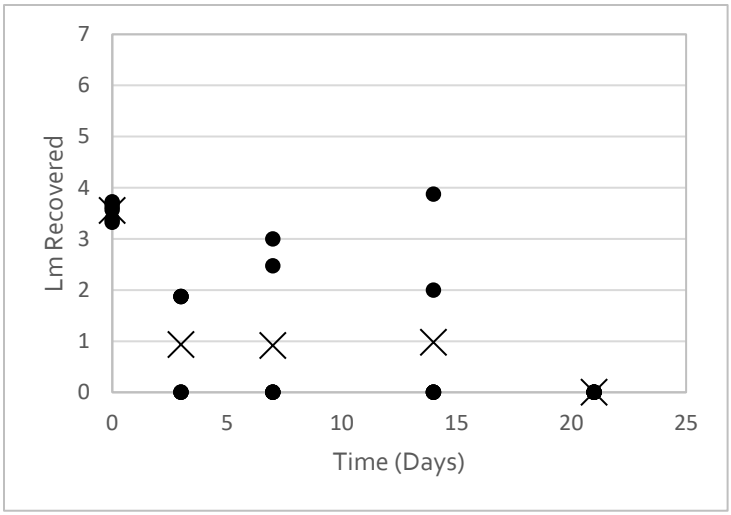


Figure 5. Levels of *Lm* recovered from the midvein of romaine leaves during a single trial. Circles represent the individual values for each leaf tested, while the "X" indicates the average of those values. Values below Log_{10} 2 are estimates.

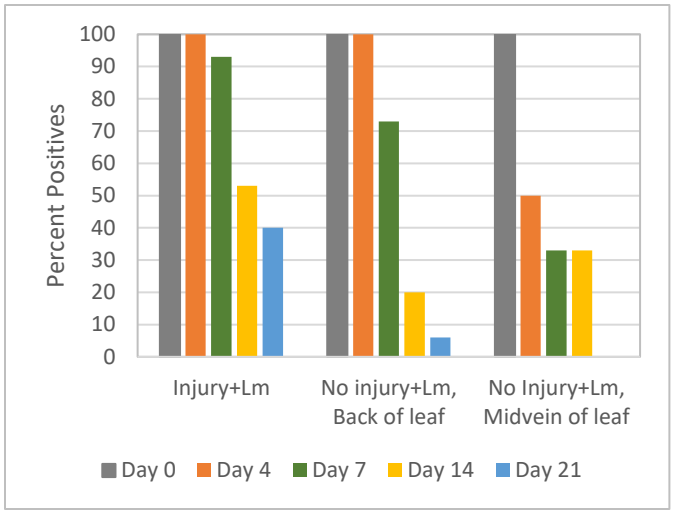
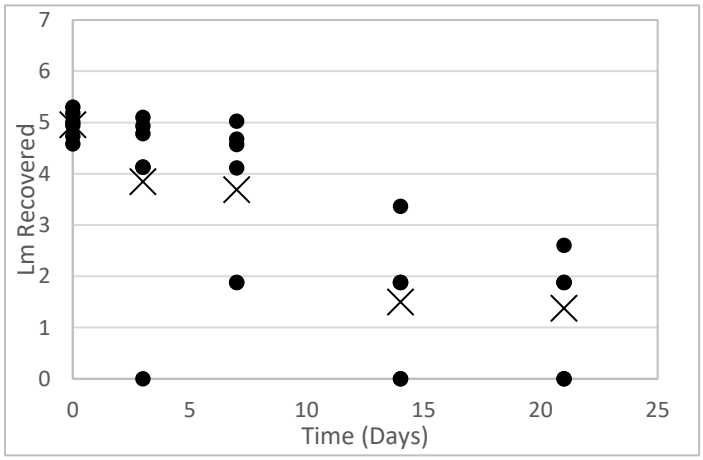


