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CPS - CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

FINAL PROJECT REPORT, DUE APRIL 30, 2010

Project Title
Fly reservoirs of *E. coli* O157:H7 and their role in contamination of leafy greens

Project Period
April 1, 2009 through March 31, 2010

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Objectives
Objective 1: Test flies in California and Oklahoma feedlots/rangeland for *E. coli* O157:H7 to determine: What proportion of tested flies are *E. coli* O157:H7 positive; what time of year are *E. coli* positive flies more prevalent; what proportion of water, manure, and feed samples are *E. coli* positive; and do feral flies transmit *E. coli* O157:H7 to plants.

Objective 2: Test plies captured in leafy green production areas for *E. coli* O157:H7 to determine: What proportion of flies captured in leafy green fields are *E. coli* O157:H7 positive; what times of year are *E. coli* O157:H7 positive flies more prevalent.

Objective 3: Examine flyspecks on spinach for evidence of bacterial colonization over time to determine: Do excreted bacteria form biofilms on the leaf surface; and how long to excreted bacteria remain viable on the leaf surface.

FLY RESERVOIRS OF *ESCHERICHIA COLI* O157:H7 AND THEIR ROLE IN CONTAMINATION OF LEAFY GREENS

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Final Report
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Abstract

Filth flies are mechanical vectors of several human pathogens. They are known to carry *E. coli, Salmonella* spp, *Shigella*, and *Campylobacter* spp. to humans via prepared foods or by contamination of surfaces. It is not known, however, what role, if any, filth flies play in contamination of pre-harvest leafy greens. In this study, flies were collected from seven areas in California in and near leafy greens production areas adjacent to animal production facilities and tested for the presence of *E. coli* O157:H7 by culturing on selective media. Less than one percent of flies captured in the Salinas valley were positive when isolated on sorbitol MacConkey plates and serological confirmation. In contrast, over 90% of the flies captured in the Imperial Valley were positive when tested by PCR. We also investigated *E. coli* O157:H7 colonization of the spinach phyllosphere after regurgitation of house fly vomitus. Fly regurgitation spots were observed by scanning electron microscopy for evidence of bacterial attachment and growth over time. Fly regurgitation spots from flies that acquired bacteria from bacterial lawns had numerous bacteria-like organisms attached to plant cell surfaces whereas very few organisms were observed for negative control fly spots. Fly regurgitation spots from flies that acquired bacteria from manure-culture slurries had few bacteria-like organisms, but this was highly variable among spots. Regurgitation spots examined one week after deposition had many more bacteria-like organisms and there was evidence of bacterial replication on the leaf surface. These data, though not conclusive, suggest that *E. coli* O157:H7 survive in the gut of flies and can colonize the spinach phyllosphere after regurgitation under laboratory conditions.

Objectives

Objective 1: Test flies in California and Oklahoma feedlots/rangeland for *E. coli* O157:H7 to determine: What proportion of tested flies are *E. coli* O157:H7 positive; what time of year are *E. coli* positive flies more prevalent; what proportion of water, manure, and feed samples are *E. coli* positive; and do feral flies transmit *E. coli* O157:H7 to plants.

Objective 2: Test flies captured in leafy green production areas for *E. coli* O157:H7 to determine: What proportion of flies captured in leafy green fields are *E. coli* O157:H7 positive; what times of year are *E. coli* O157:H7 positive flies more prevalent.

Objective 3: Examine flyspecks on spinach for evidence of bacterial colonization over time to determine: Do excreted bacteria form biofilms on the leaf surface; and how long to excreted bacteria remain viable on the leaf surface.

Difficulties encountered that resulted in a change in objectives or methods. Three major difficulties were encountered that affected our ability to complete all objectives. First was controlled access to animal samples. When the nature and purpose of our fly and animal/environmental sampling was made known to potential cooperators, access to some properties was denied. In other situations, access to animal samples was not possible because of locked gates or inability to directly contact property owners. This situation made it difficult to carry out Objective 1 under controlled conditions- we opted to complete fly collections using 2 day traps on properties or peripheral collections from roadsides and pool the fly samples from animal feedlots and leafy greens fields since most sample sites were in leafy greens fields adjacent to animal facilities. Second, the method of detection outlined in the proposal was one that required initial growth of *E. coli* O157:H7 on selective media, serological detection, then confirmation by PCR. This yielded very few positives in areas where we have previously detected large proportions of *E. coli* O157:H7 positive flies. This prompted a change in detection methodology, moving to a split sample that was simultaneously tested by PCR and recovery on selective media that was used for the final two fly collection dates in southern California. Finally, for Objective 3, we had difficulty getting accurate enumeration numbers for the fly speck bacterial titer tests- Graduate student Lakmini Wasala altered the bacterial recovery assay and also changed titer measurement to real time PCR.

