Transfer of *Escherichia coli* O157:H7 to Spinach by House Flies, *Musca domestica* (Diptera: Muscidae)

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Accepted for publication 11 February 2013.

ABSTRACT

Wasala, L., Talley, J. L., DeSilva, U., Fletcher, J., and Wayadande, A. 2013. Transfer of *Escherichia coli* O157:H7 to spinach by house flies, *Musca domestica* (Diptera: Muscidae). Phytopathology 103:373-380.

Filth flies are known mechanical vectors of pathogenic bacteria in hospital and restaurant settings, but their role as vectors for disseminating microbes to plants has not been demonstrated. *Escherichia coli* O157:H7 deposition by flies onto spinach was studied using molecular, microbiological, and microscopy techniques. Relative quantitative polymerase chain reaction studies showed that bacteria acquired by flies from contaminated cattle manure and deposited in regurgitation spots on leaves survived and multiplied. Scanning electron microscopy of the regurgitation spots of flies exposed to manure inoculated with *E. coli* suggested the multiplication of bacteria-like organisms within the spots. This finding implies that the bacteria were active and is consistent with a hypothesis that regurgitation spots serve as a nutrition source allowing *E. coli* O157:H7 to survive on the spinach phylloplane. *E. coli* O157:H7 persisted on fly body surfaces up to 13 days after exposure to acquisition sources, suggesting that fly cuticular surfaces are conducive to the growth of this pathogen. These results are consistent with the hypothesis of bioenhanced transmission of human pathogens by house flies and suggest that filth flies may affect the microbial safety of fresh produce.

There are many possible routes that lead to the contamination of fresh fruits and vegetables by organisms that are associated with foodborne illness. Brandl (4) considered the plant phyllosphere to be an intermediate niche for enteric pathogens that may ultimately reach the gut. Human pathogens are often shed in the feces of animals and transported to plants by fertilizer, irrigation water, run-off from livestock pastures, or insects (27). In contrast to the animal gut where there is an adequate supply of nutrients and moisture in an anaerobic environment, the plant phylloplane has limited amounts of nutrients and moisture, and bacteria living there are exposed to high doses of UV radiation and variable temperatures (4). Bacteria must either overcome these hurdles on the plant surface or find a way to enter plant tissue. To establish on the phylloplane, human pathogens attach to the plant surface using various adhesion methods (4).

House flies have been reported as mechanical vectors of many enteric bacterial pathogens, such as *Escherichia coli* O157:H7, *Shigella* spp., *Vibrio cholera*, *Salmonella* (5), and *Campylobacter* (19). For example, house flies transmitted *Campylobacter fetus* subsp. *jejuni* from poultry and pig farms to humans (19), and *Salmonella* and *Shigella* spp. were isolated from feral house flies in Uturu, Nigeria (28). These insects are also mechanical vectors of some protozoans that affect human health; for example, *Cryptosporidium parvum* oocytes were carried by house fly adults and larvae that developed on contaminated bovine feces. Adult fly defecation spots also carried numerous *C. parvum* oocytes after contact with contaminated bovine feces (7).

House flies carry *E. coli* O157:H7 between animals and to the neighboring environment. Flies collected from several cattle, poultry, and pig farms in Japan were positive for *E. coli* O157:H7, and the isolated *E. coli* O157:H7 colonies were positive for *stx*1 and *stx*2 genes in their virulence plasmid (8). In an outbreak of *E. coli* O157:H7-related hemorrhagic diarrhea in a Japanese nursery school, the pathogen strain responsible was traced to house flies that had originated from nearby cattle (14). In a Kansas feedlot, house flies collected from cattle feed bunks and storage sheds were positive for genes encoding *stx*1, *stx*2, *eaeA*, and *fliC* (2). The transmission of *E. coli* O157:H7 to cattle was tested experimentally by Ahmad et al. (1), who caged house flies fed with *E. coli* O157:H7 with cattle and later isolated the same strain from fly feces up to 11 days after exposure. Large numbers of *E. coli* O157:H7 were also found in the recto-anal mucosa of the cattle (3). Macovei et al. (12) showed that house flies collected from a cattle feedlot, when exposed to several livestock feed sources, contaminated the foods with different *Enterococci* species. Recent evidence that *Enterococcus faecalis* multiplies within the fly gut (6) suggests that inoculation of this bacterium by flies may occur for long periods after pathogen acquisition.

In the life cycle of the house fly, larvae consume bacteria that are necessary for their development. When larvae were artificially fed with *E. coli*, the average rate of bacterial survival in the larvae was 62% within 48 h after ingestion. The pupae and emerging adult flies were also infected with *E. coli* (18). Kobayashi et al. (11) showed that after house flies were exposed to *E. coli* O157:H7 lawns, the bacteria were acquired and could multiply within the pseudotracheae of the fly labellum. *E. coli* O157:H7 survived in the insects' intestines and were excreted for 3 days after ingestion. The authors hypothesized that the relationship between flies and bacteria was more than simple contamination but also provided a hospitable environment for bacteria to multiply on fly body surfaces, which resulted in a phenomenon called "bioenhanced transmission" (11).

House flies fed *E. coli* O157:H7 lawns and then allowed to contact different foods were able to disseminate bacteria onto food surfaces by fecal excretion for 24 h (21). When foods contaminated with defecation spots were incubated at 29°C, the bacteria proliferated 10^2- to 10^5-fold, suggesting that even a small number of bacteria in house fly excreta can serve as potential inoculum sources and lead to disease in humans (10). House fly

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http://dx.doi.org/10.1094/PHYTO-09-12-0217-FI

regurgitation may also function in the dissemination of enteric bacterial pathogens to fresh produce in the field. Talley et al. (26) documented that fly regurgitation is a potential mechanism of *E. coli* O157:H7 dissemination onto the spinach phylloplane under laboratory conditions.