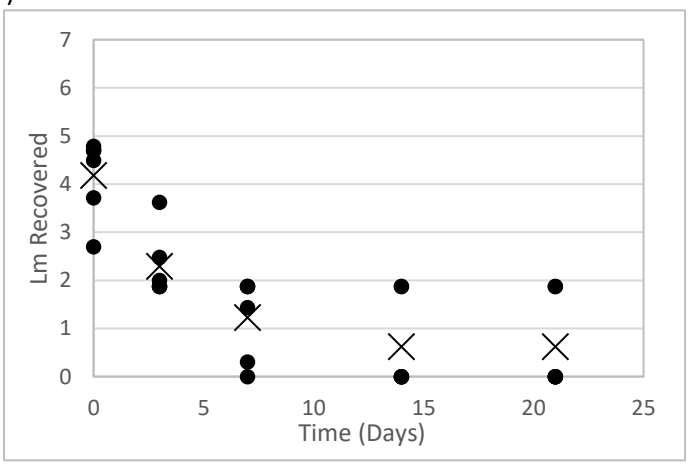
Figure 6. Percent of romaine leaves positive for *Lm* at the indicated day, based on inoculation scheme.

Evaluation of the percent of samples positive for *Lm* on romaine showed that by Day 4, none of the trial conditions indicated a marked decline (Figure 6). By Day 14, a decline to ~50% or under was observed. Injured romaine had a high percentage (≥50%) of positive samples through day 21 (Figure 6). However, given the second trial had only three plants, this may be due to sample size.

Survival of *Lm* on Iceberg lettuce. Similar to the romaine lettuce trial, the results from the iceberg lettuce trials can be evaluated in two ways. Day 0 enumeration results indicated equivalent levels of *Lm* on all leaves. The decline was negligible for injured leaves (Figure 7) between Days 4 and 7, and, conversely, leaves inoculated on the underside showed a more rapid decline with about 1-log decrease between Days 4 and 7 (Figure 8). Data from the second trial evaluating iceberg lettuce, despite a higher temperature (22°C), found similar results (Figure 9).



A



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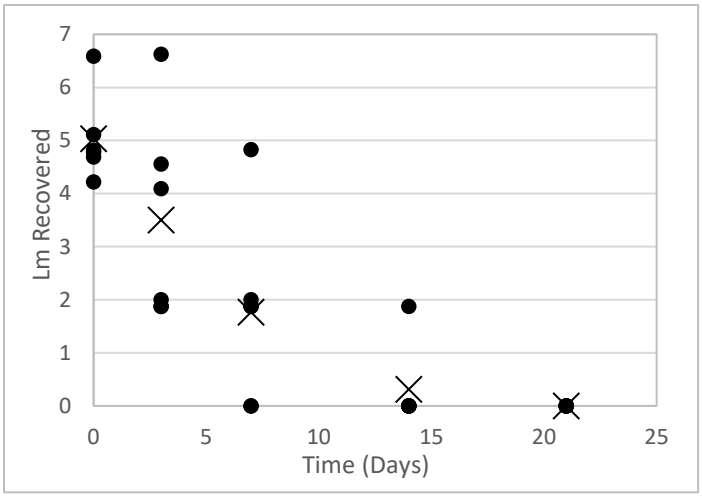
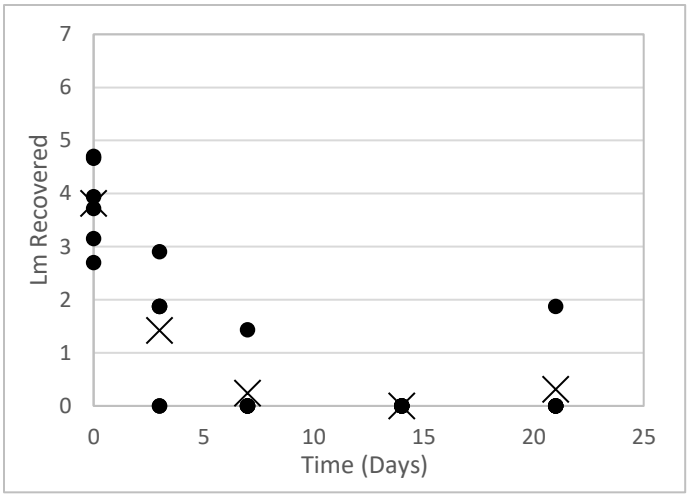
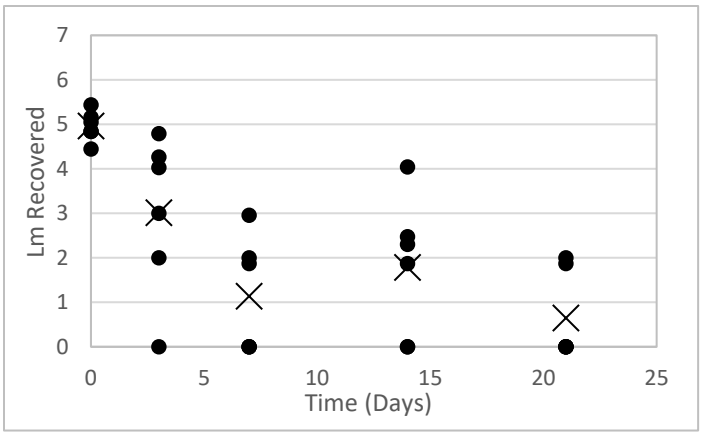


