CENTER for PRODUCE SAFETY

CPS-BARD 2009 RFP
FINAL PROJECT REPORT

Project Title

Persistence and detection of norovirus, *Salmonella* and pathogenic *Escherichia coli* on basil and leafy greens

Project Period

December 1, 2009 – November 30, 2012

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Objectives

Overall: To investigate the transmission and persistence of norovirus, *Salmonella,* and *Escherichia coli* O157:H7 (and other pathogenic *E. coli*) introduced to leafy green foliar surfaces in water using more realistic lower population levels, different irrigation regimes and other related factors.
University of Delaware: To investigate the persistence of norovirus on lettuce with viral detection and counting by plaque assay and RT-PCR, and determine the sites of adherence on produce using confocal microscopy.
Technion – Israel Institute of Technology: To determine the effect of the irrigation regime on transfer and survival of *Salmonella* in plants by dripping vs. spraying, day vs. night, summer vs. winter crops, daily vs. multiple short irrigations and other associated factors.
USDA, ARS-EMFSL (Beltsville): To determine the fate of enterohemorrhagic, avian pathogenic (APEC) and nonpathogenic *E. coli* introduced to leafy green foliar surfaces in irrigation water at levels stated in the California Leafy Green Marketing Agreement.

FINAL REPORT

Abstract

Under controlled conditions that realistically resembled environments existing in croplands and produce, this body of work conducted at the University of Delaware, Technion – Israel Institute of Technology, and the U.S. Department of Agriculture (ARS-EMFSL, Beltsville, MD), demonstrated the persistence windows of three critical pathogens of concern in the United States and Israel: Norovirus, *Salmonella* and pathogenic *E. coli*. The work added valuable information to the knowledge base regarding differences in growth conditions, seasonality, and environmental isolates. Outcomes of this work included observations that hydroponic growth conditions may pose a greater risk for virus contamination compared to plants grown in soil. Due to their small size, viruses can travel with stomata and through roots due in part to gutation and water flow. It is not yet known how long viruses can survive within plants. *Salmonella* survival on plant parts varied with season and weather conditions, and environmental *E. coli* varieties persisted on leafy greens and represent a reservoir for virulence genes. Bacterial pathogens interacted in different ways with different crops as shown by studies with environmental and outbreak isolates on leafy greens and herbs.

Background

What were the original objectives of the project? In funding, the project was accepted at a 50% reduction of budget; consequently only the first objective at each institution was retained upon revision. The original objectives of the grant proposal follow.

Overall: The goal of this research is to investigate the fate of norovirus, *Salmonella, Escherichia coli* O157:H7 and avian pathogenic *E. coli (*APEC) introduced to leafy green foliar surfaces in water using lower more realistic population levels and different irrigation regimes. In order to further understand the mechanisms of survival on the leaves, the role of selected virulence and adhesion factors will be investigated. To do this the transmission and persistence of norovirus, *Salmonella enterica* serovars Senftenberg, Typhimurium and Saintpaul, EHEC and APEC on three leafy green commodities will be evaluated. Pathogens will be introduced by contaminated water and persistence measure by culture methods and quantitative RT-PCR with localization by microscopy.

University of Delaware:

1) To investigate the persistence of norovirus on lettuce, spinach and basil with viral detection and counting by plaque assay and RT-PCR, and to determine the sites of adherence on produce using confocal microscopy.

2) To evaluate the persistence of APEC and nonpathogenic *E. coli* on produce by spot-inoculation for comparative study of *E. coli* on lettuce, spinach and basil with the USDA, ARS-EMFSL.

Technion – Israel Institute of Technology:

1) To determine the effect of the irrigation regime on transfer and survival of *Salmonella* in plants; irrigation methods that will be investigated: Dripping vs. spraying, day vs. night, summer vs. winter crops, daily vs. multiple short irrigation.
2) To determine the role of specific genetic factors in localization of *Salmonella* in produce.
USDA, ARS-EMFSL:
1) To determine the fate of enterohemorrhagic, avian pathogenic, nonpathogenic *E. coli*, and *Salmonella* introduced to leafy green foliar surfaces in irrigation water at levels stated in the California Leafy Green Marketing Agreement.
2) To assess the survival of strains of EHEC and APEC on leafy greens based on levels of expression of common virulence factors.
3) To evaluate the role of *espA* in EHEC attachment under realistic pre-harvest conditions.

The primary background issue driving this investigation was the recognition that the consumption of fecally contaminated foods is a predominant means of transmission for human enteric bacteria and viruses, which are increasingly being recognized as a significant public health threat globally. In the U.S. it is estimated that there are approximately three million cases a year of illness caused by fecally contaminated fruits and vegetables.

Describe any collaborative efforts involved in planning and implementing this project. As a joint CPS-BARD funded effort U.S. laboratories (University of Delaware and USDA, ARS-EMFSL) collaborated with an Israeli partner (Technion – Israel Institute of Technology). Due to the range of distances among the three laboratories, there was a greater level of collaboration and communication between the University of Delaware and USDA-Beltsville; however, a face-to-face meeting did occur at the University of Delaware when Dr. Yaron was visiting the U.S. in the summer of 2010. The project was discussed along with potential future collaborations.

Research Methods and Results

University of Delaware: Internalization of murine norovirus-1 by Lactuca sativa *during irrigation.*
Internalization of murine norovirus-1 (MNV-1) by lettuce was observed in two irrigation water contamination situations. In a single severe contamination situation, virus could not be removed from the plants by the replacement of fresh buffer mimicking freshwater; instead, a large quantity of MNV-1 particles were associated with the soil, and some virus particles remained suspended in the buffer. It is therefore likely that even after a flood, viruses remaining in the soil may contaminate clean irrigation water and subsequently contaminate plants. As stated by the LGMA (California Leafy Green Products Handler Marketing Agreement) accepted food safety practices (LGMA, 2010), fields in a flooded area should be left for 60 days before planting, or this may be shortened to 30 days with appropriate soil testing; however, further research and risk assessment are needed regarding the survival of virus in soil after a flooding incident due to the likelihood of viral contamination of leafy greens through root internalization or plant surface contact and the potential for viral persistence inside/on plant tissue and eventually transmission to consumers. In a situation of low levels of constant contamination, virus internalization occurred >1 day later than that in the single severe contamination; however, this still suggests that continuous use of irrigation water with a low

quantity of viruses could pose a risk of contamination. Thus, regular testing of irrigation water or groundwater for virus may help to reduce the risk of contamination; however, representative samples can be difficult to obtain due to the inconsistent presence of virus in water (Alhajjar et al., 1988).

Previous results are inconsistent among the studies that have been conducted assessing the impact of internalization of human enteric viruses or bacteriophage into produce during hydroponic or traditional growth conditions (Chancellor et al., 2006; Oron et al., 1995; Urbanucci et al., 2009; Ward and Mahler, 1982). It was reported that <2 log PFU of bacteriophage f2/g plant tissue was detected in the shoots of hydroponically growing bean (*Phaseolus vulgaris* L.) challenged with $\sim 10^{10}$ PFU/ml f2 at roots (Ward et al., 1982). Calicivirus was occasionally detected in the edible parts of romaine lettuce grown hydroponically or in soil with total virus inocula of 10^6 to 10^9 RT-qPCR U; in similar experiments using human norovirus, no virus was found in any plants, indicating that the frequency of contamination via roots was rare even when plants were exposed to high concentrations of virus (Urbanucci et al., 2009); however, Chancellor et al. (2006) found 100% positive detection of hepatitis A virus RNA inside green onions grown in soil as well as onions grown hydroponically. Poliovirus was recovered from the leaves of tomatoes grown in soil injected with only 10^3 to 10^4 PFU/mL virus once every week (Oron et al., 1995).

The inconsistent results may be a result of variant plant properties for virus penetration and uptake, and different experimental parameters of produce growth, irrigation, and soil conditions. Zhu et al. (2008) evaluated the uptake and translocation of nanoparticles in plants and found that with a size range of 0.02 to 2 μm (identical to or larger than the size of enteric viruses and bacteriophage) and with a slightly negative charge, nanoparticles were detected and distributed in pumpkin stems and leaves; however, no uptake was observed with lima bean plants (*Phaseolus limensis*) suggesting various responses of plants to nanoparticles.

Water content and water movement in soil are important for viral contamination, as indicated by the increased number of viruses recovered from produce surfaces with an increase in soil moisture content (Song et al., 2006). Low water content could increase virus particle attachment to soil-water interfaces, favor virus adsorption, and result in retention of virus movement in soil with a lesser access to plants (Torkzaban et al., 2006). To favor virus uptake, in our work saturated soil conditions were maintained with nutrient buffer during the entirety of the experiments. Nutrient solution was added to a container, and liquid moved up toward the soil through capillary force. This may explain in part why more virus internalization, especially with low inocula, was observed in our study than in some of the previous research discussed above (Oron et al., 1995, Urbanucci et al., 2009); however, further experiments are needed to monitor soil conditions, soil water content, and virus movement to evaluate their effects on virus uptake by plants.

Transpiration is the driving force for water absorption, and the majority (96%) of water is taken up by the plant through transpiration (Kramer, 1983). Humidity is a major factor controlling plant transpiration, and high humidity reduces the diffusion of water out of the leaf and lowers

the transpiration rate; transpiration will cease if humidity reaches 100% (Conger et al., 2001; Tanner and Beevers, 2001). Transpiration was assessed in our study to identify a potential role in the movement of viruses into the plant tissue. Lettuce grown under conditions of 99% humidity to minimize transpiration showed a significantly lower frequency of virus internalization than lettuce grown in a 70% RH chamber which had a 10-fold-higher transpiration rate. Thus, transpiration is likely to be a major force for virus uptake through roots.

University of Delaware: Survival of pathogenic E. coli *on basil, lettuce, and spinach.*
The contamination of lettuce, spinach and basil with pathogenic *E. coli* has caused numerous illnesses over the past decade. *E. coli* O157:H7, *E. coli* O104:H4 and avian pathogenic *E. coli* (APECstx- and APECstx+) were inoculated on basil plants and in promix substrate using drip and overhead irrigation. When over-head inoculated with 7 log CFU/ml of each strain, *E. coli* populations were significantly (P = 0.03) higher on overhead-irrigated plants than on drip-irrigated plants. APECstx-, *E. coli* O104:H4 and APECstx+ populations were recovered on plants at 3.6, 2.3 and 3.1 log CFU/g at 10 dpi (days post-inoculation), respectively. *E. coli* O157:H7 was not detected on basil after 4 dpi. The persistence of *E. coli* O157:H7 and APECstx- were similar when co-inoculated on lettuce and spinach plants. On spinach and lettuce, *E. coli* O157:H7 and APEC populations declined from 5.7 to 6.1 log CFU/g and 4.5 log CFU/g to undetectable at 3 dpi and 0.6–1.6 log CFU/g at 7 dpi, respectively.