Cooperators/collaborative efforts

This project had the assistance from two major leafy greens processors, Dole Fresh Vegetables and Fresh Express, Inc. Through them, we were able to get access to lettuce production areas in proximity to animal production areas. In addition, Mr. Jim Manassaro was able to secure permission for us to sample flies at two animal production facilities. The names and locations of the farms/animal facilities are confidential, as per agreements made before the onset of the project

Research Methods and Results

Objective 1: What proportion of flies in cattle feedlots carry E. coli O157:H7? Do feral flies transmit E. coli to plants? Combined with Objective 2. Feral flies not tested for *E. coli* transmission.

Objective 2: What proportion of filth flies captured in leafy greens carry E. coli O157:H7?

Filth flies, by definition, breed in manure, rotting vegetation, decaying animal matter, and other habitats where adequate nutrition and structural substrate is available. These flies,

primarily house flies, *Musca domestica*, are often associated with animal production facilities such as feedlots, dairies, cow-calf operations, and back yard stables. Cattle, which are considered to be a primary source of pathogenic bacteria, are grazed and confined throughout San Benito and Monterey counties, within close proximity to many leafy greens acreages. We captured and tested flies from six different locations in the Salinas / San Juan Bautista area during the summer of 2009. During the following winter months, we collected flies on two dates in southern California, near El Centro.

Methods:

Filth fly collections: Flies were collected from six locations in San Benito and Monterey counties. Three sites were near San Juan Bautista, one in northern Monterey county, and two near Gonzalez. Flies were sampled on four different dates in April, June, July, and September of 2009. In southern California, flies were collected from the Desert Research and Extension Center (a UC Riverside research feedlot) near El Centro, CA in December 2009 and January 2010.

Flies were collected using baited traps (EZ Trap, Appendix, Figure 1)) set in leafy greens fields, in areas adjacent to leafy greens, in animal production facilities, or areas adjacent to animal production facilities. Traps were set 24" from the soil line on 3' rebar posts. Twenty-four hours later, traps were collected and shipped to Oklahoma. At each location (when feasible), flies were also collected using standard 14" insect nets, placed into collection vials, and shipped on ice to Oklahoma.

Testing for *E. coli* O157:H7

Flies were macerated in groups of 10 or as individuals in Hajna broth supplemented with antibiotics cefixme, cefsoludine and vancomycin and incubated overnight at 35 C according to the protocol of Cuesta Alonzo et al. 2007. Broth was diluted 1:10 and plated on sorbitol MacConkey plates supplemented with potassium telluride, 4-methylumbelliferyl-B-D-glucoside and cefixime. Colorless bacterial colonies that did not fluoresce under UV light were tested for O157 and H7 antigens using a Remel latex agglutination kit.

PCR detection of flies: Flies were macerated in Buffer AL and proteinase K (Qiagen) and incubated overnight at 55 C and DNA was extracted according to the manufacturer's instructions (Qiagen Blood and Tissue Kit). Extracted was resuspended in Buffer AE and 2.5 ul DNA was added to each reaction tube and PCR was carried out using three separate primer sets for rfbE, eae, and stx1 genes. Resulting amplicons were run on 1.5% agarose gels and bands were visualized using Photo Doc It UVP system. Positive and negative extraction and PCR controls were included for each PCR run.

Results and Discussion: Table 1 (Appendix) shows that most of the flies captured in Salinas throughout the summer were houseflies, but we did capture blow flies, particularly early in the season. We were able to test over 1500 flies total, but this was far short of what we expected to capture and test. Of the pooled fly samples, only four were positive for *E. coli* O157:H7 on

selective media (Appendix Table 2). This was less than expected and we believe that it is an underestimation of the true number of *E. coli* positive flies. Most of the flies tested were from a single animal production facility that was distant from any leafy greens fields. All flies from that site were negative. The few fly samples that were positive came from the San Juan Bautista area, specifically from the same site tested in 2007 for which over half were *E. coli* O157:H7 positive by PCR. One positive sample came from near a feedlot. In most of the leafy greens production areas in which traps were placed within fields, we caught very few flies, even when fields were within 200-300 feet of cow-occupied pasture or a feedlot.