A

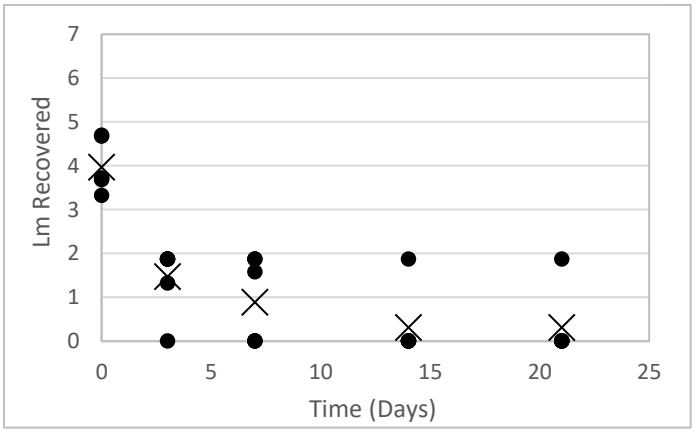


B

211 Figure 8. Levels of *Lm* recovered from the back of iceberg leaves during the first (A) and second (B) trials. Circles represent the individual values for each leaf tested, while the "X" indicates the average of those values. Values below Log_{10} 2 are estimates.



A



B

Figure 9. Levels of *Lm* recovered from the midvein of iceberg leaves during the first (A) and second (B) trials. Circles represent the individual values for each leaf tested, while the "X" indicates the average of those values. Values below Log_{10} 2 are estimates.

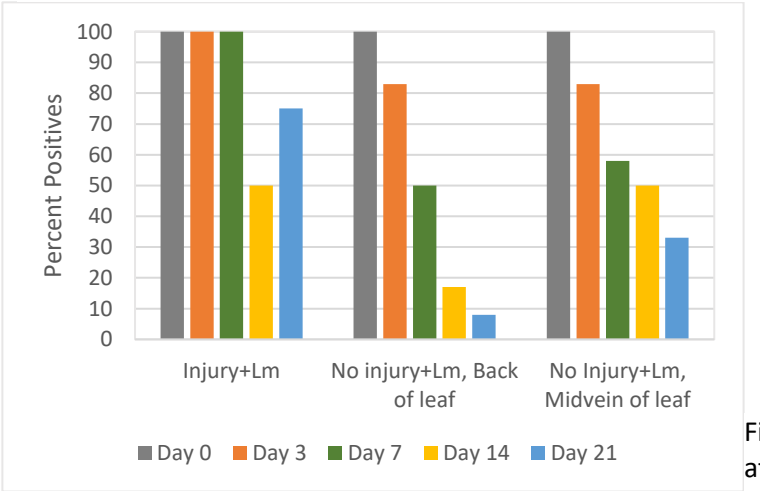


Figure 10. Percent of iceberg leaves positive for *Lm* at the indicated day, based on inoculation scheme.