Most of the research on fly movement of human pathogens is related to mechanical transport to surfaces, prepared foods, or animals, but rarely to plants. Fruit flies, *Drosophila melanogaster*, were shown to efficiently transfer *E. coli* O157:H7 to apple wounds after exposure to 10^8 CFUs on paper discs (9), but unlike filth flies, these insects are not typically associated manure or animals. The association of house flies with contamination of fresh produce in a field setting has not been documented, and only a few studies focus on the relationship between house flies and food contamination. Thus, the interaction between filth flies and human bacterial pathogens on plants is not well understood. The objectives of the present study included the documentation of *E. coli* O157:H7 survival and multiplication on the spinach phylloplane after fly regurgitation and the retention of viable bacteria on fly body surfaces.

MATERIALS AND METHODS

House fly colonies. Feral house flies from the Oklahoma State University (OSU) dairy, Stillwater, OK, were reared for up to 30 generations in the Veterinary Medical Entomology building, OSU, Stillwater. Flies were raised in 4-liter plastic tubs containing 2 liters of wheat bran and 400 g of Calf-Manna (Manna Pro Products, Chesterfield, MO) mixed with 1 liter of tap water. Newly emerged flies were maintained in the same facility with a temperature range of 25 to 28°C and a 12 h light/dark photoperiod. Two- to five-day-old adult flies were collected and transported to an arthropod containment level 2 and BSL 2 laboratory for all experiments.

***E. coli* O157:H7 cultures.** An inoculating loop of *E. coli* O157:H7 strain ATCC 43888 (Shiga toxin negative) (26) with a plasmid insert containing genes for green fluorescent protein (GFP) and ampicillin resistance was removed from frozen stock cultures and inoculated into 5 ml of Luria Bertani (LB) broth supplemented with ampicillin (100 μg/ml); the culture was incubated at 37°C overnight at 100 rpm. Five milliliters of the culture was harvested by centrifugation at 10,000 rpm, and the pellet was resuspended in sterile distilled water. The bacterial concentration was estimated visually by dark field microscopy using an Olympus BX2 microscope at 400× magnification. A 10-μl drop of bacterial suspension was placed on a glass slide covered with a 22 mm^2 coverslip. Bacterial numbers from 10 random fields were averaged, and a concentration of 10^7 cells/ml was used in all experiments. The bacterial suspension was mixed 1:1 with manure resulting in a final titer of approximately 1 to 2×10^7 CFU/g of manure, described below.

Growth and maintenance of plants. Spinach (*Spinacea oleracea*) variety Space F1 (Johnny's Selected Seeds, Winslow, ME) were grown in a greenhouse. Seeds were sown (three to four seeds per pot) in Metromix 300 (Sun Gro Horticulture, Bellevue, WA) and maintained at 27°C with a 12 h light/dark photoperiod. After seedling emergence, plants were fertilized (NPK 20:10:2, Miracle-Gro, Scotts Co., Marysville, OH) with a solution containing 100 ppm N for 5 days per week. Tap water was used the remaining 2 days. This fertilization schedule was used for 4 to 5 weeks and plants with eight to nine mature leaves were used in all studies.

***E. coli* O157:H7 acquisition sources.** House flies were exposed to four different acquisition sources: 5 g of autoclaved cow manure mixed with 5 ml of *E. coli* O157:H7 in a final concentration of 10^7 cells/ml in sterile distilled water (*E. coli*-manure, EM); 5 g of autoclaved cow manure mixed with 5 ml of sterile water (sterile water-manure, SM); a lawn of GFP-tagged *E. coli* O157:H7 on LB ampicillin agar (half of the agar removed to create space for flies; *E. coli*-plate, EP); and LB ampicillin agar (half of the agar removed; LB-plate, LB). House flies were anesthetized with CO_2 gas and then transferred to the medium-free area of the acquisition source plates. Only half of the agar plates contained the treatment, the other half provided a clean plastic surface to minimize excessive mechanical contamination of flies with the acquisition source, which could occur during transfer of anesthetized flies. Forty to fifty house flies were placed in each acquisition source plate, in five replicates. After 2 h of exposure, flies were anesthetized by pumping CO_2 for 10 s into the exposure chamber via a port cut into the lid of the petri dish. Anesthetized flies were immediately transferred into growth chambers containing spinach plants.

Relative quantification of *E. coli* O157:H7 in house fly regurgitation spots. Anesthetized flies were transferred to spinach plants enclosed in cylindrical plastic cages (3 × 8 in.). One end of the cage was embedded in growth medium and the other end was covered with a net mesh and secured by a rubber band. After a 12-h exposure to plants, flies were anesthetized and removed. Leaves were examined the following day and regurgitation spots on all spinach plants were marked. Plants were kept in the laboratory for 2 weeks and watered via the growth medium in the pot without splashing the leaves. Water was also misted about 12 in. above the cage to increase relative humidity. Thirty regurgitation spots (leaf pieces including a single regurgitation spot) were collected using a 3 mm straw punch at 0.75 (day 0.75 defined as 18 h after the beginning of the plant exposure period to flies) 4 and 8 days after exposure to the flies.