In contrast, when flies from the southern California site were tested PCR using multiple primer sets, most were positive for the *rfb*E genes which is specific for O157 strains (Appendix Table 3). All fly samples were positive for *eae* (intimin gene of O157:H7), but only three of the samples were positive for *stx*1 (shigatoxin gene). This may suggest multiple strains of *E. coli* O157 present in flies captured at that facility. Consistency of our PCR methodology is still an issue as we continue to refine the primers and add additional primers for greater specificity. It is important to note that although many flies were captured at DREC, very few were in the adjacent lettuce on the research station, suggesting little movement of flies between the feedlot and the lettuce during the winter months.

Objective 3: Does regurgitated E. coli O157:H7 colonize the spinach phyllosphere?

Filth flies obtain nutrients by a process called pre-oral digestion in which they regurgitate onto a potential food source in order to partially digest it into a liquid state. The fly then takes up the pre-digested food with its sponging mouthparts. In a concurrent study of fly regurgitation, we estimated that blow flies typically regurgitate 4-6 times and defecate 8-10 times per day onto lettuce. The fly regurgitation spots are approximately 0.5-1.0 mm in diameter when dried on a leaf surface. To understand the fate of bacteria that had been regurgitated onto spinach, Oklahoma State University graduate student Lakmini Wasala exposed house flies to different bacterial sources, then released flies onto spinach plants and recovered the regurgitation spots for examination by scanning electron microscopy.

Methods:

House flies and bacteria: Feral house flies were captured in Stillwater, Oklahoma and housed in 12” by 12”metal insect cages (BioQuip, Rancho Dominguez, CA). Flies were maintained on a diet of egg yolk protein and calf manna and subsequent eggs were used to establish permanent colonies. Resulting pupae were separated into emergence cages and the adults were obtained upon eclosion. Male and female flies were used in all experiments. Bacterial cultures of *E. coli* O157:H7 were obtained from Dr. Michael Doyle, University of Georgia and transformed with a GFP-containing plasmid. Cultures were stored at -80 F until use. ATCC strain 43888 (attenuated, lacks *stx*1 and *stx*2) was used in all experiments.

House fly exposure to bacterial sources: House fly adults were exposed to four bacterial treatments: 1. E. coli O157:H7 liquid culture mixed 1:1 v:w with autoclaved bovine manure (final bacterial titer 10^6 cfus per gram manure); 2. PBS mixed 1:1 with autoclaved manure

(negative control); 3. *E. coli* O157:H7 lawn grown on a LB-ampicillin plate (positive control); and 4. LB-ampicillin plate (negative control). All bacterial sources were in Petri plates that had been modified into exposure chambers where the lids were perforated to create a 1.5 cm port (hole) through which flies could be introduced into the chamber (Figure 2).

House flies were vacuumed from colony cages and placed into sealed holding cage. They were then anesthetized using CO_2 gas and placed in groups of ten into exposure chambers containing the four bacterial treatments. Flies remained in the exposure chambers for five hours and then were anesthetized through the port with CO_2 gas. Groups of five anesthetized flies were gently placed into 1.5" x 6" cylindrical cages containing six-leaf stage smooth-leaf spinach plants (var. "Space") where they remained for 18 hours. After plant exposure, flies were anesthetized again and removed. Regurgitation spots on the leaf surface were excised and prepared for scanning electron microscopy using standard preparation protocols.

Scanning electron microscopy of fly regurgitation spots. Excised plant tissues containing regurgitation spots were chemically fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 24 hr. After buffer washes, tissues were dehydrated in 30, 50, 60, 75, 85, 95, and 100% ethanol solutions. Samples were critically point dried and palladium-coated and then examined using a Leica environmental scanning electron microscope operated at 80 KV. Samples were processed immediately after regurgitation (Day 0) and at 7 days after regurgitation (Day 7).

Enumeration of bacteria in regurgitation spots. Regurgitation spots were excised and pooled into groups of ten spots, then macerated in PBS and plated onto LB-ampicillin plates and incubated overnight. Control spots of 10 ul of *E. coli* culture (106 cfus per ml) were similarly plated. However, recovery of fluorescing bacteria from both test and control spots was unexpectedly low. We shifted to a real-time PCR test to determine if the number of bacteria was increasing or decreasing over time.

