CPS 2012 RFP
FINAL PROJECT REPORT

Project Title
Evaluation of the level of white-tailed deer fecal colonization by *E. coli* O157:H7 and the ecological role of dung beetles with the pathogen in produce farms

Project Period
January 1, 2013 – December 31, 2013

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Objectives
1. *Use the full range of Maine's lowbush blueberry crop and prevalent white-tailed deer feces as a conceptual model system in order to develop a sampling protocol for examining the levels of pathogens in wildlife feces within agricultural systems.*
2. *Develop a model relating potentially heightened seasonal prevalence of E. coli O157:H7 found in deer feces with harvest period of lowbush blueberry.*
3. *Test for contamination of any blueberry from sample sites of any feces samples testing positive to E. coli O157:H7 in order to explore the relationship between fecal contamination and fruit contamination*
4. *Perform a lab study to determine ecologically important dung beetle/E. coli O157:H7/fruit relationships.*

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FINAL REPORT

Abstract

Wildlife as a source of microbial contamination is a concern among public health and food safety agencies. Deer feces have been determined as a point source for *Escherichia coli* O157:H7 contamination of produce. We used the lowbush blueberry agroecosystem as a model system to test food safety questions. The ecological role of a generalist dung beetle species, *Onthophagus hecate*, was explored as a biological control agent and alternatively as a pathogen vector, between deer scat and food.

A large-scale field survey confirmed that, *Escherichia coli* O157:H7 is present (6 positives out of 300 samples) within the lowbush blueberry agroecosystem. The manipulative field study indicated that, when blueberries come in direct contact with contaminated deer feces, fruit contamination is possible and that can persist for greater than 72 hours. For both the positive control and an experimental scat inoculation treatment, the levels of the bacterial population decreased over time, but at different rates ($F_{(1.9,18.8)} = 358.486$, $P < 0.0001$).

We conducted a lab study to elucidate aspects of dung beetle feeding ecology as it relates to suppression of *E. coli* O157:H7 from white tailed deer scat to lowbush blueberry fruit. Dung beetles buried the same percentage of scat whether or not the scat were inoculated with the pathogen ($F_{(1,6)} = 0.001$; $P = 0.99$ and ($F_{(2,17)} = 4.10$, $P = 0.15$). Beetles feeding on *E. coli* inoculated deer scat were not found to vector the pathogen to the fruit. In two studies, beetles lowered the amount of pathogenic *E. coli* persisting in soils ($F_{(2,9)} = 7.75$; $P = 0.01$ and $F_{(2,17)}=7.982$, $P=0.004$). Therefore, our study suggests that the generalist dung beetle species, *Onthophagus hecate*, when present in agroecosystems, has potential to contribute to suppression of *E. coli* O157:H7 on produce.

Background

Wildlife being source of foodborne microbial contamination, it is important to understand the behavior and biological interactions between wildlife and food-production units. Deer scat in multiple occasions, has been associated with outbreaks of *E. coli* O157:H7. Most recently, deer scat were determined to be the source of contamination of strawberries in Oregon. Pathogenic contamination of plants can occur directly by fecal deposition or indirectly by scat contamination. The ability of dung beetles to suppress pests and pathogens in pasture ecosystems has long been recognized. To better understand the ecological role of dung beetles and the risk of scat contamination of produce in food safety, this project explored the following questions:

- Does pathogenic *E. coli* O157:H7 occur naturally in wildlife (scat) present within the Maine lowbush blueberry agroecosystem?
- Can transmission of *E. coli* result from direct contact of contaminated wildlife scat and fruit? And if so, how long will this contamination persist on fruit?
- Do dung beetles (*Onthophagus hecate*) differentially feed on white tailed deer scat inoculated with *E. coli* O157:H7 compared to non-contaminated scat?
- What role do these dung beetles play in suppressing/vectoring *E. coli* O157:H7 from white tailed deer scat to berries as well as to soil?
- What role do dung beetles play in the persistence of *E. coli* in the soil?