Regurgitation spots were processed for relative quantification of *E. coli* O157:H7 DNA using quantitative PCR (qPCR) (22). Ten regurgitation leaf spots were pooled in a 2-ml centrifuge tube and total DNA was isolated using a QIAamp DNA Mini-kit (Qiagen, Chatworth, CA). There were three replications per treatment. Prior to DNA extraction, 10 μl of pCR 2.1 (2.5 pg/μl; TA Cloning Kit, Invitrogen Corporation, Carlsbad, CA) carrying the 200-bp target sequence was added to 200 μl of ATL buffer (tissue lysis buffer; Qiagen, Chatworth, CA) for normalization of qPCR. Two 2.3 mm metal beads (BioSpec Products Inc., Bartlesville, OK) were added, and the sample was homogenized using a Mini-Beadbeater-8 (BioSpec Products Inc., Bartlesville, OK) for 30 s. DNA isolation was carried out using the manufacturer's protocol (Qiagen). A single reaction (15 μl) contained 7.5 μl of FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany), 4.6 μl of sterile distilled water, 0.45 μl each of forward and reverse primers, and 3 μl of DNA. Samples were mixed in 96-well plates, and the reactions were performed in a Bio-Rad My iQ Optical thermocycler (Bio-Rad Laboratories, Hercules, CA). Amplification conditions were 95°C for 10 min, 50 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 20 s. The concentration of *E. coli* O157:H7 DNA was based on amplification of a 105-bp fragment of the gene encoding *eae*; this was selected as the target for detection of *E. coli* O157:H7 because this attenuated strain lacks the *stx* genes. Primer sequences for amplification of *eae,* obtained from Sharma and Dean-Nystrom (23), were as follows: forward primer 5′ GTAAGTTACACTATAAAAGCAC CGTCG 3′ and reverse primer 5′ TCTGTGTGGATGGTAATAA ATTTTTG 3′. In reactions for amplification of the 200-bp fragment (internal control–confirmed absent in bacteria or arthropods), the annealing temperature was changed to 60°C. Primers for amplification of the 200-bp target sequence were: forward primer, 5′ GTCTACCAGGCATTCGCTTCAT 3′ and reverse primer, 5′ TGTGAATGCTGCGACTACGAT 3′. The ΔCt values from all treatments (EM, EP, SM, and LB) were evaluated using analysis of variance (ANOVA) tables generated through PROC-GLM (general linear model), which utilized a complete randomized design. *P* values were analyzed through least square means (LSMEANS) for differences of ΔCt values from each treat-

ment group as well as time-dependent variables within treatments (days 0.75 to 8) (20). Statistical differences were determined at $P \leq 0.05$. Results are presented as least square means ± standard error of the mean. The data were represented as fold changes compared with day 0.75 for each treatment. Fold changes were calculated using ΔΔCt ($\Delta Ct_{treatment} - \Delta Ct_{experimental\ control}$) values, where fold change is equal to $2^{-\Delta\Delta CT}$ (22).

Quantification of *E. coli* O157:H7 in manually spotted droplets on spinach leaves using relative qPCR. This experiment served as a control to determine the response of *E. coli* O157:H7 on spinach leaf surfaces without the influence of house flies or their regurgitant. Six fully expanded leaves per plant were selected, and 10 circles (1 cm diameter) were drawn on the adaxial surface. *E. coli* O157:H7 (10^7 cells/ml in sterile distilled water) was placed in 1 μl aliquots in the center of the marked circles. Ten drops were spotted per leaf on a total of six leaves per plant (five plants total). For the negative control, drops of sterile distilled water were placed on a different set of spinach leaves in a similar manner. Treated plants were maintained in the laboratory (25°C, 12 h light/dark photoperiod) for 2 weeks. At 0, 2, 4, 6, 10, and 12 days postinoculation, 9 to 10 spots/leaf in three replicate samples (total of 30 spots) were collected from three to four leaves using a 0.6-mm-diameter straw punch and randomly selected plants. The qPCR procedure was similar to the previous experiment, except the DNA volume was reduced to 2 μl for *eae* amplification and 1 μl for the 200-bp internal standard. The ΔCt values from both treatments (*E. coli* O157:H7 and sterile water) were used to analyze significant differences between treated (*E. coli* O157:H7-spotted leaves), control groups (sterile water), and time-dependent variables within treatments (days 0 to 12).

Quantification of *E. coli* O157:H7 on external surfaces of house flies by microbiological methods. This study was conducted to evaluate the persistence and colonization of *E. coli* O157:H7 on the external body surfaces of house flies exposed to different *E. coli* O157:H7 acquisition sources. House flies were exposed to the acquisition sources for 2 h, pooled in aluminum collapsible cages (12 × 12 × 12 in.; Bioquip, Rancho Dominguez, CA), and then supplied with sugar cubes, powdered eggs, and water throughout the experiment. Samples of 20 flies were removed from each cage 0, 2, 4, 6, 8, 10, and 13 days postexposure and used for bacterial enumeration and quantification. Individual fly heads and legs were excised, placed in 100 μl of sterile water, vortexed for 20 s, inoculated to LB ampicillin agar, and incubated at 37°C overnight. Five plates were randomly selected, and 10 colonies from each plate were randomly picked and tested for the presence of *E. coli* O157:H7 using a commercially available agglutination kit (Remel Europe Ltd., Dartford, UK). The O157:H7 positive colonies were further tested by end-point PCR performed in a PTC-100 thermocycler (MJ Research Inc., Waltham, MA) using primers specific for *eae*. Amplification conditions were 95°C for 2 min, 35 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 40 s followed by 72°C for 2 min. The percentage of *E. coli* O157:H7 positives from the agglutination test and percent confirmed as *E. coli* O157:H7 by PCR analysis were used to describe differences between treatment groups (EM, EP, SM, and LB).

Scanning electron microscopy of regurgitation spots and fly body parts. Regurgitation spots on spinach were collected on days 0.75, 4, and 8 after exposure to flies, and spinach leaves spotted with *E. coli* O157:H7 were collected 0, 2, 6, and 12 days postinoculation. The labellae and tarsi of flies exposed to the four different acquisition sources were prepared for scanning electron microscopy (SEM) using the following tissue fixation procedure. Tissue pieces were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 2 h, and then washed twice for 10 min in 0.1 M phosphate buffer (pH 7.0). The samples were incubated in 1% osmium tetroxide for 1 h, and washed twice for 10 min with phosphate buffer. Fixed tissues were dehydrated through an increasing ethanol series of 30, 50, 70, 90, 95, and 100% ethanol with a 15 min incubation for each step. Samples were transported in 100% ethanol to the OSU Microscopy Laboratory and processed by critical point drying and sputter coating with Au/Pd for 1 min. Samples were examined using an FEI Quanta 600 scanning electron microscope (FEI Company, Hillsboro, OR) in high vacuum mode at 15 kV.