Results and Discussion:

Scanning electron microscopy (SEM) images of regurgitation spots resulting from fly exposure to the two control acquisition sources at Day 0 revealed very few rod-shaped bacteria-like organisms (BLOs) (Figs 3a and 3b). SEM images from regurgitation spots resulting from fly exposure to the positive control bacterial plate show a high number of rod-shaped BLOs attached to the leaf surface. Several attachment filaments are also visible at high magnification. There was some variation in the number of BLOs per spot; some spots had a few hundred BLOs, others only a few dozen (Figs 4a and 4b). The test treatment regurgitation spots from flies exposed to manure plus bacteria also had variable numbers of BLOs ranging from just a few to several dozen cells (Figs 5a and 5b). In all treatments, there were also coccoid-like organisms, but these were easily distinguished from the rod-shaped BLOs.

After 7 days, we observed similar BLOs, but in general, the numbers were higher for each of the treatments in comparison to the day 0 regurgitation spots (Figs . 6a, b). This experiment needs to be repeated before specific conclusions can be drawn, but the data show that more cells are present after seven days, suggesting that the regurgitated bacteria increased in number. We did not observe what might be termed a biofilm, but we did see large numbers of cells in arrangements that suggested crowded growth on the leaf surface.

Final summary: A very low proportion of flies in the Salinas area tested for *E. coli* O157:H7 using a standard microbiological approach were found to be positive. Only four positive flies were found in/near leafy greens in the Salinas area. We believe that our findings underestimate the number of filth flies carrying *E. coli* O157:H7 and perhaps other STECs and EHECs. A much higher proportion of flies captured in southern California and tested for the presence of *E. coli* O157:H7 specific genes by PCR were positive. The greater sensitivity and accuracy of PCR-based detection makes this diagnostic preferable to culture-based detection for the presence of bacterial DNA, but our test did not distinguish between live and dead bacteria. The primary question still remains: do flies transmit viable *E. coli* cells? Are these cells capable of colonizing the leaf surface of leafy greens? The data from examination of fly regurgitation spots would suggest that this is possible under optimal conditions. Bacteria acquired from inoculated manure survive the ingestion and regurgitation process. These data are preliminary, however, and must be confirmed with additional replications.

Although the funding period for this project has ended, this project is not yet completed. With funds from other sources, we intend to return to the Salinas area and test more flies as they become available, specifically to complete Objective 1 goals. We are working to build connections with animal production managers so that we can obtain the samples needed to answer critical questions about *E. coli* sources and fly transmission. Also, the graduate student working on Objective 3 is not yet finished with her project. We anticipate that she will complete her research and publish the findings later in 2010.

From the data collected, we made a five presentations at regional and national meetings and expect to publish two papers in professional journals.

Budget Expenditures

To date, approximately $10,600 was spent on salaries to support three months of Lakmini Wasala's graduate student stipend and two hourly workers for sample preparation assistance and fly colony care. Over $14,000 in travel was spent to support six collecting trips to California for AW and JT and post-doc Cesar Solorzano and three trips to the ESA national meeting for AW, JT, and C.S. Additionally, there were two trips to the CLGRB meetings in Seaside and Huron for AW to present research findings. Approximately $10,000 was spent for research supplies, insect cages, and collecting equipment . Another $4,000 was used for sample preparation and scanning electron microscopy at the Oklahoma State University Electron Microscopy Laboratory. $2,600 was used for growth chamber rental. Monies for

shipping and computer support were also expended. The budget was more than adequate for the work that we did, but we later learned that more extensive electron microscopy was desirable to reach more definitive conclusions. Money for additional EM time will come from other sources.

Acknowledgments

We thank Jim Lugg and Jim Manassaro for assistance in locating farmers/rancher cooperators, and Cesar Solorzano, Kaushal Maskey, and Rebecca Pace for processing fly samples. We also thank Fernando Miramontes at the DREC station for assistance with sampling. We acknowledge the confidential cooperation of several farmers and ranchers who allowed us to sample flies on their properties. Finally, we thank the Center for Produce Safety and the California Leafy Greens Research Board for funding and guidance throughout the duration of this project.

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Appendix

Figure 1. EZ trap showing large numbers of flies collected at the Desert Research Extension Center in El Centro, CA.