Technion – Israel Institute of Technology: Salmonella *and parsley.*
Microbiological and molecular-based methods were examined for their ability to precisely quantify different amounts of *Salmonella enterica* serovar Typhimurium artificially inoculated on parsley leaves. Recovery of *S.* Typhimurium from parsley by mechanical detachment using stomacher, mortar and pestle, vortex, sonicator or homogenizer followed by plating resulted in underestimation with less than 1% recovery when leaves were inoculated with 3.5 to 6.5 log CFU/g. Lower levels were undetectable by most assayed methods, and only recovery with mortar and pestle or adding of enrichment step resulted in partial detection of 300 CFU/g. Implementation of PCR-based methods with/without pre extraction of the DNA from the contaminated leaves resulted in more accurate values in estimating the population of the pathogen (about 20% of the initial inocula). Levels as low as 300 CFU/g were detected even without an enrichment step.

Short- and long-term (1 h to 28 days) persistence of *Salmonella enterica* serotype Typhimurium in the phyllosphere and rhizosphere of parsley was investigated following spray irrigation with contaminated water. Plate counting and quantitative real-time PCR (qRT-PCR)-based methods were implemented for the quantification. By applying qRT-PCR with enrichment, even irrigation with water containing as little as 300 CFU/mL was shown to result in the persistence of *S.* Typhimurium on the plants for 48 h. Irrigation with water containing 8.5 log CFU/mL resulted in persistence of the bacteria in the phyllosphere and rhizosphere of parsley for at least four weeks, but the population steadily declined with a major reduction in bacterial counts of 2 log CFU/g during the first two days. Higher levels of *Salmonella* were detected in the phyllosphere

when parsley plants were irrigated at night compared to irrigation during the morning, and during winter when compared to the other seasons.

Technion – Israel Institute of Technology: Response of Salmonella *to basil oil.*
Basil has been shown to produce relatively high levels of antibacterial compounds. The aim of this study was to observe ecological changes occurring as a result of developing resistance to ingredients of the basil oil by salmonellae. Basil plants were irrigated with contaminated water containing *Salmonella* serovars Typhimurium and Senftenberg. *Salmonella* was found to survive on the basil plants for at least 100 days. *S*. Senftenberg counts were significantly higher than *S.* Typhimurium. Moreover, *S*. Senftenberg grew on stored harvested basil plants. Susceptibility experiments demonstrated that *S*. Senftenberg was resistant to basil oil and to antimicrobial compounds in basil oil such as linalool, estragole and eugenol. The strain of *S*. Senftenberg may have adapted to the basil environment by developing resistance to the basil oil.

USDA, ARS-EMFSL: APEC, O157 and non-O157 on spinach and lettuce.
The purpose of this study was to determine the survival of APEC and pathogenic strains of O157 and non-O157 *E. coli* on leafy greens including basil, lettuce, and spinach. The survival of APEC strains and non-O157 *E. coli* strains were assessed individually and as a multi-strain inoculum on plants. Cultures were diluted 10-fold into autoclaved dairy manure slurry prior to inoculation to obtain a concentration of ~10^6 CFU/mL as confirmed by enumeration on sorbitol MacConkey agar (SMAC) supplemented with 50-µg/mL nalidixic acid (SMACN). Fresh manure was collected from the University of Delaware dairy farm, centrifuged to remove solids, and the liquid portion sterilized by autoclaving for use as the irrigation solution. Cultures of APEC and *E. coli* O157:H7 strains were combined into a single inoculum which containing 1.3 x10^5 CFU/mL of each APEC strain and 5 x 10^5 CFU/mL *E. coli* O157:H7 for a total inoculum at 1 x 10^6 CFU/mL to be applied to plants. Plants were grown in the Biosafety Level 2 (BSL-2) growth chambers. Both lettuce and spinach were grown under conditions set to 70% relative humidity for a 14-h photoperiod at 20^{o}C and 10-h dark period at 15^{o}C.

For lettuce and spinach plants, plants were harvested by using sterile scissors to cut the shoot tissue above the soil surface from each spinach or lettuce plant. The average weights of lettuce and spinach plants were 5 and 3 g, respectively. For sampling, stomacher homogenates were either plated on SMACN (Day 0) or used for MPN (Most Probable Number). On each day of analysis, either six spinach plants or six lettuce plants were harvested and microbiologically analyzed. When MPN assays yielded undetectable numbers of *E. coli*, plant material in stomacher bags was enriched and incubated at 37^{o}C for 24 h. Enriched samples were streaked for isolation to determine the presence of *E. coli* O157:H7 or APEC. APEC and *E. coli* O157:H7 were co-inoculated simultaneously on lettuce and spinach plants.

Initial populations (day 0) of APEC on spinach and lettuce were 6.1 and 4.5 log CFU/g, respectively. Initial populations (day 0) of *E. coli* O157:H7 on spinach and lettuce were 5.7 and 4.5 log CFU/g, respectively. Populations of both types of *E. coli* declined rapidly on both commodities by day 1, as APEC populations declined by 4 log MPN/g on spinach and by 2.8 log

MPN/g on lettuce. Similar declines were observed with respect to *E. coli* O157:H7 on both leafy green commodities; populations declined by 3.9 log MPN/g on spinach and 3.7 log MPN/g on lettuce. *E. coli* populations persisted for shorter durations on spinach compared to lettuce; by day 3, no APEC or *E. coli* O157:H7 was found by enrichment on spinach plants; however, on lettuce low levels of APEC strains were detectable by MPN eight days after inoculation. Both APEC and *E. coli* O157:H7 were detectable by enrichment 14 and 17 days after inoculation. APEC survived at higher levels than *E. coli* O157:H7 on 7 and 8 days post-inoculation on lettuce plants.

The lack of simultaneous survival of both APEC and *E. coli* O157:H7 strains on spinach plants compared to lettuce was surprising, given than inoculation methods were the same for both leafy green commodities. It was unclear why spinach plants supported a shorter duration of survival than lettuce plants for both APEC and *E. coli* O157:H7 strains. The interaction of the APEC and *E. coli* O157:H7 strains with bacterial aggregates on leaf surfaces may also partially explain their extended survival on the foliar tissue of lettuce plants; conversely, the potential lack of interaction of these aggregates on spinach surfaces may have also led to the more rapid decline of *E. coli* O157:H7 populations compared to lettuce surfaces. It was also possible that lower recovery of both APEC and *E. coli* O157:H7 from lettuce plants on day 0 compared to spinach plants indicated that *E. coli* attached more securely to lettuce tissue as compared to spinach tissue and fewer bacteria were able to be dislodged. Previous findings indicated variations in attachment, which occurred among closely-related *Salmonella* serovars, may have also occurred in our study with *E. coli* strains and affected recovery and enumeration from the leafy green tissue.

Outcomes and Accomplishments

Under controlled conditions that realistically resemble environments existing in croplands and produce, this work demonstrated the persistence windows of three critical pathogens of concern in the United States and Israel: Norovirus, *Salmonella* and *E. coli* O157:H7. The work adds valuable information to the knowledge base regarding differences in growth conditions, seasonality, and environmental isolates. The virus work indicated that continuous use of irrigation water with a low quantity of viruses could pose a contamination risk and that relatively low levels of virus can internalize in lettuce. Norovirus was able to survive on and within plant surfaces when grown in contaminated conditions, and this contamination is more likely to occur when plants are grown hydroponically with contaminated water than when contaminated water is used to water plants grown in soil. Avian pathogenic *E. coli* isolates survived at higher populations and for longer durations when individually inoculated onto basil plants or co-inoculated on lettuce and spinach plants compared to *E. coli* O157:H7 strains. Interestingly, the outbreak isolate of *E. coli* O104:H4, that is a combination of two different *E. coli* pathotypes, showed similar survival patterns to the APEC environmental isolates. This is a unique finding comparing different *E. coli* isolates. *Salmonella* survival on plant parts varies with season and weather conditions; seasonal variations played a role in survival of *S.* Typhimurium in plants. *S.* Senftenberg survived very well on basil plants for at least 100 days and demonstrated growth on stored harvested basil plants. Additionally, *S.* Senftenberg showed

resistance to basil oil and its antimicrobial compounds (linalool, estragole and eugenol) suggesting adaption to the basil environment. Environmental *E. coli* persisted on leafy greens and may represent a reservoir for virulence genes. *E. coli* O157:H7 in irrigation water that complied with LMGA standards did not persist for >24 h, while waters with an organic load ≥12 ppm permitted growth of O157:H7.

Summary of Findings and Recommendations

University of Delaware: MNV-1 was taken up by Romaine lettuce through the roots via contaminated irrigation water and reached edible leaf tissue. The internalization of human enteric viruses into produce during irrigation is possible under favorable conditions, and the fact that some internalized virus remained infectious poses a food safety threat. Furthermore, the virus may be taken up in a passive manner by transpiration. The exact method of virus internalization under different environmental conditions, such as soil water content and environmental relative humidity, is still unclear.

With regard to unexpected outcomes, as noted on page 4, results were inconsistent among published studies that have been conducted assessing the impact of internalization of human enteric viruses or bacteriophage into produce during hydroponic or traditional growth conditions. As noted, these conflicting results may be a result of variable plant properties affecting virus penetration and uptake, as well as influence from different experimental parameters of produce growth, irrigation, and soil conditions.

In a concluding collaborative study between the University of Delaware and the USDA-Beltsville, the detection of low populations of strains of APEC and *E. coli* O104:H4 ten days post-inoculation indicated that APEC and *E. coli* O104:H4 may be better adapted to environmental conditions than strains of *E. coli* O157:H7. This is the first reported study of *E. coli* O104:H4 on a produce commodity. These results suggest a variety of pathotypes of *E. coli* harbor potential for environmental transfer to foods.

Technion – Israel Institute of Technology: Microbiological and molecular-based methods were developed and shown to precisely quantify different amounts at low levels of *Salmonella enterica* serovar Typhimurium artificially inoculated on parsley leaves. These methods can be applied to study transfer of *Salmonella* from contaminated water or soil to plants using low and more reasonable levels of contamination.

Higher levels of *Salmonella* were detected in the phyllosphere when plants were irrigated at night compared to irrigation during the morning, and during winter when compared to the other seasons. Further elucidation of the mechanisms underlying the transfer of *Salmonella* from contaminated water to crops, as well as its persistence over time, will enable the implementation of effective irrigation and control strategies.

Susceptibility experiments demonstrated that *S*. Senftenberg was resistant to basil oil and its antimicrobial compounds and grew well on basil. This strain of *S*. Senftenberg may have

adapted to the basil environment by developing resistance to components naturally found in basil oil and thus harbor increased risk to human safety. The emergence of resistant pathogens to naturally occurring antibacterial substances may have significant potential to alter the ecology of foods and enhance the ability for pathogens to survive in new niches in the environment, such as basil and other plants.