RESULTS

Relative quantitation of *E. coli* O157:H7 in house fly regurgitation spots. There were significant differences in the relative amounts of the *E. coli* O157:H7 *eae* amplicon between treatments (df = 11, 48, F = 46.06, $P < 0.0001$). Figure 1 shows the fold-change values within treatments compared with day 0.75. Regurgitation spots deposited by flies exposed to autoclaved manure mixed with *E. coli* O157:H7 (EM) showed an 18-fold increase in the *eae* amplicon from days 0.75 to 4 ($P < 0.0001$), which suggests that the number of *E. coli* O157:H7 cells increased on spinach leaves within that time period. At day 8, the abundance of the *eae* transcript decreased compared with that at day 4, but was nevertheless significantly greater than at day 0.75 ($P = 0.001$) (Fig. 1).

For the regurgitation spots obtained from flies exposed to *E. coli* lawns (EP), the *eae* amplicon levels remained fairly level and showed no significant difference in the fold change from day 0.75 to day 4 ($P = 0.1188$), from day 0.75 to day 8 ($P = 0.0539$), and from day 4 to 8 ($P = 0.6956$), suggesting no significant change in bacterial numbers over time (Fig. 1). These ΔCt values (Table 1) were the lowest from the data set, which suggests a higher initial *E. coli* O157:H7 cell number compared with other treatments.

Quantification of *E. coli* O157:H7 in manually spotted droplets on spinach leaves using relative qPCR. This experiment was conducted to study the behavior of *E. coli* O157:H7 on spinach surfaces in the absence of fly regurgitant. The *eae* gene was detected by qPCR in leaf spots at all times sampled, but the ΔCt values showed no statistically significant changes (df = 5, 31; F = 2.1, $P = 0.0969$) (data not shown). This suggests that the levels of the *eae* amplicon, and thus the number of bacteria, did not change over time.

SEM examination of regurgitation spots. SEM revealed that housefly regurgitation spots averaged 200 to 500 μm in diameter. Some regurgitation spots were circular and others were irregular in shape (Figs. 2 and 3). The regurgitation spots of *E. coli*-manure (EM)-exposed flies contained regurgitated organic matter (termed matrix) that the flies consumed from autoclaved manure. Some images of fly regurgitant showed evidence of potential cell division of bacteria-like organisms (BLOs) in the matrix (Fig. 2B). Enterococcal-like chains were also observed in some of these regurgitation spots (data not shown). The regurgitation spots from the EP treatment also contained BLOs, but their size was smaller, and the matrix varied in appearance from the EM treatment. Bacteria were more visible and numerous in the EP matrix (Fig. 3). Some images showed potential division of BLOs, and others showed bacteria near the guard cells of stomata (Fig. 3C). Flies exposed to the SM source produced regurgitation spots similar to those in the EM treatment, but few BLOs were observed in the matrix and these exhibited variable size and morphology. Regurgitation spots of LB-exposed flies lacked the dense matrix of the other treatments.

SEM of manually spotted bacteria. On day 0, SEM of the *E. coli* O157:H7-spotted leaf surfaces showed many BLOs resembling *E. coli* (Fig. 4A). Structures potentially involved in attachment of BLOs were also observed (data not shown). The bacteria were primarily located in the interclinal junctions between epidermal cells and were present in large aggregates (Fig. 4A and B). SEM of spinach leaf surfaces spotted with sterile

water (negative control) showed a few slender, rod-shaped BLOs that were morphologically different from *E. coli* O157:H7 (data not shown). These non-target BLOs were not identified in this study.

Microbiological recovery of bacteria from fly mouthparts and tarsi. *E. coli* O157:H7-exposed flies were sampled at 2-day intervals for viable bacteria. Table 2 shows the percent positive by agglutination of the colonies sampled at each time point. The number of positives was high in the EM and EP exposed flies and low in the SM or LB (negative control) flies. When samples were retested by end-point PCR for the presence of the *eae* gene, all bacteria recovered from the SM or LB exposed flies were negative for the presence of *E. coli* O157:H7 (Table 2).

Colonies of *E. coli* O157:H7 were recovered from house flies exposed to bacterial lawns (EP) and autoclaved manure mixed with the pathogen (EM) up to 13 days postexposure (Table 2). For the EP acquisition source, initially, all colonies tested positive in PCR assays, but the percentage of positives dropped after day 6 (Table 2). A different pattern emerged from the EM-exposed flies; 100% of the tested colonies from this group were positive for *eae* on the day of exposure, but the percentage dropped to 0 on day 2, increased to 46% on day 6 and then dropped to 0% on day 8 (Table 2). On days 10 and 13, a small percentage of the tested colonies from the EM exposed flies were PCR positive for the *eae* gene.

SEM of fly parts. Fly tarsi (Fig. 5) exposed to *E. coli* lawns (EP) showed rod-shaped BLOs (1 to 2 µm in length) on the sticky hairs of the tarsal pulvilli. Numbers of BLOs were visually higher than those on flies from the EM treatment, but difficulty obtaining accurate counts of bacteria among the pulvillus hairs resulted in a statistical analysis not being performed. EM-exposed flies had only a few BLOs on the sticky hairs of the pulvilli (Fig. 5D) and on the tarsus. No BLOs were observed in the LB- and SM-exposed fly tarsi (data not shown). The labellae exposed to the EP treatment had aggregates of BLOs on the pseudotracheae, and potential cell division of bacteria was observed between the grooves of pseudotracheae (Fig. 5B). BLOs were not observed on labellae or pseudotracheae obtained from the other acquisition sources (data not shown).