Table 1. Types of filth flies caught in the Salinas area in April, June, July, and September 2009.

Location	April	June	July	September
Moss Landing	House flies	House flies	House flies	House flies
San Juan Bautista 1	Blow flies Mixed	Blow flies Mixed	No flies	Not sampled
San Juan Bautista 2	Blow flies House flies	Blow flies House flies	Flesh fly	No flies
San Juan Bautista 3	Blow flies House flies	Blow flies House flies	Blow flies House flies	House flies
Gonzales 1	House flies	House flies	House flies	Not sampled
Gonzales 2	Not sampled	Blow flies House flies	No flies	No flies

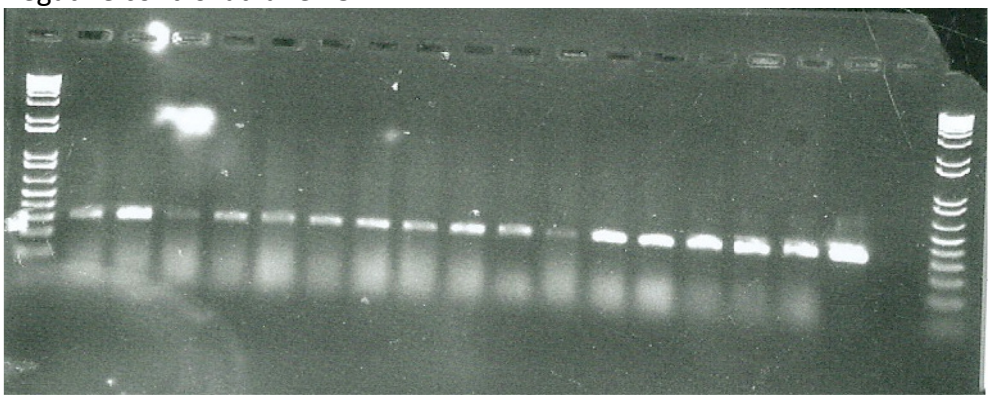
Table 2. Summary of fly samples which tested positive for *E. coli* O157:H7 using specific culture media.

Location	April	June	July	September
Moss Landing	0/100	0/480	0/130	0/330
San Juan Bautista 1	0/5	0/2	0/6	--
San Juan Bautista 2	--	0/20	0/15	0/17
San Juan Bautista 3	1/27	0/53	2/26	0/103
Gonzales 1	0/42	0/15	0/1	--
Gonzales 2	--	1/28	0/1	--

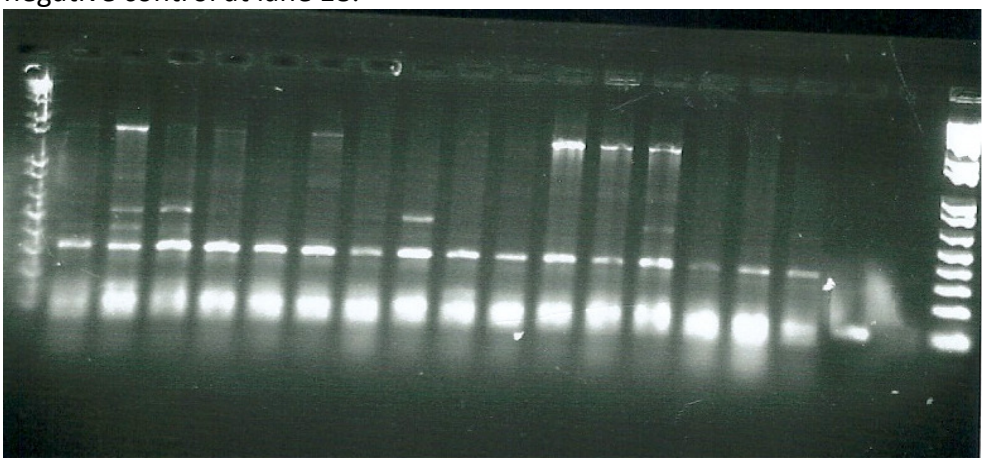
Results for *E. coli* O157:H7 detection from houseflies collected at DREC during December 2009 and January 2010.