USDA, ARS-EMFSL: APEC generally survived at population levels ~ 1 log MPN/g higher than *E. coli* O157:H7 on lettuce. Whether this difference in survival is related to an enhanced environmental fitness of APEC strains compared to *E. coli* O157:H7 is unclear. Since the APEC inoculum consisted of four strains compared to one strain for the *E. coli* O157:H7 inoculum, it is possible that the strain diversity in the APEC inoculum contained one or more strains which were more persistent than the *E. coli* O157:H7 outbreak strain used in this work. APEC strain 07-1707 is an *E. coli* O157 serotype and was used in both individual inoculation studies on basil and in simultaneous inoculation studies on spinach and lettuce. The potential survival of this strain compared to *E. coli* O157:H7 on all three commodities may indicate that persistence on foliar surfaces is less a function of serotype and more dependent on source of isolation or previous environmental exposure of the strain (*i.e*., adaptation). Previous studies demonstrated that when co-inoculated on to spinach foliar surfaces, non-pathogenic *E. coli* isolates from produce commodities survived at higher populations for up to 28 days, compared to *E. coli* O157:H7 strains from produce outbreaks, which only survived for 7 days. The specific geospatial origin of an isolate has been suggested to affect environmental fitness. Our findings indicated that *E. coli* O157:H7 strains from produce outbreaks may not survive as well in non-host environments (*e.g.,* foliar surfaces, soil, water) as *E. coli* isolated from environmental sources, where a greater opportunity exists for adaption to stresses in pre-harvest, leafy green-growing environments. These findings support the hypothesis that *E. coli* O157:H7 outbreak strains may not possess the environmental fitness of other environmentally-isolated *E. coli* isolates.

APPENDICES

Publications and Presentations

Publications

Ingram, D.T., J. Patel and M. Sharma. 2011. Effect of repeated irrigation with water containing varying levels of total organic carbon on the persistence of *Escherichia coli* O157:H7 on baby spinach. *Journal of Food Protection* 74:709-717.

Kisluk, G., D.G. Hoover, K.E. Kniel and S. Yaron. 2012. Quantification of low and high levels of *Salmonella enterica* serovar Typhimurium on leaves. *LWT - Food Science and Technology* 45:36-42.

Kisluk, G., and S. Yaron. 2012. Presence and Persistence of *Salmonella enterica* serotype Typhimurium in the phyllosphere and rhizosphere of spray-irrigated parsley. *Applied and Environmental Microbiology* 78:4030-4036.

Markland, S.M., K.L. Shortlidge, D.G. Hoover, S. Yaron, J. Patel, A. Singh, M. Sharma and K.E. Kniel. 2013. Survival of pathogenic *Escherichia coli* on basil, lettuce and spinach. *Zoonosis and Public Health* (in press).

Wei, J., Y. Jin, T. Sims and K.E. Kniel. 2010. Manure- and biosolids-resident murine norovirus-1 attachment to and internalization by Romaine lettuce. *Applied and Environmental Microbiology* 76:578-583.

Wei, J., Y. Jin, T. Sims and K.E. Kniel. 2011. Internalization of murine norovirus-1 by *Lactuca sativa* during irrigation. *Applied and Environmental Microbiology* 77:2508-2512.

Presentations

Hirneisen, K., and K.E. Kniel. Norovirus Survival on Spinach during Pre-harvest Growth. IAFP Annual Meeting, Providence, RI, July 2012. T4-01.

Ingram, D., C. Mudd, S. Feguson, D.G. Hoover, K.E. Kniel and M. Sharma. The effect of total organic carbon content and repeated irrigation on the persistence of *E. coli* O157:H7 on baby spinach. IAFP Annual Meeting, Anaheim, CA, August 2010. T4-09.

Kisluk, G. and S. Yaron. Existence of *Salmonella* Typhimurium on growing leafy greens as dictated by level of water contamination, irrigation method and type of produce. The 9th International Symposium of the Microbial Ecology of Aerial Plant Surfaces, Corvallis, OR, USA, August, 2010.

Kisluk, G. and S. Yaron. The existence of *Salmonella* Typhimurium on growing plants depends on level of water contamination, irrigation method and type of produce. The 6th FISEB (ILANIT) Congress, Eilat, February, 2011.

Kisluk, G. and S. Yaron. New insights on the interactions between the foodborne pathogen *Salmonella* Typhimurium and plants. Perspective in Phyllosphere biology. New Delhi, India, February 2012.

Kisluk, G. and S. Yaron. Quantification of low and high levels of *Salmonella enterica* serovar Typhimurium on leaves. International Conference on Environmental Security for Food and Health. Chandigarh, India, February 2012.

Kisluk, G. and S. Yaron. Pathogens in the salad or a salad of pathogens. The Annual conference for science and the environment. Tel Aviv, October 2012.

Kisluk, G. and S. Yaron. *Salmonella* in our salad or a salad of *Salmonella* – a review of a decade of research about the interaction between *Salmonella* serovars and leafy greens. The 2nd Plant/*Salmonella* Interactions workshop. Tours, France, December 2012.

Kniel, K.E. *Viral Interactions with Plants*, American Phytopathological Society Human Pathogens on Plants Workshop, College Park, MD, February 12-14, 2012.

LeStrange, K., C. Boettger, J. Wei, D.G. Hoover and K.E. Kniel. Isolation and characterization of avian pathogenic *E. coli* from Delmarva poultry. IAFP Annual Meeting, Milwaukee, WI, August 2011.P3-148.

LeStrange, K., S. Markland, K. Shortlidge, D.G. Hoover and K.E. Kniel. Evaluation of virulence profiles of environmental avian pathogenic *Escherichia coli* O157 isolates. IAFP Annual Meeting, Providence, RI, July 2012. P3-141.

Markland, S., K. Shortlidge, L. Cook, K. LeStrange, M. Sharma and K.E. Kniel. A comparison of *Escherichia coli* persistence on basil plants and soil using drip and overhead irrigation. IAFP Annual Meeting, Providence, RI, July 2012. P3-123.

Wei, J., Y. Jim, T. Sims and K.E. Kniel. Internalization of murine norovirus-1 to Romaine lettuce. IAFP Annual Meeting, Anaheim, CA, August 2010. T4-06.

Budget Summary
Brief narrative breakdown of how the funds were spent and comment on the funding.

The three different laboratories spent the funds conventionally for personnel support and supplies. The USDA, ARS-EMFSL functioned as a subcontractor in the project with the University of Delaware the funding source. There were some delays in paperwork that caused confusion, but current records should now be complete. Technion functioned independently

from the University of Delaware and USDA-Beltsville managing its funds through BARD. To our knowledge all funds have been expended in the three-year project on appropriate activities.

CPS-BARD Hoover 09 (UD budget only)	12/1/2009	11/30/2012	62,500.00
CPS-BARD Subaward USDA ARS Beltsville	12/1/2009	11/30/2012	62,500.00

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Urbanucci, A., M. Myrmel, I. Berg, C.-H. von Bonsdorff, and L. Maunula. 2009. Potential internalization of caliciviruses in lettuce. Int. J. Food Microbiol. 135:175-178.
Ward, R.L., and R.J. Mahler. 1982. Uptake of bacteriophage f2 through plant roots. Appl. Environ. Microbiol. 43:1098-1103.
Zhu, H., J. Han, J. Q. Xiao, and Y. Jin. 2008. Uptake, translocation, and accumulation of manufactured iron oxide nanoparticles by pumpkin plants. J. Environ. Monit. 10:713-717.

Applied and Environmental Microbiology

Internalization of Murine Norovirus 1 by *Lactuca sativa* during Irrigation

Jie Wei, Yan Jin, Tom Sims and Kalmia E. Kniel
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Internalization of Murine Norovirus 1 by *Lactuca sativa* during Irrigation▽

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Romaine lettuce (*Lactuca sativa*) was grown hydroponically or in soil and challenged with murine norovirus 1 (MNV) under two conditions: one mimicking a severe one-time contamination event and another mimicking a lower level of contamination occurring over time. In each condition, lettuce was challenged with MNV delivered at the roots. In the first case, contamination occurred on day one with 5×10^8 reverse transcriptase quantitative PCR (RT-qPCR) U/ml MNV in nutrient buffer, and irrigation water was replaced with virus-free buffer every day for another 4 days. In the second case, contamination with 5×10^5 RT-qPCR U/ml MNV (freshly prepared) occurred every day for 5 days. Virus had a tendency to adsorb to soil particles, with a small portion suspended in nutrient buffer; e.g., ~8 log RT-qPCR U/g MNV was detected in soil during 5 days of challenge with virus inoculums of 5×10^8 RT-qPCR U/ml at day one, but <6 log was found in nutrient buffer on days 3 and 5. For hydroponically grown lettuce, ~3.4 log RT-qPCR U of viral RNA/50 mg of plant tissue was detected in some lettuce leaf samples after 5 days at high MNV inoculums, significantly higher than the internalized virus concentration (~2.6 log) at low inoculums ($P < 0.05$). For lettuce grown in soil, approximately 2 log RT-qPCR U of viral RNA/50 mg of plant tissue was detected in lettuce with both high and low inoculums, showing no significant difference. For viral infectivity, infectious MNV was found in lettuce samples challenged with high virus inoculums grown hydroponically and in soil but not in lettuce grown with low virus inoculums. Lettuce grown hydroponically was further incubated in 99% and 70% relative humidities (RH) to evaluate plant transpiration relative to virus uptake. More lettuce samples were found positive for MNV at a significantly higher transpiration rate at 70% RH, indicating that transpiration might play an important role in virus internalization into *L. sativa*.

Human norovirus (HuNoV) is the leading food-borne pathogen in the United States, accounting for approximately 60% of food-borne disease annually (16). Recently, there have been increasing outbreaks of HuNoVs associated with vegetables and fruits (6, 8, 15). The facts that (i) fresh produce is consumed raw or with minimum preparation, (ii) postharvest biocidal sanitizing is ineffective for removal and inactivation of virus on fruits and vegetables (2), and (iii) the infectious dose of HuNoV is as low as a few particles (3) make the consumption of contaminated produce a risk to consumer health. Consumption of fresh produce, such as leafy greens, contaminated with HuNoVs is now recognized as a common cause of gastroenteritis (7). Leafy greens may be contaminated preharvest through the use of irrigation water containing fecal material (14). River, canal, pond, or well water used for irrigation may be exposed to human enteric viruses due to leakage of sewage water or from animal production zones close to produce fields. It has been shown that virus can attach to the leaf surface and internalize through stoma and cuts on the leaf during direct contact with virally contaminated water (23). However, as viral particles are small, at approximately <100 nm in diameter, another possible route of contamination through irrigation water is the internalization of human enteric viruses through roots into the edible tissues of leafy greens during plant water absorption. To date, few studies have been conducted on the uptake of enteric viruses by plants via contaminated irrigation water, and the literature is inconsistent on the likelihood and quantity of internalized virus when roots are intact or damaged (2, 21, 22).

The objective of this study was to evaluate the likelihood and concentration of murine norovirus 1 (MNV) (a widely used surrogate of HuNoV) that may be taken up by lettuce during irrigation. Two scenarios were considered which mimic (i) a severe one-time contamination (e.g., a flooding occurrence or vast amounts of fecal material brought into surface water) and (ii) irrigation water containing a lesser concentration of viruses delivered over several days (e.g., water constantly being exposed to septic tank leakage or sewage water contaminating irrigation water).

