1 Title: Impact of mulches and growing season on indicator bacteria survival during lettuce
2 cultivation

3 Running Title: Mulches effect on indicator bacteria on lettuce and soil

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ABSTRACT

In fresh produce production, the use of mulches as ground cover to retain moisture and control weeds is a common agricultural practice, but the influence that various mulches have on enteric pathogen survival and dispersal is unknown. The goal of this study was to assess the impact of different mulching methods on the survival of soil and epiphytic fecal indicator bacteria on organically grown lettuce during different growing seasons. Organically managed lettuce, cultivated with various ground covers - polyethylene plastic, corn-based biodegradable plastic, paper and straw mulch - and bare ground as a no-mulch control, was overhead inoculated with manure-contaminated water containing known levels of generic *Escherichia coli* and *Enterococcus* spp. Leaves and soil samples were collected at intervals over a two week period on days 0, 1, 3, 5, 7, 10 and 14, and quantitatively assessed for *E. coli*, fecal coliforms and *Enterococcus* spp. Data were analyzed using mixed models with repeated measures and an exponential decline with asymptote survival model. Indicator bacterial concentrations in the lettuce phyllosphere decreased over time under all treatments, with more rapid *E. coli* declines in the fall than in the spring ($p<0.01$). Persistence of *E. coli* in spring could be related to higher maximum and minimum temperatures in this season, and more regular rainfall. The survival model gave very good fits for the progression of *E. coli* concentrations in the phyllosphere over time ($R^2=0.88\pm0.12$). In the spring season, decline rates of *E. coli* counts were faster (2013 $p=0.18$; 2014 $p<0.005$) for the bare ground-cultivated lettuce compared to mulches. In fall 2014, the *E. coli* decline rate on paper mulch-grown lettuce was higher ($p<0.005$). Bacteria fluctuated more, and persisted longer, in soil compared to lettuce phyllosphere, and mulch type was a factor for fecal coliform levels ($p<0.05$),

with higher counts retrieved under plastic mulches in all trials, and higher enterococci levels under straw in fall 2014 ($p<0.05$). This study demonstrates that mulches used in lettuce production may impact the fate of enteric bacteria in soil or on lettuce, most likely in relation to soil moisture retention, and other weather-related factors, such as temperature and rainfall. The data suggest that the time between exposure to a source of enteric bacteria and harvesting of the crop is season dependent, which has implications for determining best harvest times.

Keywords: cropping practices, BoxLucas1 model, produce safety, plastic mulch, overhead irrigation, bacterial decay rates

1. INTRODUCTION

Agricultural practices are considered crucial factors impacting the bacteriological status of pre-harvest produce. Cropping methods are important not only for crop health and yield, but also to minimize food safety risks (Franz and van Bruggen, 2008). Between 1998 and 2008, produce related outbreaks were responsible for the highest number of foodborne illnesses among all food commodities, with leafy greens comprising the largest produce category (Painter et al., 2013). There exists the potential for pre-harvest contamination from direct sources such as manure (Franz et al., 2008; Johannessen, 2005) and irrigation water (Greene et al., 2008; Solomon et al., 2002). Good Agricultural Practices (GAPs) that consider methods that could introduce pathogens to the field, including soil fertilization and irrigation, are recommended to growers to minimize produce contamination risk (FDA, 2014). Assessment of pre-harvest risk, however, requires not only the identification of contamination sources, but also the interaction of these factors with cultivation practices that could affect the persistence of foodborne pathogens once introduced, and which to date, remain largely unexplored.

In Maryland, approximately 48% of farms are less than 20 ha in size (USDA, 2009) and small producers implement a variety of cropping methods. Leafy greens are more frequently irrigated by overhead application. In a study evaluating 32 leafy greens farms in the mid-Atlantic region, 81% used overhead sprinkler irrigation (Marine et al., 2015). Foliar contact with contaminated irrigation water is considered a food safety risk for produce, since water will contact edible portions multiple times over the growing season, and leafy greens are frequently consumed raw. In field-grown Iceberg and

Romaine lettuce irrigated by various methods, overhead application with contaminated water resulted in highest levels of generic *E. coli* (Fonseca et al., 2011). In mid-Atlantic and east coast states, surface water is often the primary source of irrigation water available to growers (Ragland and Tropp, 2009). In a study in New York state including 84 fresh produce growers, half reported using surface water for irrigation (Bihn et al., 2013), while in another survey in the mid-Atlantic including 313 growers, approximately half in 2010 and a quarter in 2013 reported the same (Marine et al., 2016). Surface water in this region is of inferior agricultural quality compared to groundwater (Marine et al., 2015; Pagadala et al., 2015), and may be contaminated with generic *E. coli* as well as human pathogens (Bell et al., 2015; Gu et al., 2013b; Luo et al., 2015; Micallef et al., 2012;).