Research Methods and Results

Survey of wildlife feces in Lowbush blueberry system:

In 2012, 12 blueberry farms throughout the major production region of Maine (Waldo, Hancock, and Washington counties) were surveyed for wildlife feces. Surveys took place seasonally in April, June, and August. Around 300 fecal samples were collected. Each individual sample was collected. Samples were kept separate and were immediately transferred from field sites to refrigerated storage (4° C). Initially, samples collected from each field site at one season were pooled together and screening of *E. coli* O157:H7 was carried out. Pooled fecal samples that tested positive for *E. coli* O157:H7 were tested individually. Isolation and identification of *E. coli* O157:H7 was carried out using FDA/BAM manual with some modifications. After enrichment [using modified trypticase soy broth without novobiocin (mTSB)], subsamples are isolated on selective media (using MacConkey sorbitol agar). Presumptive positive pathogens are tested by biochemical characterization and finally confirmed for O157:H7 antigen by latex agglutination. A positive control of *E. coli* O157:H7 ATCC 35150 was also plated from enrichment broth. PCR amplification of major virulence gene (*eae* gene) to all these samples was also carried out.

Two pooled sample (of 36 total) were determined to be *E. coli* O157: H7 positive with culture and confirmed by agglutination. Samples composing these pooled samples were collected from "Frankfort" field site in September and from "Harrington" field site in June.

Individual fecal samples (n=20) from positive pooled "Frankfort" sample resulted in 5 culture positive samples as identified by agglutination and 12 positive samples as identified with PCR. Three samples tested positive with both culture and PCR methods. Due to the high percentage of false positives experienced with PCR, we find that PCR is not an accurate way to sample this environment. Thus, we conclude that five individual samples from this one pooled sample are found to be *E. coli* O175:H7 positive. Positive individual samples were of white tailed deer (2), wild turkey (1), and unknown (2) origins.

Individual fecal samples (n=4) from positive pooled Harrington sample resulted in only 1 culture positive sample as identified by agglutination (also identified positive with PCR). We conclude that for the Harrington field site, one individual fecal sample was found to be *E. coli* O157:H7 positive. This sample was of white tailed deer origin.

Two field sites contained fecal samples which tested positive for pathogenic *E. coli*. In conclusion, we can state that *E. coli* O157: H7 does naturally occur, at low levels (6 of 300 individual samples) in the feces of wildlife found within Maine wild blueberry fields, across a range of multiple geographic areas. Interestingly, five of these samples were collected in the late growing season of lowbush blueberry (early September), close to harvest-time; which could pose a potential food safety risk. However, with the pathogen occurring at such low levels, it would be necessary to process thousands of samples to make a conclusive statement about the seasonality of the pathogen in the environment.

Direct transmission of pathogen field study:

In order to explore the relationship between fecal contamination and fruit contamination in field, during harvest, collected deer feces was simulated with a 9 log CFU/ml of surrogate non-pathogenic *E. coli* O157:H7 cocktail (ATCC 700728 and B6914) in the lab and these inoculated scat which had 7 log CFU/g of cocktail was dropped directly onto blueberry fruit.

Later fruit was collected at 2h, 24h, 48 and 72h time period from the field. Samples collected in the field were stored in a cooler with frozen cool packs and immediately transported to the laboratory. For the positive control group, the blueberries and the stems were sprayed with 9 log CFU/ml of non-pathogenic *E. coli* O157:H7 cocktail (to reach the final inoculum level of 7 log CFU/g) in the field. In negative control group, scats and the blueberries were not inoculated with any bacteria.

This study took place at Blueberry Hill Farm (Jonesboro, ME), a research farm managed by the University of Maine. Experimental plot consisted of 1m x 1m blocks of lowbush blueberries, separated from one another by 1 m mowed rows. Within each plot, the area with the highest density of berries was flagged. Before applying treatments to berries, a bottomless 26 cm bucket was lowered around plants to be treated to prevent bacteria drift/cross contamination. All blocks used had ripe blueberries at the time of experiment. Three treatments were applied to the experimental blocks:

1. Positive control - Surrogate non-pathogenic *E. coli* O157:H7 cocktail (ATCC 700728 and B6914) in applied directly to berries to measure persistence of the pathogen in the environment
2. Experimental treatment – white tailed deer feces artificially inoculated with non-pathogenic *E. coli* O157:H7 cocktail
3. Negative control– non-inoculated, white tailed deer feces was inoculated with sterile water instead of bacterial cocktail.