DISCUSSION

Fly dissemination of enteric bacteria to food has been documented but not extensively studied. Previous studies have documented the movement of bacteria and contamination of prepared

TABLE 1. Mean ΔCt values from quantitative polymerase chain reaction of *eae* extracted from the regurgitation spots from house flies exposed to different acquisition sources

	Days postdeposition on leaf surface		
Acquisition source[a]	0.75	4	8
E. coli-manure (EM)	12.25	8.31	9.38
E. coli-plate (EP)	3.03	4.43	4.78
Sterile water-manure (SM)	13.59	13.51	12.59
LB-plate (LB)	15.77	14.56	13.35

[a] Acquisition sources: EM, regurgitation spots from flies exposed to autoclaved manure mixed with *Escherichia coli* O157:H7; EP, regurgitation spots from flies exposed to *E. coli* O157:H7 lawns on Luria Bertani (LB) ampicillin agar; SM, regurgitation spots from flies exposed to autoclaved manure mixed with sterile water; and LB, regurgitation spots from flies exposed to uninoculated LB ampicillin agar plates. Ten regurgitation spots were pooled for each acquisition source and three technical replicates were completed.

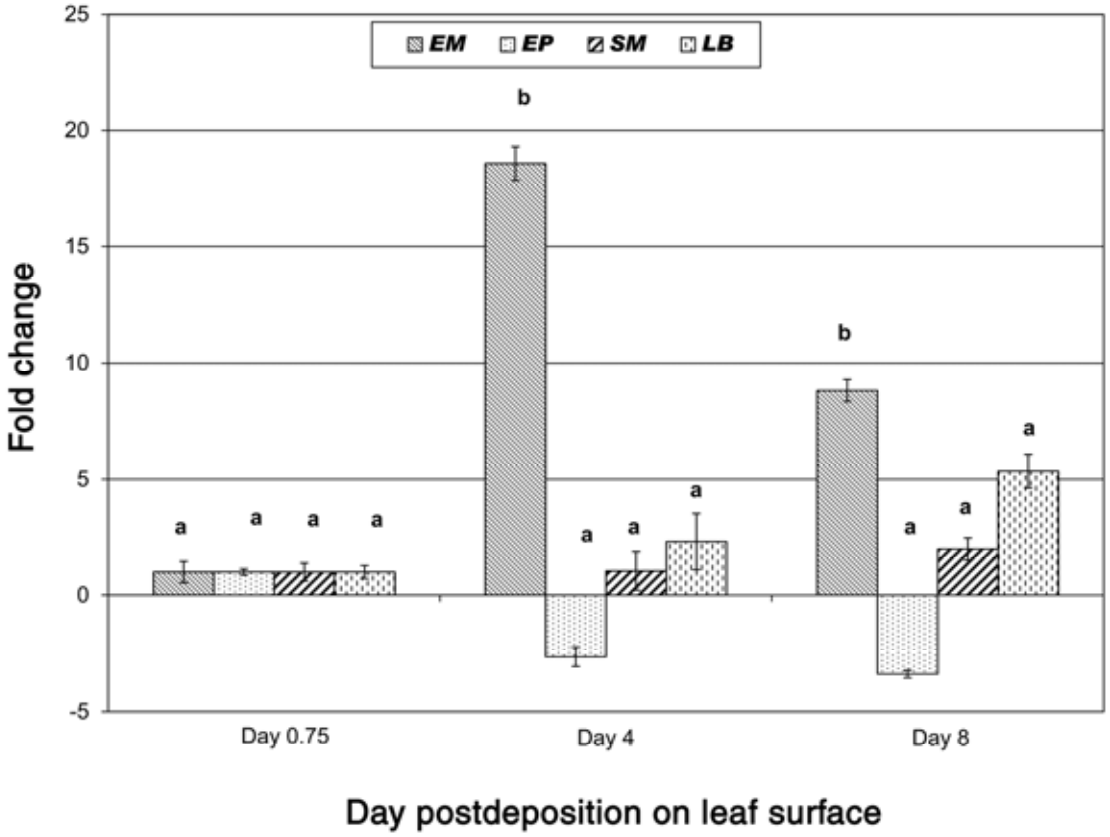

Fig. 1. Relative quantitative polymerase chain reaction analysis of *Escherichia coli* O157:H7 *eae* amplicon levels in fly regurgitation spots on spinach leaf surfaces expressed as fold change and compared with day 0.75 within different treatments. Bars represent standard error. Means with different letters are significantly different ($P < 0.05$). EM represents regurgitation spots from flies exposed to autoclaved manure mixed with *E. coli* O157:H7. EP, regurgitation spots from flies exposed to *E. coli* O157:H7 lawns on Luria Bertani (LB) ampicillin plates. SM, regurgitation spots originating from flies exposed to autoclaved manure mixed with sterile water. LB, regurgitation spots originating from flies exposed to uninoculated LB ampicillin plates.

foods and surfaces, but dissemination of bacteria by flies to fresh produce requires more investigation. In the current study, we show that flies retain live bacteria for contamination via casual contact. Our results also suggest that regurgitation may deposit viable cells on plant surfaces that are capable of replication.