MATERIALS AND METHODS

Viruses. Murine norovirus 1 (MNV) was propagated with RAW 264.7 cells cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Invitrogen Co.) as described by Wobus et al. (25). Viruses were purified from infected cells through three freeze-thaw cycles. The supernatant was recovered by centrifugation at 2,500 × *g* for 15 min and was stored at −80°C until use. MNV concentration was determined by endpoint dilution, with the highest dilution providing a positive signal on reverse transcriptase quantitative PCR (RT-qPCR), defining one RT-qPCR unit (RT-qPCR U). The titer of the initial stock of MNV was $\sim 1 \times 10^9$ RT-qPCR U/ml. The MNV plaque assay was conducted with confluent RAW 264.7 cells grown in 12-well plates for 24 to 48 h as previously described (25). The infectivity concentration of the MNV stock was $\sim 1 \times 10^8$ PFU/ml.

Lettuce (*Lactuca sativa*) plant and virus challenging. Lettuce seeds were purchased from a local store (Lancaster, PA) and surface sanitized with 70% ethanol

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for 10 min. The seeds were then washed with sterile distilled water, air dried overnight in a biosafety hood, and stored in the dark before use. Nutrient solution used to culture lettuce was prepared as described by Korkmaz et al. (10) with hydrosol (product no. 5N-4.7P-22K; Grace-Sierra, Milpitas, CA), magnesium sulfate, iron chelate (Sprint330; Ciba-Geigy, Greensboro, NC), and calcium nitrate. The nutrient solution contained 210 mg/liter K, 200 mg/liter N, 129 mg/liter C, 66 mg/liter S, 48 mg/liter P, 20 mg/liter Mg, 4 mg/liter Fe, 0.5 mg/liter Mn, 0.5 mg/liter B, 4 mg/liter Fe, 0.15 mg/liter Cu, 0.15 mg/liter Zn, and 0.10 mg/liter Mo.

For virus challenge studies, two concentrations of virus were evaluated, each representing a specific environmental scenario. To assess the situation of severe one-time contamination (e.g., flooding), 5×10^8 RT-qPCR U/ml MNV was applied to lettuce plants for 24 h, followed by removal of the virus solution, which was then replaced with virus-free nutrient solution every day for up to 5 days. To evaluate the situation of constant contamination with a relatively low quantity of virus, lettuce was grown with 5×10^5 RT-qPCR U/ml MNV and replaced with fresh nutrient solution containing the same concentration of virus every day for 5 days.

Lettuce grown hydroponically or in soil was used to evaluate the internalization of virus during irrigation. All lettuce was grown in a green house at 22 to 24°C. Lettuce seeds were sprouted and grown, and after 20 days plants were used for the virus study. For hydroponically grown lettuce, lettuce seeds were sprouted in 22-mm-square by 37-mm-deep cubes of oasis (Griffin, MA), as previously described (10), and placed in a container, and nutrient solution (50 ml; alone or with the addition of MNV) was added to the container without contact with the lettuce plant. For lettuce grown in soil, Peat-Lite (Sun Gro, Vancouver, Canada) was placed in a plant pot (Griffin, MA) and placed in a container. Nutrient solution (50 ml) was added to the container and delivered through the soil by capillary force and therefore had no direct contact with the leaf surface. MNV was diluted in nutrient solution to 5×10^8 or 5×10^5 RT-qPCR U/ml, and 50 ml of solution was applied to lettuce as described above.

To evaluate the effect of transpiration on virus uptake, lettuce growing hydroponically was challenged with 1×10^8 RT-qPCR U/ml MNV and grown at 99% relative humidity (RH) in a dew chamber (Percival, IA) or at 70% RH in a growth chamber (Conviron, Manitoba, Canada) at ~22°C for 24 h, with 12 h of fluorescent light and 12 h of darkness. Lettuce leaf samples were collected and analyzed as described below.

Sample analysis. For both high and low inoculums, nutrient solution and soil samples were collected and analyzed on days 1, 3, and 5. The viral RNA was extracted from the nutrient solution or soil samples as described by Wei et al. (24), using the QIAamp viral RNA minikit (Qiagen, CA). Leaf samples from lettuce plants were challenged with virus as described above and collected on days 1, 3, and 5. Leaf samples (50 mg) were frozen with liquid nitrogen and ground with a beadbeater (Biospec, OK) for 10 s. Then 500 μl RLT lysis buffer (RNeasy plant minikit, Qiagen, CA) was added to a homogenized lettuce sample and further bead beaten for 30 s. The RNA was extracted from lettuce samples (50 mg of leaves collected from three lettuce plants in each replicate) by using an RNeasy plant minikit in accordance with the manufacturer's instructions. Total RNA was eluted with 60 μl RNase-free water and stored at −80°C until use.

Two-step RT-qPCR was used to quantify virus concentration. The RT step was performed in 20-μl volumes, including 2.0 μl 10× buffer, 2.0 μl deoxynucleoside triphosphate (dNTP) (5 mM), 1.0 μl each primer (5 μM), 0.1 μl RNase inhibitor, 1 μl reverse transcriptase, 10.9 μl RNase-free H_2O, and 2 μl RNA, by use of the Sensiscript RT kit (Qiagen, CA) and amplified at 37°C for 60 min. The qPCR was performed in 20-μl volumes containing 10 μl 2× SYBR green mix, 1.2 μl of each primer (5 μM), 2 μl cDNA, and 5.6 μl H_2O by using a QuantiTect SYBR green PCR kit (Qiagen, CA). The amplification cycle was 95°C for 15 min, 40 cycles of 94°C for 15 s, 64°C for 30 s, and 72°C for 30 s, followed by a dissociation step of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. The primers for MNV were developed by Hsu et al. (9). To generate a standard curve, virus was serially diluted and ~10^7 to ~10^2 RT-qPCR U of virus was added to 50-mg lettuce samples, followed by RNA extraction, and applied to RT-qPCR as described above. A standard curve was generated from three independent trials of virus inoculation, RNA extraction, and RT-qPCR. The quantification of viral RNA in nutrient solution and soil samples was conducted as described by Wei et al. (24).

To evaluate the infectivity of internalized MNV, 50-mg lettuce leaf samples were homogenized in 5 ml Hanks' balanced salt solution (HBSS) by using a Tissuemiser homogenizer (Fisher Scientific, PA). The lysates were then applied to a Qiashredder (Qiagen, CA) and centrifuged at 10,000 × *g* for 2 min. The supernatant was then serially diluted with HBSS buffer and assessed in a plaque assay as described above.

Transpiration rate measurement. To measure the transpiration rate at two humidities, lettuce grown hydroponically in the oasis cube was placed at the opening of a flask. Air was applied to the water in the flask through a pipe connected with an air pump to avoid anaerobic conditions. The whole flask was covered with plastic film to prevent evaporation. The transpiration rate was measured by weight loss of the whole flask plus the plant after 1 h of transpiration (12), and the experiments were conducted in four replicates.

Statistical analysis. Statistical analysis was conducted with Tukey's test using JMP8 software (SAS, Cary, NC). For hydroponically grown lettuce or that grown in soil, experiments were replicated at least four times, with three lettuce plants in each replicate. For samples in the RH study, experiments were replicated eight times, and leaf samples were collected from one lettuce plant in each replicate. Statistical differences were considered when P values were <0.05.

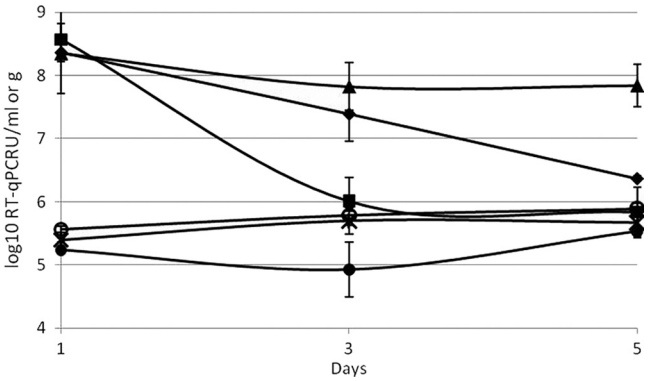

FIG. 1. MNV left in the soil or nutrient solution for 5 days of virus internalization under two conditions: one-time severe contamination (5×10^8 RT-qPCR U/ml MNV added into nutrient solution at day 1 and replaced with virus-free solution afterward) (hydroponic solution [◆], soil [▲], and nutrient solution for soil [■]) and low levels of constant contamination (5×10^5 RT-qPCR U/ml MNV added into nutrient solution every day) (hydroponic solution [X], soil [●], and nutrient solution for soil [○]).

RESULTS

Concentration of MNV in nutrient solution or soil after 5 days of virus challenge. Fresh nutrient solution (50 ml with or without added virus) was added to a container holding the hydroponic oasis cubes or the soil pots every day, and approximately 10 to 30 ml remained after 24 h, depending on evaporation rates, relative humidity, and environmental conditions within the green house. The remaining volume was collected for analysis of virus concentration and replaced with fresh nutrient solution, to the original volume of 50 ml. Soil samples were also collected to evaluate the virus associated with soil particles.

For the one-time severe contamination situation of hydroponically grown lettuce, ~7.4 and 6.4 log RT-qPCR U/ml MNV were detected in nutrient solution after 3 and 5 days, respectively. This was true even after the removal of the original solution containing 5×10^8 RT-qPCR U/ml MNV after 1 day, which was replaced with virus-free solution every day until day 5 (Fig. 1). In this situation, some of the original virus solution may have been held in the oasis cube and mixed with virus-free buffer over the 5 days, and thus the virus could not be completely removed but steadily reduced during 5 days. For soil challenged with the solution of 5×10^8 RT-qPCR U/ml MNV, ~8.3 log RT-qPCR U/g MNV was detected in soil after 1 day and had no significant reduction after 5 days (7.8 log) ($P < 0.05$). However, only ~6 log RT-qPCR U/ml MNV was found in nutrient buffer for the plants grown in soil after

TABLE 1. MNV viral genome detected in lettuce leaves grown hydroponically[a]

Day	Control	Log RT-qPCR U MNV (±SD)/50 mg lettuce sample (no. of positive samples/no. of total replicates) for:	
		5×10^8 MNV/ml	5×10^5 MNV/ml
1	ND	3.9 (±2.0) (2/5)	2.3 (±0.05) (2/6)
3	ND	3.8 (±1.4) (6/6)	2.3 (±0.5) (4/6)
5	ND	3.4 (±0.5)(4/6)	2.6 (±0.2) (2/6)

[a] ND, not detected. Detection limit is ~1.5 log RT-qPCR U MNV/50 mg lettuce tissue. Each replicate contained three lettuce plants.