Mulching (use of soil covers such as plastic or straw) is a widely used cropping approach to enhance the growth of fresh produce. Mulches are applied to the soil surface for retention of soil moisture, regulation of soil temperature and suppression of weed growth. Mulching also has a positive effect on crops with improved lettuce head, leaf and stem growth, and total yields compared with bare ground (Khazaei et al., 2013; Verdial et al., 2001). Plastic mulch is the most common type and can directly affect the plant micro-climate by decreasing soil water loss and modifying the surface radiation budget (Liakatas et al., 1986), thus providing high productivity and increased nutrient accumulation in lettuce (Verdial et al., 2001). Starch-based biodegradable films (mainly from corn, potato and rice crops) have been introduced as an alternative to conventional plastic mulch to reduce environmental impact. Other biodegradable mulches include straw, hay, paper and compost. Organic mulches reduced soil temperature and

maintained higher soil moisture levels compared to black plastic mulch (Schonbeck and Evanylo, 1998).

Different types of mulches have been reported to have different effects on plant pathogen risks. Straw mulch reduced levels of center rot caused by the bacterium *Pantoea ananatis* on sweet onion, while black plastic mulch had the opposite effect, speeding up the onset of the epidemic (Gitaitis et al., 2004). Fresh-market tomatoes grown using a cover bed of hairy vetch mulch or polyethylene plastic suffered markedly lower foliar disease than tomatoes grown in uncovered beds (Mills et al., 2002). Few studies have addressed mulching from a food safety perspective, and little is known about its effects on the survival of foodborne pathogens in soil and on produce. Counts of mesophilic aerobic and psychrotrophic bacteria were higher on plastic mulch-cultivated lettuce compared to lettuce grown in bare ground (Agüero et al., 2008), and splash dispersal of *Salmonella* during a simulated rain event on plastic mulch enhanced the dispersal of *Salmonella* to tomatoes (Cevallos-Cevallos et al., 2012a). To further our understanding of mulch influence on lettuce and soil microbiota, the objective of this study was to assess the effect of various mulches on the fate of soil and epiphytically-associated fecal indicator bacteria, introduced via overhead application. The indicator microorganisms tested were *Escherichia coli*, fecal coliforms and *Enterococcus* spp. Lettuce was cultivated during two different growing seasons (spring and fall) over a two year period to additionally assess the impact of season-related effects.

2. Materials and Methods

2.1. Field site and plot design

The field experiment was conducted at the Wye Research and Education Center (Wye REC) of the University of Maryland, Queenstown, MD. Experiments were conducted during the spring and fall seasons of 2013 and 2014. Bacteria were introduced via overhead application, to simulate introduction via spray irrigation, to lettuce organically grown on different mulches. There were five treatments of different ground covers: bare ground (BG) (no mulch control), black polyethylene plastic mulch (PP), biodegradable (corn-based) plastic mulch (CP), paper mulch (PM) and straw mulch (SM) (year 2 only). Plots were arranged in a randomized complete block design with four replications per treatment. Each treatment consisted of three or four 4.6 m by 0.6 m double-row beds, with 1.2 m uncovered spacing between the beds. A buffer row with polyethylene plastic mulch and planted with lettuce was laid on the southwestern side of the plot to separate it from other research fields. Twelve lettuce heads were planted 0.3 m apart in rows in each bed.

The soil at Wye was a loam soil with a pH of 5.8 to 6.2. Soils in the plot area were chisel-plowed. Raised planting beds were formed with a rototiller and bed shaper. The plots used drip irrigation and were irrigated with well water. Drip irrigation T-tape, 200 μ m thick with 30 cm emitter spacing, with a flow rate of 300 LPH per 100 m tape at 0.83 bar, was buried 2.5-5.0 cm deep. One line of drip tape was used per bed and was placed midway between two rows of plants. Pelletized chicken manure pellets (Purdue AgriRecycle, Seaford, DE) were applied at a rate of 3,360 kg/ha.

2.2. Lettuce cultivation

Romaine lettuce cultivar ‘Parris Island Cos’ seeds were started in the greenhouse in 2.5 cm pots (April or September) and transplanted 30 cm apart in the field three weeks

post germination. Overhead inoculum application to lettuce was performed 8 weeks post transplantation. Rainfall data were also recorded (see section 2.6).

2.3. Inoculum Preparation

To simulate lettuce contamination by overhead irrigation, water from a secondary lagoon receiving liquid dairy manure was collected from the University of Maryland's Central Maryland Research and Education Centre, Clarksville Facility (Clarksville, MD) two days before inoculation. The lagoon water was enumerated for generic *E. coli*, fecal coliforms and *Enterococcus* spp. before and after being supplemented with *E. coli* (when levels were low), and on the morning of inoculation day. Supplementation was with non-pathogenic *E. coli*, previously isolated from liquid manure from the same site, which was cultured in Tryptic Soy Broth (TSB) (BD, France) for 24 h at 37°C to a concentration of 8 log CFU/ml and 20 ml broth was added to the inoculum (24 L). For enumeration, a serial dilution of 1:5, 1:50 and 1:500 in PBS were made up, and 100 µl of each dilution were spread for enumeration on Tryptone Bile Glucuronic Agar (TBX) plates (HiMedia Laboratories Pvt. Ltd, India) for *E. coli* and fecal coliform and Enterococcosel Agar plates (EA) (BD, Germany) for *Enterococcus* spp. The plates were incubated at 44°C for 24-48 h (TBX) and 37°C for 24-48h (EA).