A GFP-tagged strain of *E. coli* O157:H7 was used in this study, but we discovered that the plasmid carrying the GFP insert was lost fairly quickly in the absence of ampicillin selection pressure on plant and insect surfaces, thus recovery of fluorescing colonies or visualization by fluorescence microscopy was difficult beginning 2 to 4 days after fly exposure to bacterial sources. This necessitated a change to a different bacterial quantification measure. Relative qPCR allows estimation of the quantity of target DNA relative to some standard. Although this method amplifies DNA from both live and dead cells, qPCR measures changes in target DNA quantity compared with the internal control DNA which does not increase or decrease. In this study, we employed qPCR to measure the relative amount of change (increase or

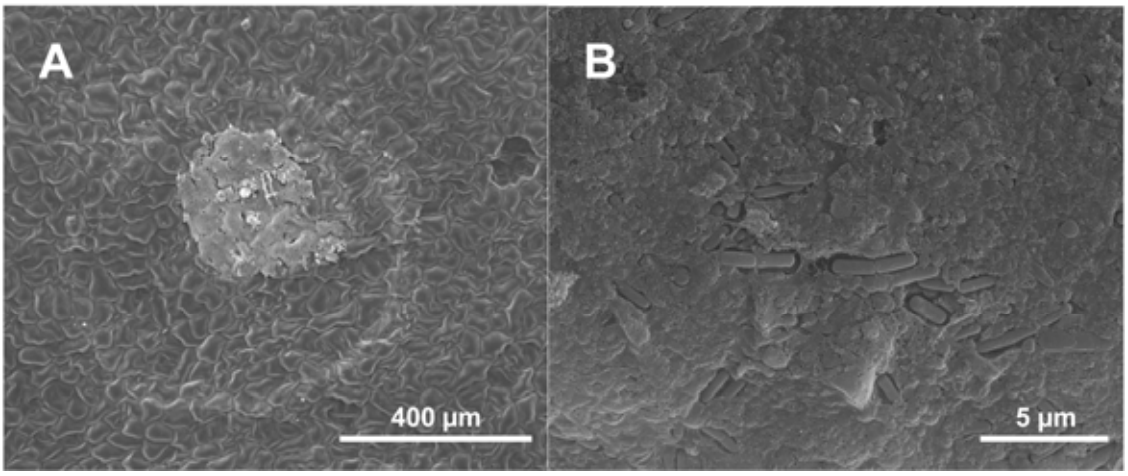

Fig. 2. Scanning electron microscopy of a regurgitation spot deposited by house flies exposed to *Escherichia coli* O157:H7-contaminated manure (*E. coli*-manure acquisition source). **A,** Low magnification (400 µm) showing a diffuse ring around the regurgitation spot. **B,** Higher magnification (5 µm) of spot showing bacterial-like organisms presumably in the process of cell division.

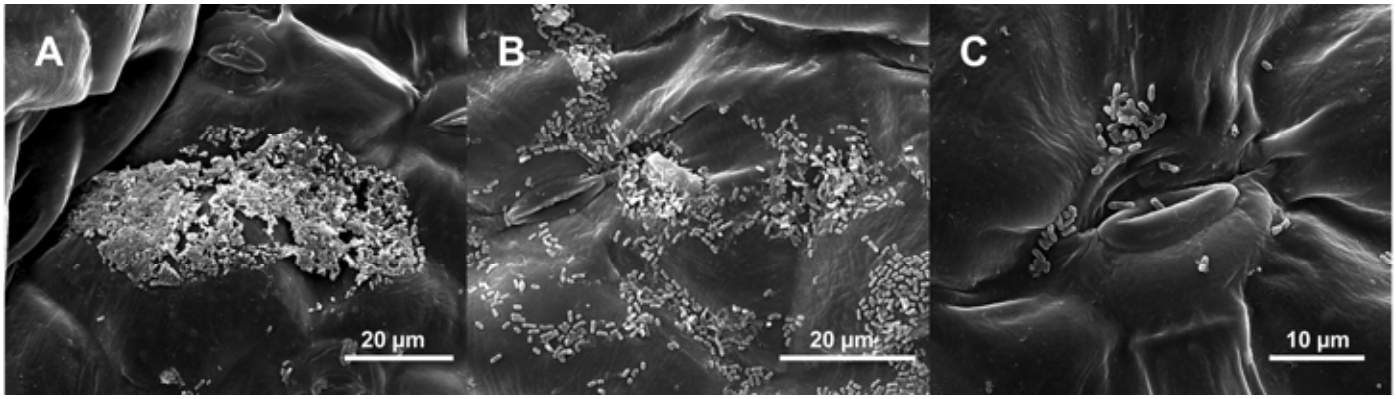

Fig. 3. Scanning electron microscopy of house fly regurgitation spots deposited on spinach leaves after feeding on *Escherichia coli* O157:H7 plates (EP acquisition source). **A,** Well-defined regurgitation spot with clear edges (20 µm). **B,** Diffuse regurgitation spot with cells dispersed over a larger area (20 µm). **C,** Bacteria-like structures in close proximity to a spinach stomate (10 µm).