Fresh white tailed deer scat was collected from the University of Maine experimental forests to use in this study. Experimental treatment scat pellets were artificially inoculated with non-pathogenic *E. coli* O157:H7 cocktail by a dipping method. Around Twenty-eight grams experimental treatment scat pellets were artificially inoculated with non-pathogenic *E. coli* O157:H7 cocktail and were shaken for 3 min. After 3min of shaking, the inoculum from the scat was drained and they were dried in the hood for 2hrs. For the negative control group, to scat samples sterile water was applied to keep them moist instead of a bacterial cocktail.

For the experimental group, 28 grams of inoculated and benign scat were dumped directly onto blueberry fruit within randomly assigned blocks of blueberry plants, taking care to initiate as much contact between feces and berries as possible. Environmental variables were recorded at the time of experiment initiation.

One hour after materials were delivered to wild blueberry blocks (and control treatments had the opportunity to dry), 15 g treated berries were collected from each treatment area. Blueberries were harvested by clipping stems and avoiding contact with berries, ensuring no cross-contamination between replicates occurred. Berries were immediately transferred to a cooler with ice packs for transport back to the lab. This collection process was repeated at 24, 48, and 72 hours.

Bacterial enumeration for non-pathogenic *E. coli* O157:H7 was performed in the lab as soon as they are delivered. Blueberry fruit was separated from stems with a sterile forceps which was dipped in 80% ethanol in between the samples (to avoid cross contamination). Approximately to 15g of blueberries, 0.1% peptone water was added and was shaken for 3 min. After shaking, serial dilutions were done and samples were plated on CT-SMAC agar. The plates were incubated at 37^{o}C overnight and plates were counted for viable cells of *E. coli* O157:H7. All colorless colonies on CT-SMAC are finally confirmed for O157:H7 antigen by latex agglutination.

No *E. coli* O157:H7 were seen on the negative control plates during all these time-periods (with detection limit < 1.0 log CFU/g). When analyzed with a repeated measures MANOVA (independent factors: time and time*treatment) both the time ($F_{(1.9,18.8)}$ = 678.884, P < 0.0001 (Greenhouse-Geisser ε= 0.627), and the interaction term is significant ($F_{(1.9,18.8)}$ = 358.486, P < 0.0001 (Greenhouse-Geisser ε = 0.627). This analysis indicates that not only are the treatments different than each other in log CFU *E. coli* O157:H7 levels, but also that these bacterial population differences are influenced by the sampling time. There is a marked decay of the bacteria over each time period. This trend is not seen as strongly in the experimental treatment group as times 2, 24, and 48 hours are similar. A large decrease in logarithm CFUs is observed beginning with the 72 hour collection. At this last collection date, the logarithm CFU's of the pathogen surrogate positive control had decreased to a level similar to the initial (2 hours) experimental group CFU counts. For both treatment groups, positive control and experimental inoculation, as time from inoculation increases, persistence of the bacteria decreases, but at different rates.

Role of dung beetle in pathogen transmission/suppression

To better understand the role of the *Onthophagus hecate* in the potential suppression/transmission of *E. coli* O157:H7 from infected scat to blueberries and soil, two projects were completed. Both studies introduced three treatment combinations into microcosms. Treatments were:

- Treatment 1: beetles + deer pellets (scat) inoculated with *E. coli* O157:H7
- Treatment 2: beetles + deer pellets NOT inoculated with *E. coli* O157:H7
- Treatment 3: deer pellets inoculated with *E. coli* O157:H7 (with no beetles)

The first project took place in 37.9-liter glass microcosms (Grreat Choice™, PetSmart, Phoenix, AZ) filled with fruit bearing lowbush blueberry plants and intact soil from blueberry fields (this provided a very close simulation to field conditions. The second project took place in 1-litre beakers with autoclaved soil, as opposed to blueberry plants/soil. This provided a more acute examination of soil/beetle/pathogen interactions.