Like romaine, iceberg trials did not fall to 50% or lower positives until day 14, and that was only for leaves inoculated on the back. Injured iceberg and midvein had >30% of the samples positive for *Lm* through day 21 (Figure 10). Evaluation by enrichment on day 21 found 75% of injured lettuce samples were positive for *Lm* while only 8% of leaves contaminated on the back were positive. Conversely, contamination on the midvein of the iceberg leaf yielded positives throughout the trial, with 33% yielding *Lm* on day 21.

DISCUSSION

This is an observational study, and more data is being generated to obtain statistical analysis to further the conclusions of the study. Furthermore, the intention is to create a document that can be potentially offered as a peer-review publication in the future. This study has added valuable data on the survival of *Lm* on lettuce during cultivation. These findings showed the potential for *Lm* to persist through 21 days post-contamination on lettuce. Critically, it showed that survival varied depending on the contamination site. Injured leaves, whether iceberg or romaine, were more likely to have *Lm* persist than uninjured leaves. A previous study showed that, when lettuce seeds were inoculated with *Lm,* it internalized in the leaf tissues and was found up to 20 days later highlighting the persistence of *Lm* (Shenoy et al. 2017). Furthermore, the use of the outbreak strain LS1061, instead of a surrogate revealed that *Lm* can readily survive past 14 days, which a prior review evaluating *L. innocua* noted rapid *Lm* decline compared to this surrogate (Milillo et al. 2012). Additionally, for iceberg lettuce, contamination on the midvein of the leaf appeared to aid persistence with at most 0.5-log reduction average between Days 7 and 14. The leaves of iceberg lettuce presumably created a more protected region and an environment with increased humidity. This protection may have allowed *Lm* to survive longer. Throughout the study, there were no visible differences between control plants and *Lm*-spiked plants, whether they were injured or not, consistent with what is generally known about the effect of *Lm* visually on contaminated commodities.

One of the drawbacks of simulated growth environments is the difficulty in replicating outdoor environments. It is not possible to duplicate all environmental conditions like rain events, sun exposure, and wind. These environmental factors likely impact *Lm* contamination events and possible persistence on plants. In this experiment, there were stable conditions but in production fields it varies. One way to mimic wind would be to use fans. This would replicate the constant air movement that occurs in outdoor environments. Rain would be more difficult to replicate indoors, but spraying leaves with water could be an option. A major difficulty would be attempting to reproduce a strong enough wind or rain to generate conditions replicative of the outdoors while still maintaining a safe laboratory space, leading to a decision on whether use of surrogates would be better as they may permit replication of these conditions, while understanding the potential limitations in their survival, highlighted by the data in this study.

Reducing the risk of contamination in field grown lettuce may be difficult, because of the current FDA guidelines for harvesting post irrigation event (U.S Food and Drug, 2011). However, this data suggests that *Lm* can survive past 14 days with greater prevalence among injured lettuce and on the midvein of iceberg. In fields with drip irrigation, more intense solar radiation, and minimal rainfall, pathogen contamination events may be less common and a lower risk because contamination by splashing events may not be as likely. Additionally, this is a small study, and more work is needed to verify these results. Further research needs to be done on whether injured lettuce contaminated with

Lm can transport or infect other parts of the plant. This research showed there is a potential risk for *Lm* to persist on lettuce, increasing the risk of introducing *Lm* into facilities or other products and this information will be important when developing risk-based guidance.

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