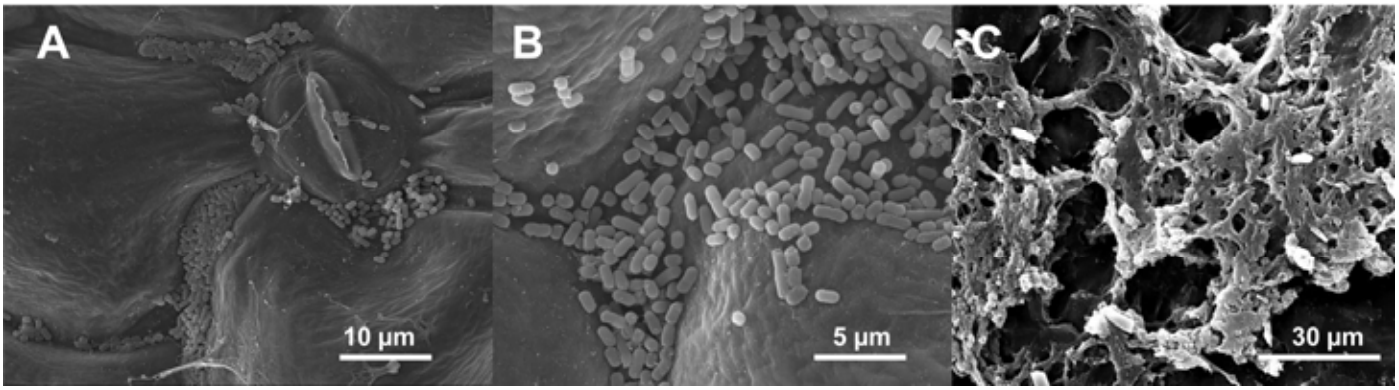

Fig. 4. Scanning electron microscopy of control spots. **A,** Manually spotted *Escherichia coli* O157:H7 on spinach leaves. **B,** Higher magnification showing individual bacterial cells in interclinal spaces. **C,** Regurgitation spot from a house fly exposed to a mixture of manure and sterile water (sterile water-manure acquisition source).

decrease) in *E. coli* DNA over time, from the point of deposition of bacteria on the spinach surface at time point 0.75 to several days later. As expected, regurgitation spots from flies that were fed on "control" acquisition sources (SM and LB) showed no significant differences in fold change, although there was a slight amplification of *eae* at 4 and 8 days postdeposition (Fig. 1). It is plausible that trace amounts of the *eae* gene were present in the digestive tract of flies and in the autoclaved cattle manure. The ΔCt values from the SM and LB treatments were the highest (Table 1), suggesting very low levels of the *eae* amplicon in the sample. We postulate that fly regurgitant may provide a nutrition source for bacteria since manually spotted cells showed no evidence of replication. It is possible that the manually spotted *E. coli* O157:H7 did not survive on the leaf surface and died between days 2 and 4, but the DNA remained intact and was detected by qPCR (Fig. 1).

Our study provides the first investigation of house fly regurgitation spots on spinach leaves by SEM and documents the survival and replication of *E. coli* O157:H7 within those spots on the leaf surface. Mitra et al. (13) reported that spotting of a cocktail

TABLE 2. Percentage of randomly selected colonies obtained by plating from house flies exposed to various acquisition sources and identified as *Escherichia coli* O157:H7 by polymerase chain reaction

	Days postexposure[b]						
Acquisition source[a]	0	2	4	6	8	10	13
E. coli-manure (EM)	100 (0)	0 (0)	20 (20)	46.67 (22)	0 (0)	2.5 (2)	6 (4)
E. coli-plate (EP)	100 (0)	100 (0)	100 (0)	100 (0)	15.33 (13)	0 (0)	25 (22)
Sterile water-manure (SM)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
LB-plate (LB)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] Acquisition sources: EM, flies exposed to autoclaved manure mixed with *E. coli* O157:H7; EP, flies exposed to *E. coli* O157:H7 lawns on Luria Bertani (LB) ampicillin agar; SM, flies exposed to autoclaved manure mixed with sterile water; and LB, flies exposed to uninoculated LB ampicillin agar plates.
[b] Percentages were generated from 10 colonies picked from five randomly selected plates (N = 5) per treatment on each day. Numbers in parentheses are standard error values.