For project 1, twelve sections of mature, fruiting, lowbush blueberry "sod" carefully cut from fields at Blueberry Hill University Research Farm (Jonesboro, ME). Plant and soil structure was kept intact. Dung beetles were collected using live pitfall traps and all collected beetles were verified as Onthophagus hecate using Howden and Cartwrite's definitive dichotomous key. Fresh deer feces were collected from the University Experimental Forest (Orono, ME) and, to simulate the scat of an infected white tailed deer, they are inoculated with an *E. coli* O157:H7 cocktail to achieve a concentration of 10^6 CFU/g on scat. For blueberry plants, twelve sections of mature, fruiting, lowbush blueberry sod were carefully cut from fields at Blueberry Hill University Research Farm (Jonesboro, ME). The plant and soil structure was kept intact, as this has been shown to influence dung beetle competitive interactions and feeding. Blueberry plants were fitted into sterilized, 10 gallon, glass terrariums of equal dimensions of blueberry sod. Beetles were allowed 10 days to feed on/bury scat, within a microcosm including fruiting lowbush blueberry plants after which the harvestable fruit, soil, and leftover scat were tested for *E. coli* O157:H7 presence.

After beetles were allowed the opportunity to feed on feces for 10 days, soil cores were taken immediately adjacent to where scat had been placed until 25g of soil were obtained. All ripe/harvestable blueberries were harvested from each enclosure, from which 25g were randomly selected for analysis. All soil and blueberry samples were then tested for the presence of *E. coli* O157:H7 by direct selective plating on sorbitol MacConkey agar (SMA) and later confirmed by agglutination using Remel *E. coli* O157:H7 Latex test. Uneaten scat, remaining on soil surface within inoculated treatment group tanks, was also tested for presence of *E. coli* O157:H7 on day 10.

For project 2, soil was collected from a blueberry field at Blueberry Hill Farm in Jonesboro, ME. Rocks and roots were removed. Soil was autoclaved. Aluminum foil covered microcosms for the duration of the experiment. In order to minimize the amount of microflora present on beetles, dung beetles were surface-sterilized with chlorine, reducing background flora more efficiently without influencing mortality or behavior of the beetles. Beetles inoculated with 7log CFU/g pathogenic *E. coli* O157:H7 were allowed to feed on feces for 10 days and monitored daily. After beetles were allowed to feed, amount of feces removed by beetles was calculated and the entire amount of soil (300 ml), as well as scat was analyzed for levels of the pathogen. Beetles were also collected from microcosms for analysis.

Our data from project 1 showed that, no *E. coli* O157:H7 was detected on blueberries from any of the treatment groups. We can conclude that dung beetle, *Onthophagus hecate,* does not play any role in vectoring the *E. coli* O157:H7 from inoculated white tailed deer feces to ripe, pre-harvested, lowbush blueberries. Beetles removed identical amounts of scat whether they had, or had not, been inoculated wit*h E. coli* O157:H7. There was a greater amount of colorless bacterial colonies remaining in the soil samples from within treatment tanks containing "*E. coli* O157:H7 inoculated scat and NO beetles" than within tanks containing "*E. coli* O157:H7 inoculated scat WITH beetles" ($F_{(2,9)} = 7.76$; $P = 0.05$). As both treatments had *E. coli* O157:H7 inoculated scat, these data suggests beetles are playing a role in decreasing the amount of bacterial colonies, including *E. coli* O157:H7, persisting in the soil. Interestingly, there was no significant difference in the levels of bacterial colonies found within the tanks with "*E. coli* O157:H7 inoculated scat with beetles" and tanks with "No *E. coli* scat with beetles" ($F_{(2,9)} = 7.76$; $P = 0.58$). These data indicate that beetles may have the ability to bring the amount of bacteria, including *E. coli* O157:H7, in contaminated soils down to levels not different from soils with no *E. coli* O157:H7 contamination. Lastly, there were significantly fewer colonies persisting in soil from microcosms including dung beetles than in microcosms without dung beetles ($F_{(2,9)} = 7.75$; $P = 0.01$). This is not surprising in the light of the first two findings. These data suggest that *E. coli* O157:H7 can move into the soil without the presence of beetles and that, in the absence of beetles, levels of bacterial colonies remain the highest.