2.4. Overhead inoculum application and sampling

Overhead inoculation was carried out by sprinkling lagoon water of known indicator bacterial concentrations onto lettuce. In spring and fall 2013 and 2014, respectively, *E. coli* counts were 7.7 log CFU/ml, 4.2 log CFU/ml, 6.00 log CFU/ml and 6.0 log CFU/ml; fecal coliforms counts were 7.7, 3.6, 3.6 and 3.4 log CFU/ml; and *Enterococcus* spp. counts were 3.2, not measured, 1.0 and 1.6 log CFU/ml. Aliquots of 100 ml of inoculum

were measured out and applied to each lettuce plant. A colander was placed over the lettuce heads to disperse the inoculum evenly over the plant. Care was taken not to sprinkle any inoculum away from the lettuce. Inoculations were performed on windless days to avoid drift. Lettuce and soil samples were collected prior to inoculation and 30 min after inoculation. Thereafter, samples were taken at 1, 3, 5, 7, 10 and 14 days post inoculation. During sampling, two to four inner leaves of lettuce were harvested randomly from separate lettuce heads within each bed using sterile scissors and collected into a labeled sterile bag. Soiled outer leaves were avoided. Simultaneously, 200 g composite soil samples from the surface to 10 cm depth from lettuce root zones within each bed were collected using sterile scoops. The samples were temporarily stored in a cooler containing ice packs during transportation to the lab located at the Department of Plant Science and Landscape Architecture of University of Maryland (60 miles from the research field), and subsequently refrigerated until analyzed. .

2.5. Sample processing

Samples were processed within 48 h of collection. For each sample, 20 g of leaves were weighed from each sample into a sterile Whirlpak bag (Nasco, Jackson, WI). Eighty ml phosphate-buffered saline (PBS) were added to each bag and stomached in a laboratory stomacher (Seward, Stomacher 400 circulator, U.K.) for 2 min at 250 rpm at room temperature. From the resultant washate, dilutions were made of 1:50 and 1:500 using PBS, and 100 µl of original washate (1:5) and each prepared dilution were spread on TBX and EA plates. The plates were incubated at 44°C for 24-48 h (TBX), and 37°C for 24-48h (EA) and plates counted.

Ten g soil samples were weighed from each composite sample into a sterile Whirlpak bag. To each bag, 90 ml of PBS were added and bags shaken for 2 min. From the washate, a 1:100 dilution was made using PBS and 100 µl of each dilution were spread on TBX and EA agar plates. The plates were incubated at 44°C for 24-48 h (TBX) and 37°C for 24-48 h (EA).

2.6. Weather data collection

Air temperature and rainfall data for the period of time trials were being conducted was obtained from the Weather Station at the Wye REC. Precipitation data was collected using a NOAA IV Precipitation Gauge (ETI Instrument Systems, Fort Collins, CO). Air temperature was recorded with a HMP45C-L, Vaisala temperature and relative humidity probe (Campbell Scientific Inc., Logan, UT), in an aspirated radiation shield at a height of ~1.8 m. Daily sums of UV-A and UV-B irradiance were obtained by the UV-B Monitoring and Research Program (UVMRP), a data collection and research program of the United States Department of Agriculture (USDA), which has a climatological site at the Wye REC. Data is available at <http://uvb.nrel.colostate.edu/UVB/index.jsf>. In 2014, soil surface temperature under the mulches was recorded using a thermocouple data logger with probe (DataQ Instruments Inc., Akron, OH). Daily amounts of rain (cm).

2.7. Data analysis and modeling of microbial data

A logarithmic transformation was performed on the raw data using the formula $\log_{10}(1+c)$ to keep all the counts positive after transformation before statistical analyses, where c is the measured concentration in CFU/g. Quantile-quantile plots were used to test the normality of the data. Data points below the detection limit were designated a value equal to half the detection limit. Bacterial count data were analyzed using mixed models with