TABLE 3. MNV viral genome detected in lettuce leaves of lettuce grown in soil[a]

Day	Control	Log RT-qPCR U MNV (±SD)/50 mg lettuce sample (no. of positive samples/no. of total replicates) for:	
		5×10^8 MNV/ml	5×10^5 MNV/ml
1	ND	2.0 (±0.1) (2/4)	ND (0/4)
3	ND	2.3[b] (1/4)	1.7[b] (1/4)
5	ND	2.0 (±0.05) (3/4)	2.4 (±0.5) (2/4)

[a] ND, not detected. Detection limit is ~1.5 log RT-qPCR U/50 mg lettuce tissue. Each replicate contained three lettuce plants.
[b] No standard deviation was obtained because one sample showed positive results.

both 3 and 5 days, which is significantly lower than the virus concentration in soil ($P < 0.05$). Thus, compared to plants grown hydroponically in oasis cubes, virus was likely to be adsorbed to soil particles, with a small portion of particles dissociated and suspended in nutrient buffer.

In the situation of low levels of constant contamination (soil or nutrient solution with fresh samples of 5×10^5 RT-qPCR U/ml virus every day), MNV concentration remained constant over the 5-day period and ~5 to 6 log RT-qPCR U/ml or U/g virus was detected in both nutrient solution and soil after 5 days (Fig. 1).

MNV internalization into lettuce grown hydroponically or in soil. For lettuce grown hydroponically, virus was detected in some replicates of leaf samples for both high and low MNV inoculums at days 1, 3, and 5 (Table 1). At days 3 and 5, lettuce challenged with 5×10^8 RT-qPCR U/ml MNV had significantly higher virus internalization than lettuce grown with 5×10^5 RT-qPCR U/ml MNV every day ($P < 0.05$). This may have resulted from (i) a higher MNV concentration in nutrient solution during the 5-day period (Fig. 1) and (ii) virus sustained in lettuce after uptake. Cell culture assays indicated that MNV internalized into lettuce leaves was still infectious when high virus inoculums were used (Table 2); however, the concentration of infectious MNV was significantly lower than that determined using qRT-PCR detection of the viral genome.

For lettuce grown in soil, approximately 1.7 to 2.4 log RT-qPCR U/50 mg MNV was detected in some leaf samples of lettuce grown at both high and low inoculums at days 1, 3, and 5, except for samples with 5×10^5 RT-qPCR U/ml at day 1 (Table 3); there was no significant difference detected concerning the concentrations of internalized viruses between lettuce plants grown at high and low inoculums at days 3 and 5. As mentioned above, virus particles may have been adsorbed to soil particles and therefore may be less accessible to uptake into lettuce leaf tissues than to the virus suspended in the nutrient buffer. Although approximately 8.7 RT-qPCR U/ml MNV was inoculated in the simulation of a one-time severe contamination event, ca. 6 log/ml was detected in the nutrient solution at days 3 and 5. This amount was similar to the concentration of virus in buffer in the situation of low levels of constant contamination, which may explain the similar internalizations at both inoculums. In cell culture infectivity assays, infectious MNV was detected in three replicates of lettuce samples grown in soil at high inoculums after 5 days, indicating a potential risk to food safety.

Effect of RH on MNV internalization into lettuce grown hydroponically. To evaluate the effect of transpiration on the uptake of human viruses, lettuce grown hydroponically was challenged with 1×10^8 RT-qPCR U/ml MNV under conditions of very high RH (99%) (to minimize transpiration) and low RH (70%) (to favor transpiration). The transpiration rate at 70% RH was approximately 10-fold that corresponding to 99% RH (Table 4), and only one out of eight lettuce samples showed positive internalization of MNV at 99% RH, with ~2.7 log RT-qPCR U/50 mg (Table 4). However, for plants grown at similar temperatures but at 70% RH, MNV was detected in seven out of eight lettuce samples, with ~2.6 log RT-qPCR U/50 mg observed. This indicated that transpiration may play an important role for virus uptake through the roots.

TABLE 2. Infectivity of MNV internalized into lettuce grown hydroponically or in soil

Day	PFU (±SD)/50 mg lettuce tissue of lettuce grown[a]:			
	Hydroponically		In soil	
	5×10^8 MNV/ml	5×10^5 MNV/ml	5×10^8 MNV/ml	5×10^5 MNV/ml
1	10 (±14)	0 (±0)	0 (±0)	ND
3	323 (±589)	0 (±0)	0[b]	0[b]
5	11 (±13)	0 (±0)	3 (±6)	0 (±0)

[a] The means presented are from the leaf samples in which virus was detected. ND, no infectivity data because no viral RNA was detected in lettuce leaves.
[b] No standard deviation was obtained because there was one replicate of sample.

TABLE 4. MNV internalization into hydroponically grown lettuce at two humidity levels (RH) for 24 h[a]

% RH	Control	Log RT-qPCR U MNV (±SD)/50 mg lettuce sample (no. of positive samples/no. of total replicates) for 1×10^8 MNV/ml	Transpiration rate (±SD) (g/cm^2/h)
99	ND	2.7 (1/8)	0.0031 (±0.002)
70	ND	2.6 (±0.09) (7/8)	0.032 (±0.009)

[a] ND, not detected. Detection limit is ~1.5 log RT-qPCR U/50 mg lettuce tissue. Each replicate contained one lettuce plant.

DISCUSSION

Internalization of virus by lettuce was observed in two irrigation water contamination situations. It was demonstrated that in a one-time severe contamination situation, virus could not be removed from the plants by the replacement of fresh buffer mimicking fresh water; instead, a large quantity of MNV particles were associated with the soil, and some virus particles remained suspended in the buffer. Thus, it is likely that even after a flood, viruses remaining in the soil may contaminate clean irrigation water and subsequently contaminate plants. As stated by the LGMA (California Leafy Green Products Handler Marketing Agreement) accepted food safety practices (13), fields in a flooded area should be left for 60 days before planting, or this may be shortened to 30 days with appropriate soil testing. However, further research and risk assessment are needed regarding the survival of virus in soil after a flooding incident, due to the likelihood of viral contamination of leafy greens through root internalization or plant surface contact and the potential for viral persistence inside/on plant tissue and eventually transmission to consumers. In a situation of low levels of constant contamination, virus internalization occurred >1 day later than that in the one-time severe contamination; however, this still suggests that continuous use of irrigation water with a low quantity of viruses could pose a risk of contamination. Thus, regular testing of irrigation water or groundwater for virus may help to reduce the risk of contamination; however, representative samples can be hard to obtain due to the inconsistent presence of virus in water (1).

Previous results are inconsistent among the studies that have been conducted assessing the impact of internalization of human enteric viruses or bacteriophage into produce during hydroponic or traditional growth conditions (4, 17, 21, 22). It was reported that <2 log PFU of bacteriophage f2/g plant tissue was detected in the shoots of hydroponically growing bean (*Phaseolus vulgaris* L.) challenged with $\sim 10^{10}$ PFU/ml f2 at roots (22). Calicivirus was occasionally detected in the edible parts of romaine lettuce grown hydroponically or in soil with total virus inoculums of 10^6 to 10^9 RT-qPCR U; in similar experiments using human norovirus, no virus was found in any plants, indicating that the frequency of contamination via roots was rare even when plants were exposed to high concentrations of virus (21). However, Chancellor et al. (4) showed 100% positive detection of hepatitis A virus RNA inside green onions grown in soil as well as hydroponically. Poliovirus was recovered from the leaves of tomatoes grown in soil injected with only 10^3 to 10^4 PFU/ml virus once every week (17).

The inconsistent results may be a result of (i) variant plant properties for virus penetration and uptake and (ii) different experimental parameters of produce growth, irrigation, and soil conditions, etc. Zhu et al. (26) evaluated the uptake and translocation of nanoparticles in plants and found that with a size of 0.02 to 2 μm (identical to or larger than the size of enteric viruses and bacteriophage) and with a slightly negative charge, the nanoparticles were detected and distributed in pumpkin stems and leaves. However, no uptake was observed with lima bean plants (*Phaseolus limensis*), indicating the various responses of plants to nanoparticles.

Water content and water movement in soil are important for viral contamination, as indicated by the increased number of viruses recovered from produce surfaces with an increase in soil moisture content (18). Low water content could increase virus particle attachment to soil-water interfaces, favor virus adsorption, and result in retention of virus movement in soil with a lesser access to plants (20). To favor the virus uptake, in this study saturated soil conditions were maintained with nutrient buffer during the entirety of the experiments. Nutrient solution was added to a container, and liquid moved up toward the soil through capillary force. This may explain in part why more virus internalization, especially with low inoculums, was observed in our study than in some of the previous research discussed above (17, 21). However, further experiments are needed to monitor soil conditions, soil water content, and virus movement to evaluate their effects on virus uptake by plants.

Transpiration is the driving force for water absorption, and the majority (96%) of water is taken up by the plant through transpiration (11). Humidity is a major factor controlling plant transpiration, and high humidity will reduce the diffusion of water out of the leaf and lower the transpiration rate, and transpiration will cease if humidity reaches 100% (5, 19). Transpiration was assessed in this study to identify a potential role in the movement of viruses into the plant tissue. In this study, lettuce grown under conditions of 99% humidity in order to minimize transpiration showed a significantly lower frequency of virus internalization than lettuce grown under conditions of a 70% RH chamber which had a 10-fold-higher transpiration rate. Thus, transpiration is likely to be a major force for virus uptake through roots.

In conclusion, MNV was taken up by romaine lettuce through the roots via contaminated irrigation water and reached edible leaf tissue. The internalization of human enteric viruses into produce during irrigation is possible under favorable conditions, and the fact that some internalized virus remained infectious poses a threat to food safety. Furthermore, the virus may be taken up in a passive manner by transpiration. The exact method of virus internalization under different environmental conditions, such as soil water content and environmental relative humidity, is still unclear.

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Manure- and Biosolids-Resident Murine Norovirus 1 Attachment to and Internalization by Romaine Lettuce

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Manure- and Biosolids-Resident Murine Norovirus 1 Attachment to and Internalization by Romaine Lettuce▽

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The attachment of murine norovirus 1 (MNV) in biosolids, swine manure, and dairy manure to Romaine lettuce and internalization of this virus were evaluated. The MNV in animal manures had behavior similar to that of pure MNV; however, MNV in biosolids had significantly higher levels of attachment and internalization than pure MNV or MNV in manures. The incubation time did not affect the attachment of MNV in biosolids or manure. Confocal microscopy was used to observe MNV on lettuce after SYBR gold-labeled MNV was added directly to lettuce or after lettuce was submersed in labeled virus. MNV was observed on the lettuce surface, inside open cuts, and occasionally within stomata. In general, lettuce pieces with a long cut on the edge and short cuts on the stem was more likely to contain internalized MNV than intact lettuce pieces, as observed by confocal microscopy; however, while the difference was visible, it was not statistically significant. This study showed that the presence of MNV in biosolids may increase the risk of fresh produce contamination and that the MNV in open cuts and stomata is likely to be protected from sanitization.