In project 2, no significant differences in the levels of bacteria were found between the bodies of those dung beetles feeding on inoculated scat and those beetles feeding on non-inoculated scat ($F_{(1,11)} = 1.922$, $P = 0.1958$). Dung beetles buried the same percentage of feces whether or not it was artificially inoculated with the pathogen ($F_{(1,6)} = 0.001$; $P = 0.99$ and ($F_{(2,17)} = 4.10$, $P = 0.15$). Soil from the treatment group with "*E. coli* O157:H7 inoculated scat and no beetles" has more counts of *E. coli* O157:H7 compared with "*E. coli* O157:H7 inoculated scat and beetles group. Lastly, there were fewer *E. coli* O157:H7 colonies persisting in soil from group including dung beetles than in groups without dung beetles. Dung beetles lowered the amount of pathogenic *E. coli* O157:H7 persisting in the soil ($F_{(2,9)} = 7.75$; $P = 0.01$ and $F_{(2,17)}=7.9821$, $P=0.0044$). Therefore, this study suggests that the generalist dung beetle species,

Onthophagus hecate, when present in agroecosystems, has the potential to contribute to the suppression of *E. coli* O157:H7.

Outcomes and Accomplishments

All the goals were completed as designed for this grant. Efforts were done in determining the relationship between pathogen and the scat in the blueberry fields through a series of field and lab studies and this provided valuable information. This project helps better assess risk of food contamination due to wildlife fecal contamination as well as provides sound evidence for potential dung beetle-mediated biological control of this risk

Summary of Findings and Recommendations

While finding lower levels of the pathogenic *E. coli* O157:H7 in the presence of dung beetles was a novel and interesting finding, regarding food safety, the fact that the beetles manipulated and fed on the scat without transmitting the pathogen to the fruit, is equally as important as the suppression of the pathogen in the soil. Resulting lower levels of pathogenic *E. coli* O157:H7 in the soil due to dung beetle activity would not be such a useful ecosystem service if beetles were acting as a vector of the pathogen from scat to the fruit.

Major findings:

- A wildlife feces survey was done to examine the levels of E. coli O157: H7 within lowbush blueberry crop systems and it confirmed that *E. coli* O157: H7 is present at low levels (6 of 300 individual samples) in the feces of wildlife found within Maine wild blueberry fields.
- As five of the positive samples were collected in the late growing season of lowbush blueberry (early September), close to harvest-time we can say that, though the risk of infection in the wildlife population appears to be low, the risk for food contamination remains.
- To test the potential for infected deer to directly transmit *E. coli* O157:H7 to lowbush blueberries, a field study was performed and it indicated that, when blueberries come in direct contact with contaminated deer feces, fruit contamination is possible and that can persist for greater than 72hours.
- Studied were done to understand the role of dung beetles in the potential suppression/transmission of *E. coli* O157:H7 and our results showed that the dung beetles did not play a role in vectoring pathogenic *E. coli* O157:H7 to the lowbush blueberry fruit and they did not show any feeding preference between pathogen-inoculated feces and non-pathogen inoculated feces.
- Dung beetles when they feed on scat, *E. coli* O157:H7 can be moved into the soil. But, the lower levels of pathogenic *E. coli* O157:H7 recovered from microcosms containing beetles (as opposed to those without beetles) indicates that some aspects of dung beetle biology are contributing to this decrease.

APPENDICES

Publications and Presentations

- M. Jones, F. Drummond, V.C.H. Wu and S. Tadepalli. 2013. Suppression of the human pathogen, *Escherichia coli* 0157:H7, by dung beetles (Coleoptera:Scarabaeidae) using the lowbush blueberry (*Vaccinium angustifolium)* agroecosystem as a conceptual model system. The Entomological Society of America, Minneapolis, Aug 2013.

- S. Tadepalli, M. Jones, F. Drummond, and V.C.H. Wu. 2014. Evaluation of the level of white-tailed deer fecal colonization by *Escherichia coli* 0157:H7 and the ecological role of dung beetles with the pathogen in blueberry farms. IFT Annual Meeting & Food Expo, 2014

- M. Jones, S. Tadepalli, F. Drummond, and V.C.H. Wu. 2014. Suppression of the human pathogen *Escherichia coli* O157:H7 by dung beetles (Coleoptera: Scarabaeidae) using the lowbush blueberry agroecosystem as a conceptual model system. Manuscript in preparation.

Budget Summary

All funds have been utilized except those on travel. Funds received by UMaine were mainly used for labors, material costs, and indirect cost (5% of personnel). The details of expenses could be found from submitted UMaine finical reports (# 25178, 25471, 25485, and 25621 as Jan. 31, 2014).