repeated measures, using JMP® Pro 11. Fixed effects were mulch type and day post inoculation, while subject was treated as a random effect, due to repeated measures and hence non-independence of the observations and residuals. For *E. coli* data from lettuce samples, the relationship between indicator bacterial counts and post-inoculation time was modeled with the BoxLucas1 survival model, describing exponential decline with asymptote, using Matlab (R2013a, Mathworks). The model takes the form of $c(t) = a*[1 - \exp(-b*t)] + c(0-)$, where c is the measured bacterial counts in the unit of log CFU/g, t is the post-inoculation time in the unit of days, $c(0-)$ is the measured bacterial counts before inoculation. The performance of the survival model was compared to a null model that predicts bacterial counts do not depend on post-inoculation time using F-test. The BoxLucas1 model outperformed the baseline null model for every single case ($p < 0.05$). The model contains two parameters. The a parameter reflects the overall magnitude of decline and the b parameter reflects the decline rate. In a few cases, the indicator bacteria become undetectable after day 0. In these cases, the b parameter becomes undetermined and the optimization routine returns an arbitrarily large b parameter. We estimated the b parameter for these cases by assuming that the indicator bacteria count was just below the detection limit at day 1, and remained at the lower limit of detection for the duration of the experiment. The differences in b parameters among mulches were assessed with ANOVA, and t -test was used to compare b parameters across seasons and years.

3. Results

3.1. Indicator bacterial survival in the lettuce phyllosphere

The progression of indicator bacterial counts in the lettuce phyllosphere as a function of time after inoculation is shown in Fig. 1. The bacterial counts declined over time and exhibited similar decline patterns in the same season. Time was a significant factor for all bacterial populations in both seasons (at $p < 0.0001$ for *E. coli*; at $p \leq 0.05$ for *Enterococcus* spp.; and at $p < 0.005$ fecal coliforms (excluding fall 2013, not significant). Mulch type was a significant factor for *E. coli* on lettuce in fall 2014 ($p < 0.0001$), with paper mulch-grown lettuce having lowest counts.

E. coli was only detectable on 1.6% of samples (1/63; spring: 1/32, fall: 0/31) prior to inoculation (Day 0). There was a rapid increase in *E. coli* counts as a result of inoculation (Day 0), followed by progressive decreases over time in all four trials (Fig. 1). At the end of the experiment (Day 14), *E. coli* was detected on 34.4% (11/32) of the samples in the spring season, and none of the samples in the fall season (0/31). Since temporal progression was clearest for *E. coli*, data for this species were used for subsequent modeling.

Fecal coliforms (excluding *E. coli*) were detected on 3.2% (spring: 1/32, fall: 1/31) of lettuce samples prior to inoculation. There was an increase in fecal coliform counts immediately after inoculation. In the spring season, fecal coliform levels generally decreased over time after inoculation, but were still detectable on 43.8% (14/32) of the samples at the end of the trial (Fig. 1). In the fall, peak values for fecal coliform bacteria were achieved on day 3 (2013) and day 1 (2014), declining promptly afterwards. Fecal coliforms were not detectable on any of the samples at the end of the experiment in the fall season.

Before inoculation, native *Enterococcus* spp. were recovered from 59.4% (19/32) of lettuce samples in the spring season, and 22.6% (7/31) of the lettuce samples in the fall. The progression of *Enterococcus* spp. counts over time fluctuated considerably over the course of the experiment, and declines were only observed in the fall in both years, but not the spring. At the end of the experiment, spring lettuce exhibited a 65.6% (21/32) *Enterococcus* spp. positive rate, compared to 3.2% (1/31) in the fall (Fig. 1).

3.2. *E. coli* progression on lettuce

3.2.1. Modeling *E. coli* survival on lettuce

The exponential decline model was used to mathematically describe the survival of *E. coli* after inoculation (Fig. 2). The model produced very good fits for the progression of *E. coli* count data ($R^2=0.88\pm0.12$). The model parameters are given in Table 1. The a parameter reflects the asymptote in population density and the b parameter represents the rate of decline over time. Since *E. coli* was undetectable at the end of the experiment for the majority of lettuce samples, the a parameter was largely determined by the lower limit of detection of the methods used for enumeration. For this reason, the b parameter was used in subsequent analysis to investigate decline rate dependency on various factors.

3.2.2. Differences in *E. coli* survival on lettuce grown on various mulches or bare ground

Decline rates (b parameter) for *E. coli* populations on lettuce differed among mulch types in different seasons (Fig. 3). In the spring, *E. coli* populations from bare ground-grown lettuce had a higher decline rate than from lettuce grown on mulches. This

difference was significant in 2014 ($p<0.005$), but not in 2013 ($p=0.18$). For the fall trials, no differences in decline rates among mulch types were observed in 2013 ($p=0.89$). On the other hand, populations retrieved from paper mulch-grown lettuce in fall 2014 had a significantly higher decline rate than lettuce cultivated with other ground covers ($p<0.0005$).

3.2.3. Differences in *E. coli* survival on lettuce across seasons

E. coli decline rates on lettuce differed across seasons and between the two years (Fig. 4). The decline rate for the fall season was significantly larger than the spring season ($p<0.01$), possibly associated with differences in temperature and rainfall. The decline rate also differed between years for the same season. For both spring and fall, the decline rate was higher in 2014 compared to 2013 (spring: $p<0.01$, fall $p=0.13$).