To detect *E. coli* O157:H7, we used five primer sets (rfbE, flicH7, eae, stx1, and stx2) of which three (rfbE, eae, and stx1) have worked well under our standard PCR protocol conditions. For the rfbE gene, 87/97 samples resulted positive, all 97 resulted positive for eae gene, and 3/97 for the stx1 gene.

rfbE gene (292bp); Samples are 619-651, positive control was 43888 at 0.1ng/uL, negative control at lane 18.



eae gene(106bp); Samples are 600-615, positive control was 43888 at 0.1ng/uL, negative control at lane 18.



stx1gene (~190bp); Samples are # 635-650, positive control was *E. coli* O157:H7 strain 4546 at 0.5ng/uL, negative control at lane 18.

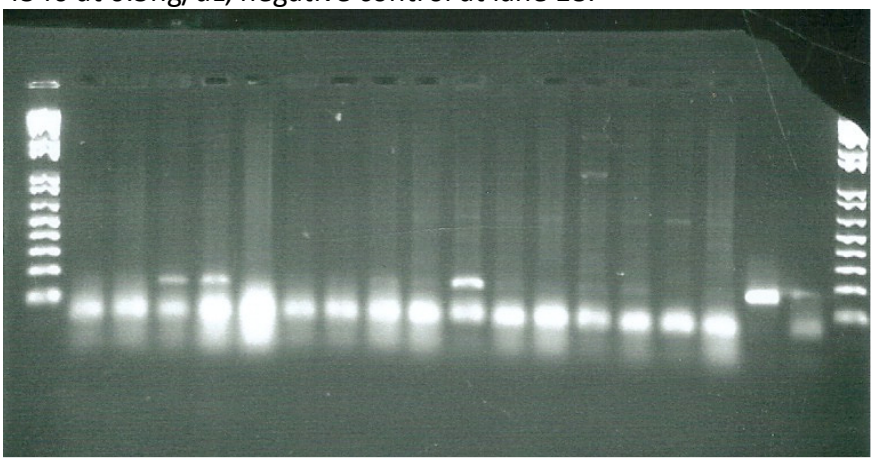


Table 3. Samples and gene sets used to evaluate presence/absence of *E. coli* O157:H7 from house flies collected from El Centro, CA. Each sample is a pool of 10 flies

Sample	rfbE	Flich7	eae	Stx1	Stx2
571	+	Optimization required	+	-	Optimization required
572	+	Optimization required	+	-	Optimization required
573	+	Optimization required	+	-	Optimization required
574	+	Optimization required	+	-	Optimization required
575	+	Optimization required	+	-	Optimization required
576	+	Optimization required	+	-	Optimization required
577	-	Optimization required	+	-	Optimization required
578	-	Optimization required	+	+	Optimization required
579	-	Optimization required	+	-	Optimization required
580	+	Optimization required	+	-	Optimization required
581	-	Optimization required	+	-	Optimization required
582	+	Optimization required	+	-	Optimization required
583	+	Optimization required	+	-	Optimization required
584	+	Optimization required	+	-	Optimization required
585	+	Optimization required	+	-	Optimization required
586	+	Optimization required	+	-	Optimization required
587	+	Optimization required	+	-	Optimization required
588	-	Optimization required	+	-	Optimization required
589	+	Optimization required	+	-	Optimization required
590	+	Optimization required	+	-	Optimization required
591	+	Optimization	+	-	Optimization required

		required			
592	+	Optimization required	+	-	Optimization required
593	-	Optimization required	+	-	Optimization required
594	+	Optimization required	+	-	Optimization required
595	+	Optimization required	+	-	Optimization required
596	+	Optimization required	+	-	Optimization required
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612	+	Optimization required	+	-	Optimization required
613	+	Optimization required	+	-	Optimization required
614	+	Optimization	+	-	Optimization required

		required			
615	-	Optimization required	+	-	Optimization required
616	+	Optimization required	+	-	Optimization required
617	-	Optimization required	+	-	Optimization required
618	+	Optimization required	+	-	Optimization required
619	+	Optimization required	+	-	Optimization required
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621	+	Optimization required	+	-	Optimization required
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		required			
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660	+	Optimization	+	-	Optimization required