Noroviruses (NoVs) are leading food-borne pathogens, accounting for over 60% of food-borne disease in the United States (15). They are the most common cause of nonbacterial gastroenteritis, and an estimated 23 million cases occur annually in the United States. NoVs are prevalent in the environment and can be found in waste treatment plant influent and effluent (7), biosolids (4), and animal feces (14). Due to these facts, the use of biosolids and animal manure on agricultural land may disseminate human pathogens in the environment and subsequently increase the chance of crop contamination (22). Recently, increasing outbreaks of NoV infection have been associated with salads and vegetables (9, 11, 13). Fresh produce could be contaminated from preharvest to postharvest at any point in the chain of production, and one of the major routes with a high likelihood of contamination is the use of contaminated water for irrigation and washing (12). Water could be contaminated by the use of biosolids and manure as organic fertilizer on United States farms or by runoff from animal production zones close to produce fields. Changes in processing, including more cutting and coring performed in the field during harvest, also increase the potential risk of microbial contamination. Furthermore, postharvest sanitizing regimens used by industry have a limited effect on the removal or inactivation of enteric viruses on lettuce (1), and since it only takes a few infectious particles to cause an infection, consumption of fresh produce continues to be a public health risk.

Intensive studies of the behavior of bacteria such as *Escherichia coli*, *Pseudomonas*, and *Salmonella* on fresh produce have been conducted (2, 3, 16, 17), but little work has been conducted with viruses. It has been reported that *E. coli* and *Pseudomonas* can grow on lettuce surfaces (17). While *Pseudomonas* tended to adhere to intact leaf surfaces, *E. coli* cells were entrapped in stomata and preferentially penetrated through the cut edge, which protected them from disinfection by chlorine treatment. For *Salmonella enterica* serovar Typhimurium, attachment preferentially occurred at the plant cell wall junction, suggesting that there might be a receptor site at this location for bacterial attachment (16). Virus adsorption to lettuce has also been found to vary depending on the strain and surface properties of the virus. Feline calicivirus (FCV) had a higher level of attachment to lettuce when the pH was above its isoelectric point (pI), while for bacteriophage MS2, strong adsorption to lettuce was observed at a value below its pI (21). As viruses are small particles that most likely are associated with feces when they are present in biosolids or animal manure, it is important to understand the mechanism of their attachment to and internalization by leafy greens if biosolids or manure is used in vegetable production. The objective of this study was to evaluate murine norovirus 1 (MNV), a widely used surrogate for human NoV, to determine its adsorption to and internalization by lettuce after the virus was stored in manure or biosolids for up to 30 days, and confocal microscopy was also used to observe virus on lettuce.

MATERIALS AND METHODS

Viruses. MNV-1.CW1 was propagated in the RAW 264.7 cell line cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Invitrogen, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% glutamine, 1% HEPES buffer, and 1% glutamate (25). Virus-infected cell lysates were purified by three freeze-thaw cycles, and the supernatant was recovered after centrifugation at 2,500 × *g* for 15 min and stored at −80°C for further study. A plaque assay was conducted as described previously (25). In brief, virus was diluted and inoculated onto confluent monolayers of RAW 264.7 cells grown in 12-well plates, and after 2 h of agitation at 37°C, the inoculum was aspirated and the cells were overlaid with 1 ml of 1.5% SeaPlaque agarose in 2× DMEM containing 2% FBS. The plates were incubated at 37°C with 5% CO_2 for 48 h, and plaques were visualized by staining with 0.5 ml complete minimum essential medium Eagle containing 0.5% neutral red per well for 6 to 8 h.

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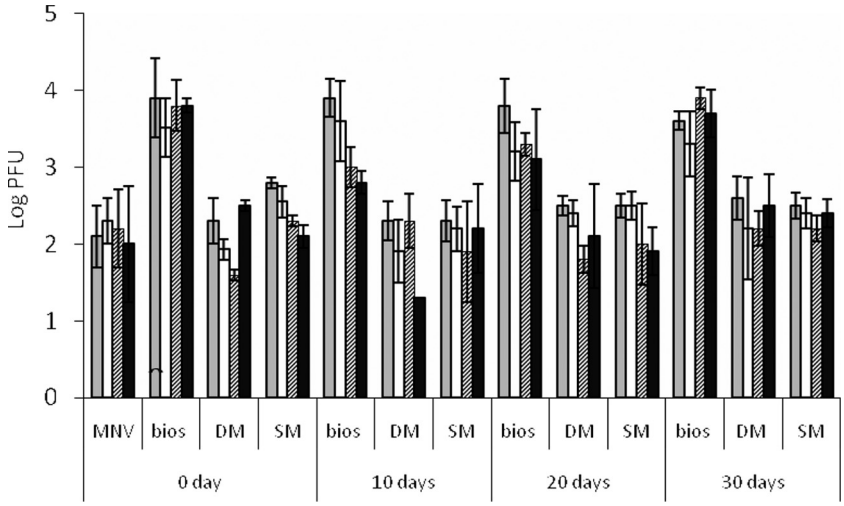

FIG. 1. Quantity of virus on lettuce after lettuce pieces were agitated in a pure MNV solution or a biosolids, SM, or DM suspension (after manure or biosolids were incubated for 0, 10, 20, and 30 days at 20°C) and virus stability after lettuce pieces were removed from the virus solution or manure suspension and incubated at 4°C for up to 24 h. The incubation times were 0.5 h (gray bars), 6 h (open bars), 12 h (bars with diagonal lines), and 24 h (black bars). bios, biosolids.

Biosolids and animal manure. Biosolids were obtained from the Back River Wastewater Treatment Plant (WWTP) (Baltimore). Solid swine manure (SM) was collected from a local farm in Kent County, DE. Liquid dairy manure (DM) was collected from the University of Delaware College of Agriculture and Natural Resources farm. Manure and biosolids were autoclaved and stored at 4°C before use.

Attachment of MNV in biosolids or animal manure to lettuce. Romaine lettuce was purchased from a local supermarket (Newark, DE) and cut into pieces that were 1 by 1 cm. Four pieces were submerged in 5 ml of a suspension containing pure virus ($\sim 2 \times 10^5$ PFU/ml) and agitated for 5 min. The lettuce pieces were then removed from the viral suspension and incubated at 4°C for 0.5, 6, 12, and 24 h. To analyze attachment, lettuce pieces were vortexed with 5 ml Hanks' balanced salt solution (HBSS) for 10 min, and the concentration of attached MNV was determined by the plaque assay as described above. For liquid DM, MNV was inoculated into 5 ml of a manure suspension containing $\sim 2 \times 10^5$ PFU/ml (final concentration), and the MNV-contaminated DM suspension was then agitated with lettuce pieces. For biosolids and SM, 1 ml of an MNV suspension was added to 2 g biosolids or manure, dried for 15 min in a biosafety cabinet, vortexed for 30 min with 10 ml Na_2HPO_4 (0.15 M, pH 9.5), and diluted with HBSS to obtain a final concentration of $\sim 2 \times 10^5$ PFU/ml. Lettuce pieces were submerged in diluted biosolids or SM samples and incubated as described above. To study whether the survival of virus in manure or biosolids affected their attachment to lettuce, MNV was also incubated in biosolids, SM, and DM for 10, 20, and 30 days at 20°C, after which solid manure and biosolids were diluted as described above for the attachment study.

Internalization of MNV in biosolids or manure by lettuce. Intact whole pieces of Romaine lettuce were dipped into MNV (~ 25 cm^2 of leaf was submerged in a virus solution) and incubated for 5 min. Each lettuce piece was removed and incubated at 4°C for 30 min before it was analyzed for virus attachment as described above. Similar lettuce pieces were cut so that they had an approximately 10-cm cut at the leaf edge and two 2-cm cuts at the stem, and they were analyzed in the same manner. To differentiate possible internalization from attachment, lettuce pieces were wiped with 1% Virkon for 3 min using a Q-tip to inactivate attached viruses but not internalized viruses. The internalized viruses were then recovered by vortexing in HBSS for 10 min, and the virus titer was determined by the plaque assay. The internalization ratio was calculated as follows: (quantity of MNV recovered from Virkon-wiped lettuce)/(quantity of MNV recovered after lettuce was removed from virus suspension) $\times$ 100. MNV in biosolids, SM, and DM on day zero were also used in the viral internalization study.

Virus particle purification. Cesium chloride (CsCl)-purified MNV was used for confocal microscopy analysis of virus on lettuce. Purification of virus particles was performed as described previously (25), with little modification. RAW 264.7 cells were infected with MNV and incubated for 48 h; next, cellular debris was removed by three cycles of freezing and thawing and centrifugation at 2,500 $\times$ *g* for 15 min. The supernatant was layered on top of 3 ml 30% sucrose and centrifuged at 90,000 $\times$ *g* for 3 h using a Sorvall WX ultracentrifuge (Thermo Scientific, NC). The debris was then washed with phosphate-buffered saline (PBS), mixed with CsCl to obtain a final density of ~ 1.336 g/cm^3, and centrifuged at 115,000 $\times$ *g* for 22 h using the Sorvall WX ultracentrifuge (Thermo Scientific, NC). The gradient was fractionated, and the density of each fraction was determined to locate the virus particles. The density of MNV was 1.36 ± 0.04 g/cm^3 (25). RAW 264.7 cells not infected with MNV were purified in the same way and used as a control.

Virus staining with SYBR gold. The original SYBR gold stock solution (Invitrogen, CA) was diluted 1:1,250 with PBS, mixed with CsCl-purified MNV ($\sim 1.5 \times 10^8$ PFU/ml) or a control solution at a 1:1 ratio, and agitated in the dark for 30 min. The SYBR gold-labeled MNV was then transferred to 100,000-molecular-weight Microcon centrifugal filter devices (Millipore, MA) and washed with PBS three times using centrifugation at 10,000 $\times$ *g* for $\sim$5 min. The MNV was then recovered from the membrane in PBS. Lettuce was cut into pieces that were 1 by 1 cm or 1 by 0.2 cm, added to 0.5 ml of a SYBR gold-labeled MNV suspension or a control solution, and agitated in the dark for 5 min, or 100 μl of an MNV suspension or a control solution was directly pipetted onto a lettuce piece that was 1 by 1 cm. The lettuce samples were analyzed by confocal light microscopy as described below.

Confocal light microscopy. Confocal images were acquired with a Zeiss LSM 510 NLO laser scanning microscope (Carl Zeiss, Inc., Germany) using a Zeiss 40× C-Apochromat (1.2NA) water immersion objective lens. Multichannel images of SYBR gold fluorescence and autofluorescence were acquired in fastline-switch mode using the 488-nm laser line of a 25-mW argon laser (LASOS, Ebersberg, Germany) and 543-nm helium neon laser lines (LASOS) with a 560 long-pass emission filter. The SYBR gold fluorescence was green, and the plant autofluorescence was red. The confocal images were captured either as two-dimensional single optical sections or as three-dimensional Z stack optical sections.

Statistical analysis. All experiments were performed with three replicates. The statistical analysis was conducted using an analysis of variance single-factor test with Office 2007 software to assess the significance of variations. Data were considered to be statistically significantly different if the *P* value was <0.05.