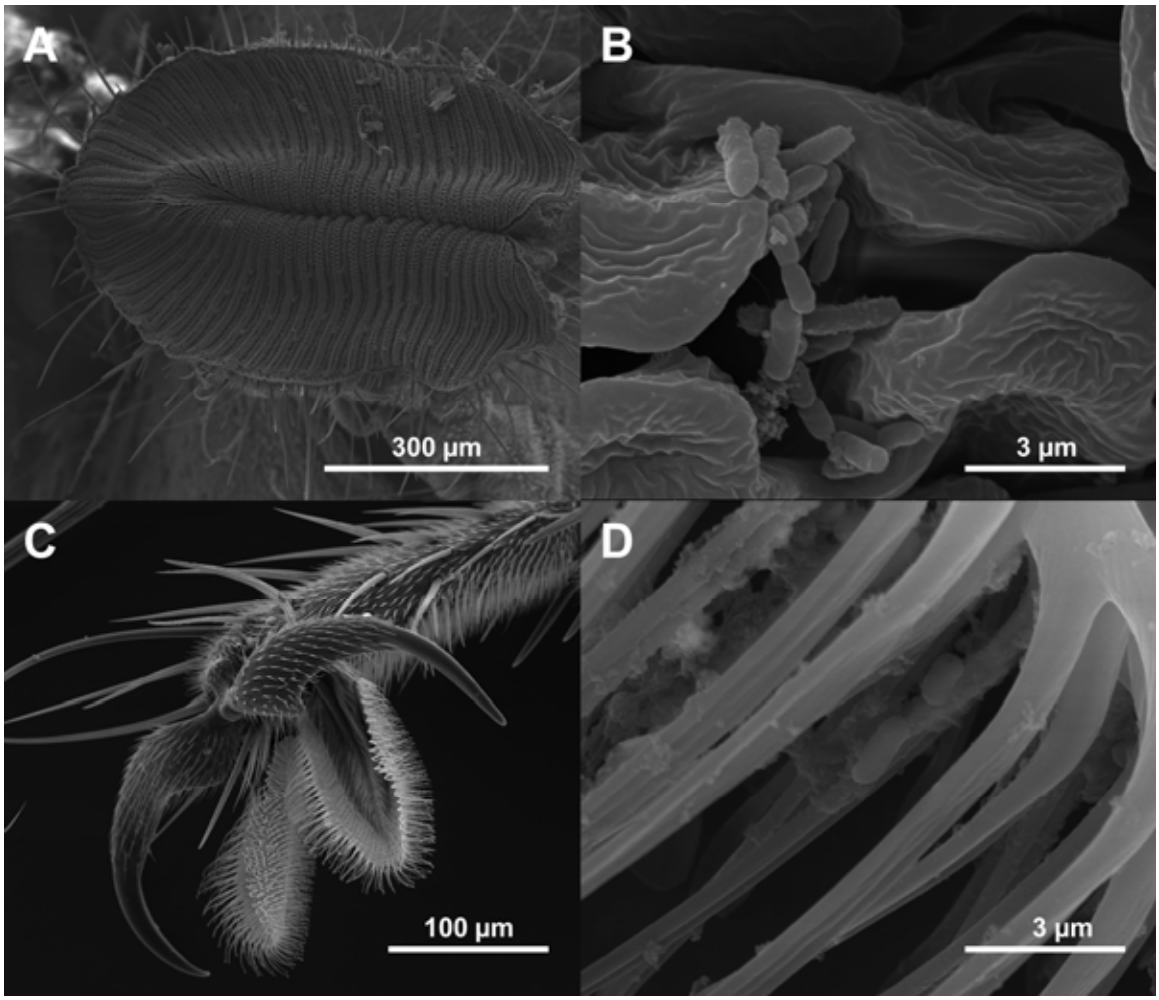

Fig. 5. Scanning electron micrographs of fly body parts. **A,** House fly labellum. **B,** Higher magnification showing bacteria adhering to and dividing on pseudotracheae. **C,** House fly tarsus. **D,** Higher magnification of glandular hairs on the pulvillus of the tarsus showing bacteria adhering to individual hairs.

of five *E. coli* O157:H7 strains on the abaxial surface of spinach leaves resulted in bacterial survival on the phylloplane for 14 days postinoculation. They also documented that the bacterial titer and colonization of leaf surfaces increased over time. In contrast, the *eae* target of manually spotted *E. coli* O157:H7 used in this study (ATCC 43888) did not go up over time, whereas the *eae* target DNA in the EM regurgitation spots increased dramatically in 4 days (Fig. 1). This suggests that fly regurgitation spots serve as a nutrient source for bacteria, allowing them to survive and multiply on the spinach phylloplane. The composition of house fly regurgitant and its potential as a nutrient source for bacteria remain to be determined.

Our results have implications for increased risk of disease due to fly transmission of pathogenic *E. coli*. What can be inferred from this work is that bacterial numbers on fly body parts increased after an initial decline, suggesting that *E. coli* O157:H7 growth (replication) may occur and/or fluctuate on the cuticular surface of flies. More importantly, viable *E. coli* O157:H7 persisted on fly body surfaces for up to 13 days after the initial exposure. These results support the hypothesis of "bioenhanced transmission" (11), which states that the relationship between flies and bacteria is more specific than simple contamination of fly surfaces.

The BLOs that were observed by SEM in the regurgitation spots and on fly body parts are interpreted to be derived from the same *E. coli* O157:H7 cells that the flies were exposed to under our experimental conditions. Few to no bacterial cells of the same dimensions as *E. coli* were observed in the two negative control treatments, although we cannot rule out the possibility that the colony flies used in the study were already carrying some *E. coli.* Flies require bacteria for development and oviposition, so working with aseptic flies would be impractical. SEM observations of fly body parts in this study revealed that the pseudotracheae of the labellae, body hairs, and the glandular setae of fly tarsi provide a hospitable environment for *E. coli* O157:H7 multiplication. Sukontason et al. (25) studied the ultrastructure of pulvilli from different fly families by scanning and transmission electron microscopy. The electron-lucent area of house fly setae (distal end) played an important role in fly attachment to surfaces and could serve as an adhesive surface for microorganisms (25). The results from this research and the Sukontason study suggest that bacteria associated with fly feet could contaminate plant surfaces. In addition to regurgitant, fly body hairs, sticky glandular hairs on the pulvilli, and pseudotracheae on the labellum are additional niches where bacteria can survive.

Micrographs of the BLOs on spinach clearly show filaments between the cells and the leaf surface. In a study of biofilm formation by *E. coli* strains, Prigent-Combaret et al. (16) showed that *E. coli* K-12 strains formed curli, or fimbriae, on coverslips and this enabled them to attach to the glass surface and to each other. These fimbriae were confirmed as curli by anti-curlin immunogold labeling (16). Shaw et al. (24) showed that *E. coli* O157:H7 encodes genes for the type III secretion system (T3SS) for adherence to the spinach and lettuce leaves and that EspA filaments allowed the bacteria to attach to surfaces. Similarly Xicohtencatl-Cortes et al. (29) documented that mutations of the adhesin intimin (*eae*) and flagella major subunit (*fli*C) reduced the colonization and leaf invasion capability of EHEC strains to spinach and lettuce. It is not clear whether the BLO attachment structures observed in this study might be curli or flagella, but further studies to investigate these possibilities are underway.

We have shown that house flies carry and deposit *E. coli* O157:H7 onto spinach under laboratory conditions using contaminated sources with inocula at levels higher than typically found in nature. The inoculum that the flies were exposed to was approximately 1 to 2×10^7, one log unit higher than levels documented in manure of a small proportion of super shedding cattle (15,17). Many factors influence the rate of acquisition of bacteria by flies, including initial bacterial load in the contaminated source, behavior of the fly, and exposure time. Although the bacterial load in the acquisition sources of this study was high, this was done to increase the probability of successful transmission. Further tests using lower bacterial loads and fly transmission under field conditions await completion for a more accurate estimate of the risk of fly transmission of pathogens to preharvest plants.

ACKNOWLEDGMENTS

We thank Xiaoping Guo for support and assistance with the qPCR. A. Wayadande acknowledges support from the National Integrated Food Safety Initiative (grant 2009-5110-05856) and the Oklahoma Agricultural Experiment Station (project OKL02668). This research was also partially supported by the Center for Produce Safety, Davis, CA.

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