3.3. Indicator bacterial survival in soil

Indicator bacteria were also enumerated in soil from around lettuce plants, under mulches. The progression of indicator bacterial populations in soil as a function of time for the various treatments is shown in Fig. 5. Populations of fecal coliforms and *Enterococcus* spp. in soil mostly became established and continued to be recovered up until the end of the time course on day 14 in both seasons. *E. coli* populations, on the other hand, persisted only in bare ground soil in spring and fall 2013, and paper mulch-covered soil in spring 2013, attaining peak size around day 3, after which declines were observed. In 2014, *E. coli* populations declined rapidly in both seasons following inoculation. Time was a significant factor (at $p<0.005$; Table 2) for *E. coli* population dynamics in both seasons (Table 2). Soil under corn-based plastic had the highest *E. coli*

population levels in spring 2013 and 2014, but this impact was not significant (Fig. 5). Bare ground soil had significantly higher levels of *E. coli* right after inoculum application (day 0+). This is attributable to the protection provided by mulches compared to uncovered soil, and is not being considered as a biologically significant observation. Mulch was a significant factor for fecal coliform populations only, in both seasons (at $p<0.05$; Table 2). In the fall of both years, soil under polyethylene plastic and corn-based plastic harbored higher populations of fecal coliforms (Fig. 5). Soil under corn-based plastic and polyethylene plastic had the highest fecal coliform levels in spring 2013 and 2014, respectively (Fig. 5). No discernible impact of mulches on *Enterococcus* spp. in soil could be identified, except for higher levels under straw mulch in fall 2014 ($p=0.04$) (Table 2).

3.4. Soil and air temperature, UV irradiation and precipitation

Soil temperature under mulches was recorded during the second year of the study. Temperature varied significantly by season ($p<0.001$), with spring and fall average temperatures recorded at 26.1°C and 17.0°C, respectively. In both seasons, bare ground (no mulch cover) maintained a 1.7°C lower temperature than soil with mulch cover ($p=0.21$, Spring: $p=0.22$). Precipitation data, recorded on a daily basis, is shown in Fig. 6. Total rainfall for the duration of the experiment was recorded as 94.0 mm and 22.4 mm in spring 2013 and 2014, respectively, and 12.2 mm and 55.6 mm in fall 2013 and 2014, respectively. There was a considerable amount of rainfall on day 10 in spring 2013 (55.9 mm), and on day 2 in fall 2014 (35.6 mm). No direct correlations between bacterial count and rainfall were observed (Fig. 6). UV-A irradiance (Fig. 7A) was measured at an average of 1.2 and 1.5 MJ/m² day (spring 2013 and 2014, respectively) and 0.4 and 0.6

MJ/m² day (fall 2013 and 2014, respectively). In the same order, average UV-B irradiance (Fig. 7B) was measured at 29.7, 30.6, 6.3 and 9.5 KJ/m² day. Maximum and minimum air temperatures for the trial periods in spring and fall 2013 and 2014 are shown in Fig. 7C and 7D. Average maximum and minimum temperatures recorded were 29.3, 20.7; 28.4, 18.4°C in spring 2013 and 2014, respectively, and 14.0, 3.9; 19.3, 10.0°C in fall 2013 and 2014, respectively.

4. Discussion

This study presents novel data on the influence of various mulches, used in lettuce cultivation during two seasons, on the survival of three indicator bacterial groups for foodborne and waterborne pathogens. Enteric bacteria were introduced into the system via overhead application, simulating contamination through water used in overhead spray irrigation, the most popular type of irrigation used in leafy greens production in the mid-Atlantic states. Following inoculation, bacteria survived longer in soil, but declined rapidly in the phyllosphere, with a considerable seasonal effect resulting in prolonged survival in the spring. On the other hand, bacterial survival on lettuce appeared less influenced by ground cover type, although some significant differences were observed, with faster declines on lettuce grown on bare ground in the spring, and paper mulch in the fall. *E. coli* on lettuce provided the most tractable data, amenable to modeling. Day to day fluctuations in bacterial counts hindered this type of analysis in soil. However, fecal coliform levels were more abundant under plastic mulches, and enterococci populations were highest under straw mulch in one trial. These findings suggest that mulching may

promote the persistence of enteric pathogens under favorable meteorological and edaphic conditions.

We expected that mulches would modify bacterial survival in the phyllosphere and rhizosphere of crops by virtue of their effect on soil temperature and moisture. Although we could not model soil bacterial data, phyllosphere *E. coli* levels dropped more rapidly on bare ground-grown spring lettuce, compared to mulch-grown lettuce. In our study, soil temperature was lowest in the bare ground plots in both spring and fall, with an average soil temperature differential between bare ground soil and mulched soil of 1.7°C. This difference therefore could be attributed to factors such as higher temperature and evaporative losses and increased exposure to UV irradiation in uncovered beds.