		required			
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662	+	Optimization required	+	-	Optimization required
663	+	Optimization required	+	-	Optimization required
664	+	Optimization required	+	-	Optimization required
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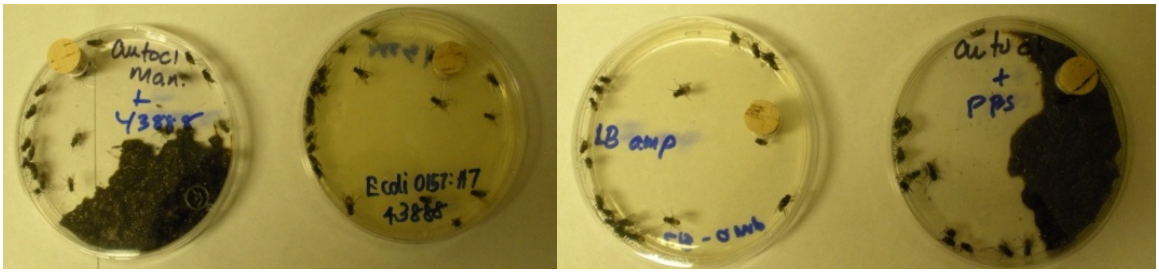
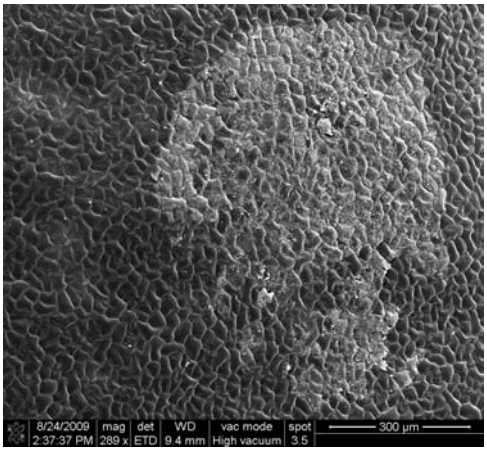
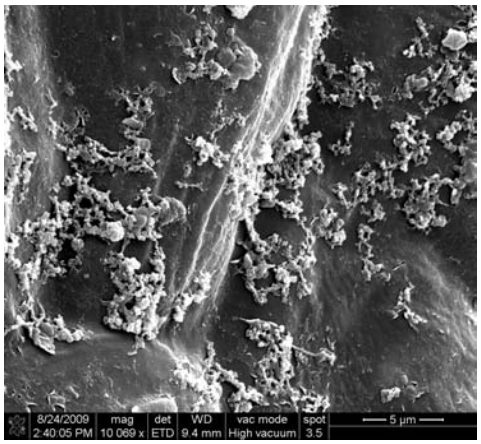


1 2 3 4

Figure 2. Four fly exposure chambers and treatments: 1. O157:H7 mixed 1:1 with autoclaved bovine manure (test); 2. E. coli O157:H7 plate (positive control); 3. LB ampicillin plate (negative control); 4. PBS mixed 1:1 with autoclaved manure (negative control).

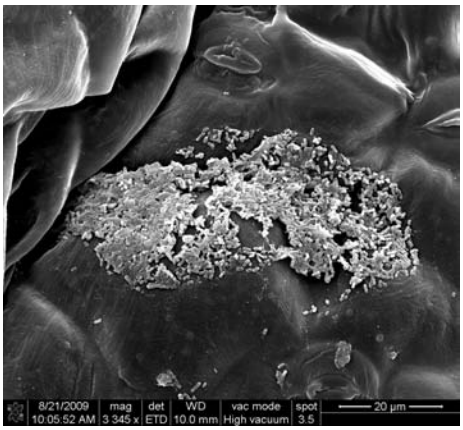


A

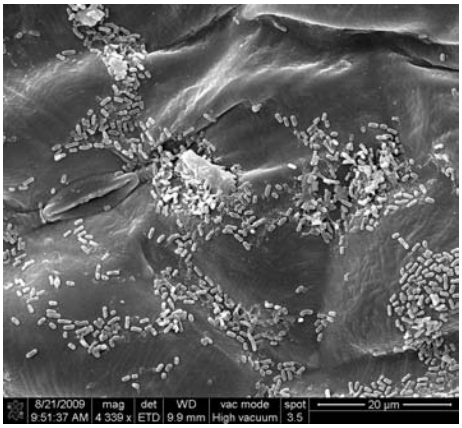


B

Figure 3. A. Low magnification scanning electron micrograph of a house fly regurgitation spot obtained after fly fed on PBS-manure. B. Higher magnification showing lack of bacteria-like organisms