RESULTS AND DISCUSSION

Attachment of MNV in biosolids or animal manure to lettuce. For pure virus or MNV in animal manure at day zero, $\sim$2 to 2.5 log PFU MNV attached to the lettuce pieces, while for MNV in biosolids, $\sim$4 log PFU virus attached, a value that was significantly higher than the values for the other three samples

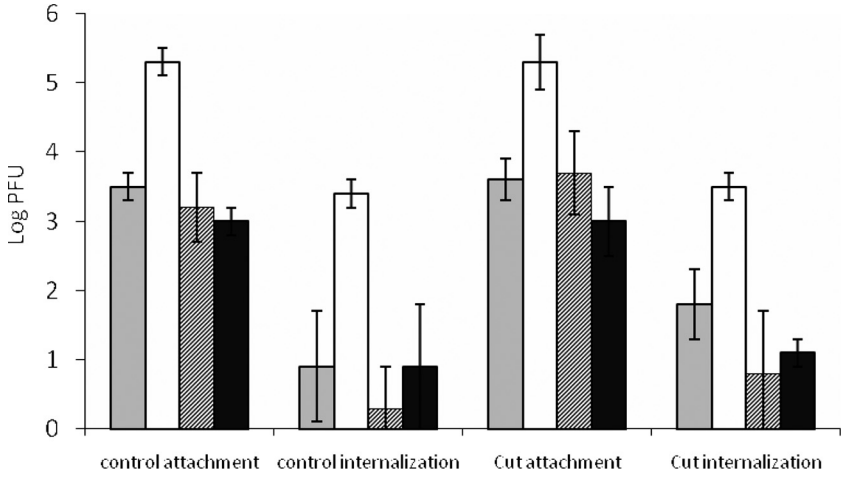

FIG. 2. Internalization of pure MNV or MNV in biosolids, DM, or SM by lettuce. Intact lettuce pieces or lettuce pieces with a long cut on the edge and short cuts on the stem were submerged in a manure suspension for 5 min. Each lettuce piece was then wiped with 1% Virkon to eliminate the attached viruses but not the internalized viruses. Gray bars, pure virus; open bars, MNV in biosolids; bars with diagonal lines, MNV in DM; black bars, MNV in SM.

(Fig. 1). For storage in manure or biosolids, MNV was stable in the biosolids and SM, as there was no loss of infectious virus after 30 days of incubation at 20°C. For DM, there was a 1-log loss at 10 days, and then the infectivity titer remained at ~4 log PFU/ml at 20 and 30 days. For MNV in SM and DM stored for 10, 20, and 30 days, ~2 log PFU MNV was attached to the lettuce in all samples. For MNV in biosolids at all incubation times, ~4 log PFU MNV was attached, and there was no significant difference from attachment at day zero (Fig. 1). These findings provide evidence that the length of incubation of biosolids or manure does not affect the attachment of virus to lettuce, as long as the virus remains infective during storage. After attachment to the lettuce, MNV was quite stable for both pure virus samples and all of the biosolids and manure samples, and no significant loss of infectious virus was observed after incubation for 24 h at 4°C (Fig. 1). While it is not known exactly why the biosolids enhanced MNV attachment to lettuce, $FeCl_3$ is added to the Back River WWTP biosolids for phosphate control and the biosolids contain a significantly larger amount of iron than DM and SM (19). The presence of iron oxides has been shown to improve adsorption of virus (MS2 and ϕX174) to sand particles (26), and it was possible that more MNV particles aggregated on biosolids than on DM and SM and thus were concentrated on biosolids particles, which led to increasing virus attachment to lettuce.

Internalization of MNV in biosolids or manure by lettuce. Virkon was used to inactivate attached MNV but not internalized MNV, and our preliminary study showed that wiping lettuce with 1% Vikon for 3 min could inactivate >4 log MNV/cm^2 (23). For both control (noncut) and cut lettuce samples, ~3 log PFU of virus was attached for pure MNV or MNV in SM and DM, while for biosolids, ~5 log PFU was attached (Fig. 2). For MNV in biosolids ~3 log PFU of virus was internalized by both control and cut lettuce pieces, a value which was significantly higher than the value for pure MNV or MNV in DM or SM (~1 log PFU). However, there was no significant difference in the internalization ratio between intact and cut lettuce pieces dipped in the same MNV sample ($P < 0.05$); also, there was no significant difference in the internalization ratios for all four MNV samples (Table 1). This indicates that the significantly higher level of internalization of MNV in biosolids resulted from the large number of attached virus particles. There were no significant differences in either attachment or internalization between control and cut lettuce pieces for all four MNV samples, implying that MNV may be internalized by lettuce through some mechanism other than entry through open cuts. However, the level of internalization of pure MNV was a bit higher for cut lettuce based on both raw data and confocal microscopy, and the lack of statistical differences between cut and noncut samples could also have resulted from variations due to virus behavior (8).

Observation of virus on lettuce by confocal microscopy. SYBR gold is a sensitive fluorescent dye for detecting double- or single-stranded DNA or RNA, and it has been widely used to enumerate viral particles collected from natural seawater with epifluorescence microscopy (6, 18, 24). However, since our virus samples were obtained from cell lysates, cell debris could also bind to the SYBR gold dye and emit fluorescent signals. Purification of the virus with CsCl greatly reduced the contamination from cellular debris, as few fluorescent dots were observed with the control, and the control sample was

TABLE 1. Internalization ratios for pure MNV and MNV in biosolids or manure for lettuce pieces

Virus	Internalization ratio[a]	
	Control (intact lettuce)	Lettuce with cuts
Pure virus	0.5 ± 0.5	3.0 ± 2.9
MNV in biosolids	0.1 ± 0.1	0.65 ± 1.0
MNV in SM	1.7 ± 1.8	1.5 ± 1.2
MNV in DM	1.7 ± 1.8	1.9 ± 1.1

[a] For each sample, the mean and standard deviation were calculated based on the results for three replicates. The internalization ratio was calculated as follows: (quantity of MNV recovered from Virkon-wiped lettuce)/(quantity of MNV recovered after lettuce was removed from virus suspension) × 100.

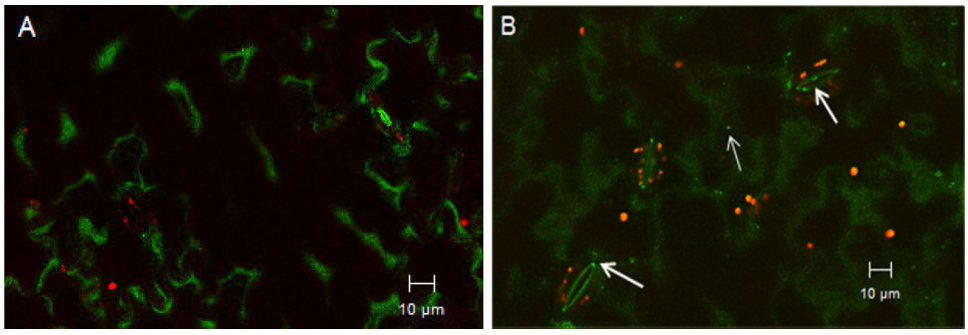

FIG. 3. Confocal microscopy images of control (A) and MNV attached to Romaine lettuce (B) after 100-μl drops were pipetted on lettuce leaves. The arrows indicate MNV on the lettuce surface or inside stomata. Green indicates plant cell walls, and red indicates autofluorescence from plant chlorophyll.

significantly different from purified virus samples (data not shown). We observed that SYBR gold-labeled viruses were attached to lettuce surfaces after virus was pipetted directly onto lettuce or after lettuce was agitated in virus suspensions (Fig. 3 and 4). Furthermore, with both treatments, viral particles were occasionally seen inside stomata (~1 to 2 μm inside stomata), suggesting that viruses could internalize through the guard cells in lettuce. Viruses were also observed on the cut edges of lettuce pieces; however, from the front view of a lettuce surface, it was difficult to differentiate whether the viruses were on the surface or inside the cut edge (Fig. 4D). Front views of the cut edges showed that viruses were inside the cut (under the epidermis) about 3.5 μm from the cut edge (Fig. 5), which could be protected from washing and sanitization. With control samples, no viral particles were observed on lettuce surfaces or inside the cut edges. As viruses could internalize in lettuce through both the stomata and the cut edges, these observations may explain why cut samples had more MNV particles but there was no statistical difference in internalization compared to noncut lettuce, as shown in Fig. 2.

It was reported previously that stomata and damaged areas or cuts were important in protecting food-borne microorganisms such as *E. coli* from different sanitizers (10, 17, 20). After submersion of a leaf in an *E. coli* suspension, *E. coli* cells were found in most stomata without penetration (10, 17). Compared with intact surfaces, *E. coli* also seemed to preferentially attach to cut edges, and during a 24-h incubation period, *E. coli* was found to penetrate cut edges, while little penetration was observed for bacteria on intact surfaces (17). Since viruses are different from bacteria and are considered nonliving when they

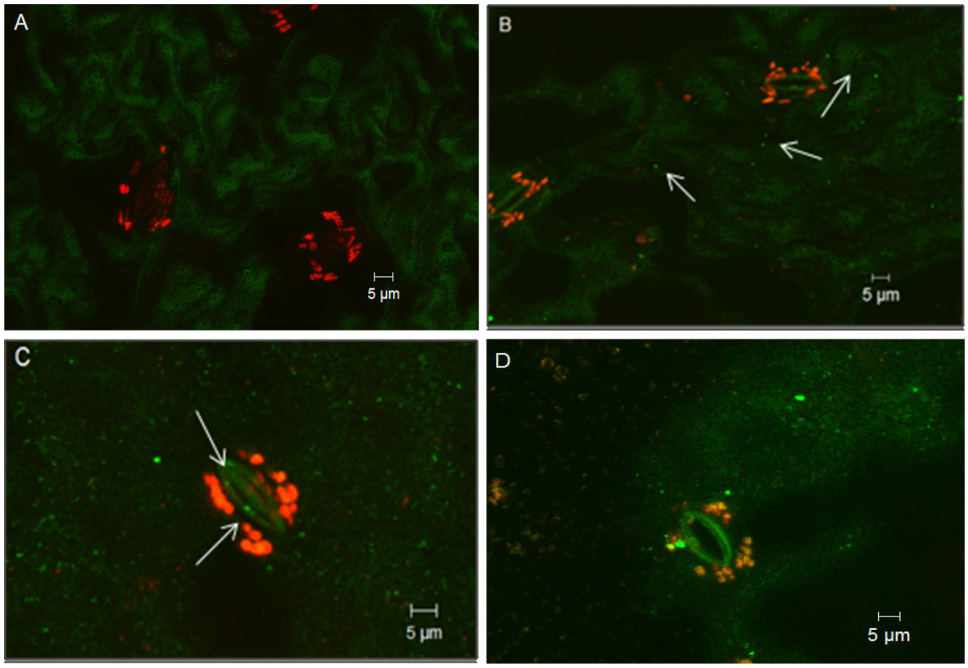

FIG. 4. Confocal three-dimensional stack images of lettuce pieces agitated in a control solution (A) and an MNV suspension (B) for 5 min. (C and D) Virus in stoma (C) and attached to a cut edge (D) after 5 min of agitation. In panel D, the green fluorescence in the middle indicates the cut edge, and the red fluorescence on the left indicates cellular leakage. The arrows indicate MNV on lettuce surfaces (B) or in stomata (C).