Indirectly, UV irradiation, and heat and moisture losses from bare ground could also be related to less vigorous, slightly smaller plants than those produced on mulched beds. Evidence for reduced survival of pathogens as a result of drier conditions has been shown for *E. coli* O157:H7 which survived longer in rhizosphere and bulk soil following slurry injection compared to manure broadcasting (Semenov et al., 2009). The association however did not hold in fall 2014, when the most rapid decline rate was observed for *E. coli* on paper mulch-grown lettuce. Paper mulch however was the hardest to keep in place, being susceptible to tearing and deterioration following heavy rains, and portions of the beds became uncovered. We therefore are interpreting this result with caution, as the observation could be related to increased evaporation and heat loss due to mulch tearing, and therefore consistent with higher decline rates on bare ground-grown lettuce. In any case, the variation among mulches was subtle and not easily discernible. The temperature under polyethylene mulches has been reported to be higher than

biodegradable mulches (Moreno and Moreno, 2008). We also observed this with polyethylene plastic mulch in both seasons, and biodegradable plastic mulch in the spring. Yet, the difference in trapped warmth alone could not account for variations in decline rates among mulches in 2014. Hay, compost and paper mulches have also been reported to reduce afternoon soil temperature, while organic mulches maintained higher soil moisture levels than other treatments (Schonbeck and Evanylo, 1998). In this study, soil temperatures were recorded in the morning and soil under plastic mulches had higher soil temperatures than other mulches, although paper and straw-covered soil exhibited a wide variation.

Although the difference among mulch types was not consistently observed in the fall, seasonal differences were robust. Decline rates for bacteria on lettuce were higher in the fall than in spring. This could not be attributed to UV-irradiation, as levels were significantly higher in the spring compared to fall in both years (Figs. 7A and B). Simultaneously, while maximum and minimum air temperature ranges were equivalent for the two seasons ($\sim 10^{\circ}\text{C}$), averages were higher in spring, with maxima and minima of around 29°C and 20°C . These temperatures are conducive to *E. coli* growth, and contrast with fall averages of 17°C and 7°C . With a considerable number of hours with temperatures below 10°C , and three nights recording subzero minima (Fig. 7D), retarded bacterial growth is expected. Although warm temperatures appear to explain bacterial persistence in spring compared to fall, variation between the two years within a season is confounding. Spring 2013 reported higher minimum temperatures, possibly contributing to the slower decline rate in this trial compared to the spring of the following year. However, fall 2014 was warmer than fall 2013, but the opposite trend was observed

(albeit not significant). Growth promoting temperatures probably have more influence on phyllosphere bacterial population dynamics than minimum temperatures, whereby impaired growth would occur below a certain threshold, regardless of the air temperature drop. The second factor which might explain the abrupt fall decline rates could be a very dry spell in 2013 resulting in rapid bacterial deactivation, and a substantial rainfall event in 2014 early in the experiment, depleting populations. This contrasts to the more regular rainfall occurring during both spring trials, suggesting that increased moisture levels also positively influenced epiphytic bacterial survival.

Other factors, such as soil moisture and the added carbon source provided by straw, paper and biodegradable plastic could also possibly contribute to variations in bacterial counts. Organic mulches serve as a nutrient source for many microorganisms in the soil. By regulating the temperature and moisture of the soil and attenuating fluctuations, microbial activity may continue at a more uniform rate. Mulching likely increases the activity of many microorganisms in the soil, and may reshape the bacterial and fungal community structure. Organic mulch has been shown to have long-term effects on soil microbial activity and community structure in the top few centimeters of the soil profile (Yang et al., 2003), and the effect is dependent on mulch types. The observed differences in bacterial population progression between soil and phyllosphere, with more persistence in soil and increased die-off aboveground, could be explained by micro-niche diversity. Soil is an extremely heterogeneous habitat at the micro-scale, providing a wider array of micro-niches compared to the phyllosphere. Taxa introduced to the phyllosphere may not possess the specialized strategies needed for this unique habitat, while soil may provide a wider range of physico-chemical conditions allowing

for a larger diversity of taxa to become established. Discrepancies between bacterial survival in lettuce versus soil have been previously reported for *E. coli* O157:H7 (Islam et al., 2004). This is advantageous from a food safety perspective, whereby pathogens introduced to the crop phyllosphere may not persist until harvest. At the same time, introductions to soil may result in recalcitrant populations, which could in turn serve as an inoculum source for crops (Monaghan and Hutchison, 2012). Mulches could play a role in hampering or promoting dispersal of pathogens from soil via airborne routes or rain splash. Plastic mulch was reported to affect *Salmonella* dispersal onto tomatoes via rain splash or aerosols (Cevallos-Cevallos et al., 2012a; 2012b). In this study, moderate rain was succeeded by about a 0-1 log increase in lettuce bacterial counts, counteracting the overall decline in bacterial levels over time. This can be observed, for instance, with rainfall depths of less than 20 mm, with population growth on days seven to ten in spring 2013, and days five to seven in spring 2014 - especially for plastic mulch-grown lettuce - and population levelling between days two and three in spring 2013 and days seven and ten in fall 2014 (Fig. 6). An increase in water availability probably results in enhanced dissolution of nutrients, promoting bacterial growth. Conversely, rainfall depths greater than 35 mm in a day, in spring and fall 2013, did not follow this trend, maybe as a result of epiphytic bacterial depletion following intense rainfall. As mentioned earlier, the substantial rain event on the second day in fall 2014 may in fact explain why *E. coli* decline rates were higher in this trial compared to fall 2013, and seemingly unexplained by temperature data. Fluctuations in fecal coliform and enterococci counts in soil were also observed. These oscillations in bacterial dynamics in soil have been previously described as ‘waves’ of population explosions and crashes as a direct consequence of