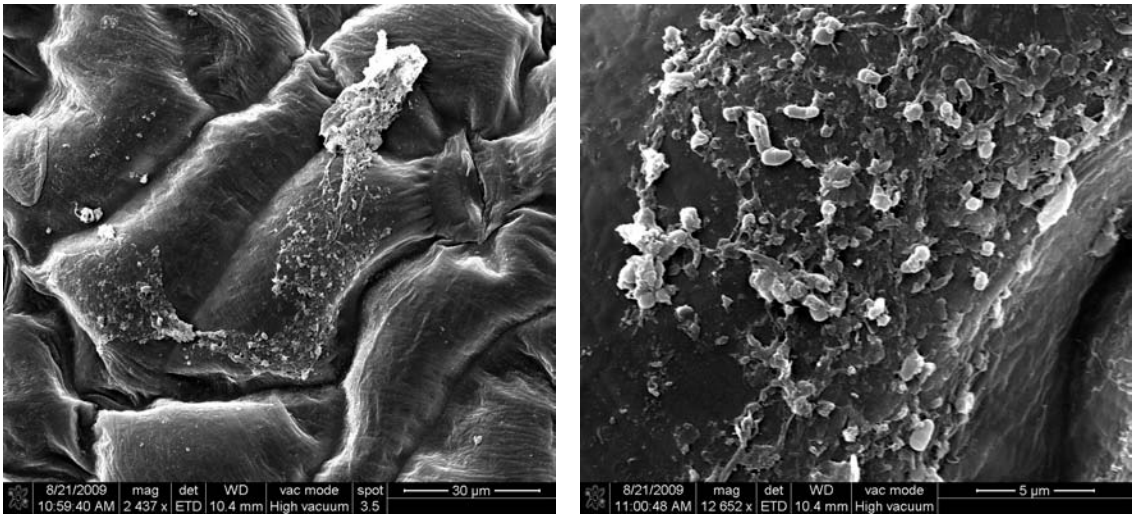


A



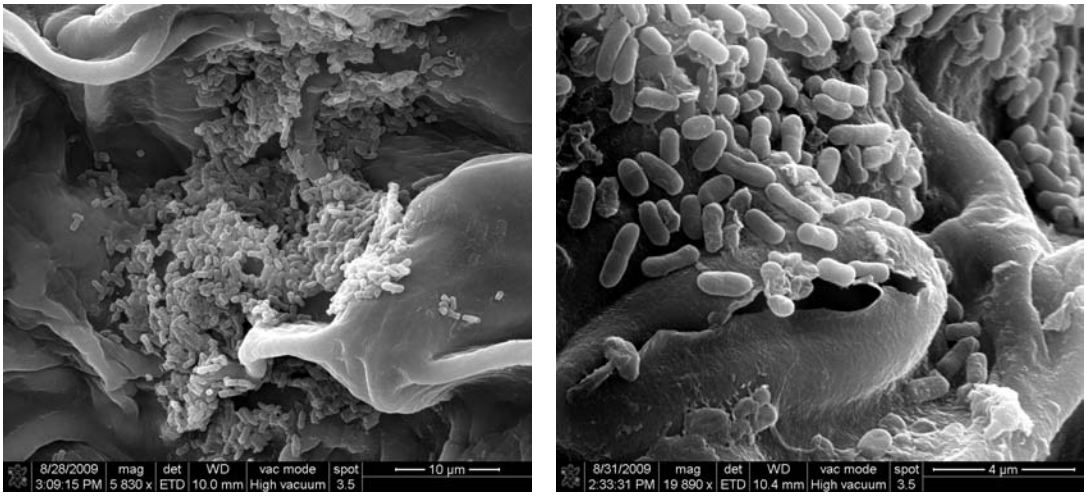
B

Figure 4. A. Low magnification scanning electron micrograph of a house fly regurgitation spot obtained after fly fed on an *E. coli* plate and collected at Day 0. B. Higher magnification showing BLOs on leaf surface.



A B

Figure 5. A. Regurgitation spot obtained at Day 0 after fly exposure to *E. coli* inoculated manure. B. Higher magnification showing a few BLOs embedded in fly vomitus.



A B

Figure 6. A. lower magnification SEM image of a fly regurgitation spot collected on Day 7 from a fly that fed on E. coli inoculated manure. B. higher magnification SEM image of a fly regurgitation spot collected on Day 7 from a fly that fed on an *E. coli* plate.