nutrient availability and depletion (Zelenev et al., 2000). The cycle continues as dead cells release nutrients, which supports the next wave of growth. Fluctuations in response to plant residue have also been reported (Zelenev et al., 2005). Here we observed the most marked fluctuations in coliforms and *Enterococcus* spp. in rhizosphere soil, the site of root exudation of phytochemicals and sloughing off of root cells, as well as in the phyllosphere. Bacterial population dynamics in the latter are less understood.

Data on different cropping practices is necessary to devise measures that reduce pre-harvest risk during crop production. Improved understanding of the effect of mulching on the fate of epiphytic enteric bacteria on leafy greens provides an important step in determining which cropping practices result in the best food safety outcomes. By assessing the effect of several different mulches on the survival of indicator bacteria for foodborne pathogens, both in the lettuce phyllosphere and in soil, this study provides evidence of a low but significant mulch impact on lettuce microbiota. Warmer temperatures and increased moisture levels in mulched soil could promote bacterial survival, when compared to bare ground. The impact of mulching on foodborne pathogens is worth exploring further in a system that is receiving contaminated overhead irrigation water during an entire growing season. These types of studies are typically based on bacterial indicators and surrogates to foodborne pathogens. The adequacy of these taxa in lieu of pathogens in any studied system is crucial for the generation of meaningful and tractable data. Three types of indicators were assessed here, with *E. coli* yielding the data most amenable to analysis, followed by fecal coliforms (excluding *E. coli*), which exhibited similar trends as *E. coli*, but had more variation, possibly as a consequence of encompassing several species. Although enterococci are useful bacterial

indicators in water, they may be members of a plant's microbiome (Müller et al., 2001) and have been found naturally occurring on other produce crops (Micallef et al., 2013). In this study, the utility of *Enterococcus* spp. in the phyllosphere and soil was found to be limited, due to this taxon being found naturally prevalent in the lettuce phyllosphere prior to inoculation, and the ease with which it established populations, often persisting beyond the study duration. On the other hand, these same characteristics could make enterococci adequate for assessing long term impacts, since their persistence allows for longitudinal analysis.

Based on our findings, *E. coli* and fecal coliform data may be signaling risks associated with mulches. In the spring, mulch-grown lettuce appeared more supportive of *E. coli* persistence than bare ground-grown lettuce, possibly as a result of increased moisture and warmer air and soil temperatures, while fecal coliforms were more abundant in plastic-covered soil. The slower general decline rate observed in the spring, often at a time when manure is applied to fields prior to summer crop planting, could result in enhanced pathogen survival. This suggests that the interval needed between exposure to a source of enteric bacteria and harvesting of the crop to attain adequate log reductions may be weather-dependent and has implications for determining best harvest times. More data is needed to assess the interactive effects of temperature and moisture on pathogen persistence to support possible recommendations on manure drying times before covering with mulch.

Tables

Table 1. Model parameters for *E. coli* on lettuce grown on various mulches – bare ground (BG), polyethylene plastic (PP), corn-based biodegradable plastic (CP), paper mulch (PM) and straw (SM) – over two seasons in two consecutive years. For each mulch type, the mean a and b parameters and standard errors, the mean R -squared value and root mean squared error (RMSE) of the Box Lucas model fit are shown in the table. The survival model gives significantly better fit than a null model that assumed no relationship between bacterial count and time for every single case ($p<0.05$).

<i>E. coli</i> populations in lettuce phyllosphere						
Parameters	BG	PP	CP	PM	SM	
<i>a</i>	5.46±0.85	6.69±1.1	6.57±0.8	6.62±2		Spring 2013
<i>b</i>	0.56±0.37 _a	0.21±0.07 _a	0.31±0.12 _a	0.18±0.13 _a		
<i>R</i> ²	0.73	0.85	0.91	0.73		
<i>N</i>	3	3	3	3		
<i>RMSE</i>	1.18	0.92	0.75	1.32		
<i>a</i>	1.76±0.11	1.39±0.09	2.0±0.13	1.58±0.09		Fall 2013
<i>b</i>	0.68±0.08 _a	0.51±0.09 _a	0.55±0.15 _a	0.6±0.1 _a		
<i>R</i> ²	0.92	0.93	0.93	0.93		
<i>N</i>	3	3	3	2		
<i>RMSE</i>	0.17	0.13	0.22	0.14		
<i>a</i>	3.91±0.52	4.56±0.3	4.02±0.42	4.26±0.41	4.4±0.46	Spring 2014
<i>b</i>	0.89±0.69 _a	0.6±0.21 _b	0.46±0.19 _b	0.63±0.29 _b	0.33±0.11 _b	
<i>R</i> ²	0.66	0.90	0.84	0.83	0.88	
<i>N</i>	4	4	4	4	4	
<i>RMSE</i>	1.03	0.53	0.63	0.72	0.55	
<i>a</i>	4.21±0.29	4.03±0.47	3.81±0.14	4.21±0.0	3.34±0.1	Fall 2014
<i>b</i>	0.46±0.12 _b	0.43±0.19 _b	0.82±0.1 _b	1.4±0.0 _a	0.88±0.08 _b	
<i>R</i> ²	0.93	0.85	0.97	1.00	0.97	
<i>N</i>	4	4	4	4	4	
<i>RMSE</i>	0.42	0.67	0.23	0.0001	0.17	

Table 2: Mixed model analysis of variance for effect of mulch type and day and factor interaction on populations of bacterial indicators (*E. coli*, fecal coliforms (FC) and *Enterococcus* spp.) in soil under lettuce cultivation.

	Spring 2013			Spring 2014			Fall 2013			Fall 2014		
	df	F	<i>p</i>	df	F	<i>p</i>	df	F	<i>p</i>	df	F	<i>p</i>
<i>E. coli</i>												
Mulch	3	0.97	0.45	4	0.61	0.66	3	0.85	0.49	4	3.95	0.02*
Day	1	24.05	<0.0001*	1	9.16	0.003*	1	9.89	0.004*	1	10.60	0.002*
Mulch x Day	3	1.55	0.21	4	0.47	0.76	3	1.89	0.15	4	2.30	0.06
FC												
Mulch	3	4.08	<0.05*	4	4.11	0.02*	3	3.89	0.04*	4	4.23	0.01*
Day	1	34.09	<0.0001*	1	0.001	0.97	1	5.38	0.03*	1	12.04	0.001*
Mulch x Day	3	2.15	0.10	4	1.54	0.20	3	1.46	0.24	4	4.40	0.003*
<i>Enterococcus</i>												
Mulch	3	0.59	0.64	4	2.03	0.14	3	0.95	0.44	3	3.30	0.04*
Day	1	13.35	0.0005*	1	0.12	0.73	1	0.09	0.76	1	0.01	0.97
Mulch x Day	3	0.14	0.94	4	0.32	0.86	3	0.51	0.68	3	0.80	0.53

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Figure Captions

Figure 1. Levels of indicator bacterial counts over time in the lettuce phyllosphere; generic *E. coli* (Ecoli), fecal coliforms (FC), *Enterococcus* spp. (Ent), in log CFU/g under different ground covers; bare ground (BG), polyethylene plastic (PP), corn-based biodegradable plastic (CP), paper mulch (PM) and straw (SM). Dashed line indicates lower limit of detection.

Figure 2. Modeling *E. coli* count data over time using the Box-Lucas model, for lettuce grown on different mulches – bare ground (BG), polyethylene plastic (PP), corn-based biodegradable plastic (CP), paper mulch (PM) and straw (SM). Spring and fall decline curves for 2013 and 2014 are shown. Dashed line indicates lower limit of detection.

Figure 3. Differences in decline rates, based on the b parameter from the Box-Lucas model, across mulches for two seasons over two years are shown. Error bars indicate standard deviation. * indicates significant different at $p<0.05$.

Figure 4. Differences in decline rates, based on the b parameter from the Box-Lucas model, across two seasons. Error bars indicate standard deviation. * indicates significant different at $p<0.05$.

Figure 5. Levels of indicator bacterial counts over time in the rhizosphere; generic *E. coli* (Ecoli), fecal coliforms (FC), *Enterococcus* spp. (Ent), in log CFU/g under different ground covers; bare ground (BG), polyethylene plastic (PP), corn-based biodegradable plastic (CP), paper mulch (PM) and straw (SM). Dashed line indicates lower limit of detection.

Figure 6. Levels of indicator bacterial counts over time in the lettuce phyllosphere with rain precipitation. Bars represent average bacterial levels for all mulch-grown lettuce and bare ground-grown lettuce for generic *E. coli* (Ecoli), fecal coliforms (FC) and *Enterococcus* spp. (Ent), in log CFU/g. and line graph represents daily rainfall in mm. Horizontal dashed line indicates lower limit of detection.

Figure 7. Daily sums of (A) UV-A and (B) UV-B irradiance representing daily exposures, and (C) maximum and (D) minimum air temperatures during the 15 day trial periods in spring and fall 2013 and 2014. Day 0 represents day of inoculum application.

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