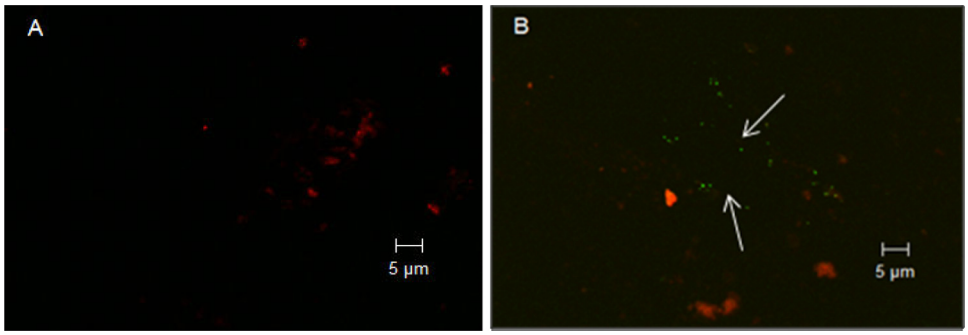

FIG. 5. Confocal microscopy images of front views of cut edges after lettuce was agitated in a control solution (A) and in an MNV suspension (B). MNV is indicated by arrows. The virus in panel B was ~3.5 μm inside the cut edge.

are outside their hosts, the attachment of viruses to stomata or to a cut edge is a matter of probability rather than preference. As shown in our study, viruses were found on lettuce surfaces but only occasionally in stomata, while *E. coli* preferred to gather inside the stomata (17). The major driving force behind virus attachment should be physicochemical forces that control the interactions between viruses and plant surfaces. But little is known about such interactions, and different viruses and phages have exhibited viable attachment patterns (21). Since enteric viruses are frequently associated with feces and biosolids in the environment, information on interactions between solids, viruses, and leaf surfaces can contribute to reliable methods that prevent attachment or remove attached viral particles.

In conclusion, this study showed that biosolids could promote the attachment of MNV to lettuce and resulted in an increased number of virus internalized in lettuce, which may pose a food safety risk. Also, it was found that MNV, like bacteria (17), could internalize in lettuce through cut edges as well as stomata. Since the infectious dose of human norovirus is as low as <100 particles (5), the viruses that escape from sanitization during washing due to protection by stomata or cut edges could pose a threat to food safety as well as to human health.

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Presence and Persistence of Salmonella enterica Serotype Typhimurium in the Phyllosphere and Rhizosphere of Spray-Irrigated Parsley

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Salmonella enterica **is one of the major food-borne pathogens associated with ready-to-eat fresh foods. Although polluted water might be a significant source of contamination in the field, factors that influence the transfer of *Salmonella* from water to the crops are not well understood, especially under conditions of low pathogen levels in water. The aim of this study was to investigate the short- and long-term (1 h to 28 days) persistence of *Salmonella enterica* serotype Typhimurium in the phyllosphere and the rhizosphere of parsley following spray irrigation with contaminated water. Plate counting and quantitative real-time PCR (qRT-PCR)-based methods were implemented for the quantification. By applying qRT-PCR with enrichment, we were able to show that even irrigation with water containing as little as ~300 CFU/ml resulted in the persistence of *S.* Typhimurium on the plants for 48 h. Irrigation with water containing 8.5 log CFU/ml resulted in persistence of the bacteria in the phyllosphere and the rhizosphere for at least 4 weeks, but the population steadily declined with a major reduction in bacterial counts, of ~2 log CFU/g, during the first 2 days. Higher levels of *Salmonella* were detected in the phyllosphere when plants were irrigated during the night compared to irrigation during the morning and during winter compared to the other seasons. Further elucidation of the mechanisms underlying the transfer of *Salmonella* from contaminated water to crops, as well as its persistence over time, will enable the implementation of effective irrigation and control strategies.**

Over the past few decades, fresh fruits and vegetables, and in particular leafy greens, sprouts, and herbs, have been increasingly recognized as significant reservoirs of food-borne pathogens (10, 11, 22). Such ready-to-eat foods are consumed raw with minimal or no processing aimed to destroy human pathogens. Hence, the presence of food-borne pathogens at the time of consumption hides potential health risks (10, 43). In the United States, for instance, the proportion of outbreaks associated with fresh produce, out of all reported food-borne outbreaks with an identified food source, has increased from 0.7% in the 1970s to 6% in the 1990s (51), to 13% in the 2000s (22), and to 33% in 2011 (15). *Salmonella enterica* is one of the most commonly identified food-borne pathogens associated with fresh produce. Between 2006 and 2008 *S. enterica* was the confirmed etiologic agent in 31 of 170 produce-related outbreaks reported to the Centers for Disease Control and Prevention (CDC) (16). It was also reported that ca. 16.5% of total *Salmonella* cases of illness and death in the United States in 2009 were associated with fresh produce (8). Recent surveys performed in different countries to investigate the occurrence of *Salmonella* spp. in fresh herbs and leafy vegetables identified *Salmonella* in up to 28% of the samples, prior to consumption (reviewed in reference 63). Since herbs and leafy vegetables are frequently traded internationally and are used as ingredients in many ways year-round, contaminated products can cause outbreaks with a wide geographic distribution (53).

The increase in reported food-borne illness cases linked to fresh produce can be attributed to increased consumption of ready-to-eat foods, the appearance of new minimally processed ready to eat products, globalization of the produce industry, and more effective surveillance (11, 43). In addition, recent evidence obtained by food microbiologists and plant pathologists supports the hypothesis that enteric pathogens have adapted to persist on or in plants as part of their natural life cycle between infecting hosts (57). It has been indicated that the connections between enteric pathogens such as *Salmonella* and plants may be more complicated than simple passive transfer, because the bacteria can attach, survive, and even colonize and grow on and in plants by affecting their immune defense response (5, 6, 18, 28, 32, 37, 39, 50).

Contamination of raw fruits and vegetables by human pathogens takes place along the "farm-to-fork" food production chain (13). For many years, water has been a *Salmonella* carrier (34) and, in fact, introducing *Salmonella* to leafy vegetables through irrigation water can be a major way of contamination (39, 50). However, little is known about environmental parameters that influence the rates of such transfer, especially under low levels of pathogens in irrigation water. Furthermore, there have been relatively few contradictory studies with regard to the survival of *Salmonella* and other enteric pathogens in the phyllosphere and the rhizosphere over extended periods (61). Therefore, the aim of the present study was to investigate the short-term (up to 2 days) and long-term (up to 4 weeks) persistence of *S. enterica* serotype Typhimurium in the phyllosphere and the rhizosphere of parsley plants following spray irrigation with contaminated water during the day and night and during different seasons.

MATERIALS AND METHODS

Bacterial strains and preparation of bacterial suspension. *S. enterica* serotype Typhimurium ATCC 14028 (*S.* Typhimurium) was used for

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contamination. *S.* Typhimurium is one of the most predominant *Salmonella* serotypes found in developed countries. It was the causative agent in food-borne outbreaks associated with different fruit and vegetables worldwide (17, 20) and has been detected in herbs and dried spices (48). *Salmonella* cells were transformed with pGFP plasmid (Clontech) by electroporation using a MicroPulser electroporator (Bio-Rad Laboratories), to obtain green fluorescent protein (GFP)-labeled cells. Transformants were selected by plating onto Luria-Bertani (LB) agar plates supplemented with ampicillin (100 μg/ml), and stored at −80°C in LB medium supplemented with 20% glycerol. The stability of GFP expression by *S.* Typhimurium ATCC 14028 on parsley leaves has been previously investigated and described (39). For contamination experiments, *S.* Typhimurium was cultured overnight in LB broth supplemented with ampicillin (100 μg/ml) at 37°C with aeration. Cells were harvested by centrifugation (4,000 × g for 20 min at 4°C), washed, and resuspended in sterile saline (0.85% NaCl). The culture was diluted to prepare 150-ml bacterial suspensions at concentrations ranging from ~10^1 to ~10^8 CFU/ml.

Growth of parsley plants. Parsley plants (*Petroselinum crispum* var. *neapolitanum*) were grown in a glass greenhouse (3 by 6 by 2.5 m). Portions (2 g) of parsley seeds (~800 seeds) were disseminated in each planter containing ~12 liters of commercial nonsterile potting soil (Avital 11; Tuff Marom Golan, Marom Golan, Israel). Planters were automatically watered with tap water using drip irrigation. The amount of irrigation water was designed following a pilot soil capacity experiment aimed to keep the soil in the planter wet with minimal excess of water (39). The greenhouse has hatches to adapt the indoor conditions (temperature and humidity) to the seasonal weather changes in Haifa. Temperature and relative humidity in the greenhouse were automatically logged by a data logger (D-logMateTHD; MRC). At 10 to 12 weeks after dissemination, which is the common period of time for the seed to develop to a harvestable plant (3), parsley plants were used for irrigation assays. Preliminary experiments showed that plants of this age (and up to 4 weeks afterward) provide enough mass for sampling and processing.

Irrigation procedure with contaminated water. Each planter was manually spray irrigated by spraying onto the phyllosphere three times during a 9-day period (3-day intervals) with 150 ml of freshly prepared contaminated water. Irrigation was performed by a hand sprayer from a distance of ~20 cm from the phyllosphere. Each planter was spray irrigated by water harboring GFP-expressing *S.* Typhimurium at different bacterial concentrations, ranging from ~10^1 to ~10^8 CFU/ml. Day irrigations were conducted during late-morning hours, 10 to 11 a.m. Night irrigations were conducted between 10 to 11 p.m. To study the impact of seasonality on contamination, day irrigations were conducted in different seasons of the year. For safety reasons, the irrigation of each planter was conducted inside a glove box, from which the plant was removed 1 h after irrigation. In each experiment the plants in the control planters were grown and treated in a similar way using *Salmonella*-free water.

Sample collection and recovery of GFP-expressing *S.* Typhimurium for plate counting. After the irrigation challenge, samples of leaves (20 g), stalks (20 g), and roots (10 g) were aseptically collected from each planter. Soil samples (20 g) were collected from 1 to 5 cm below the surface with a sterile spoon. Sampling was conducted in triplicates 48 h after the last irrigation step. For the study of persistence of *S.* Typhimurium in the plant environment, samples were collected at different time points (1 h, 10 h, 1 day, 2 days, 7 days, 2 weeks, and 4 weeks) after the last irrigation step. All samples were placed in sterile stomacher bags, brought immediately to the laboratory, and stored at 4°C until processing. For the recovery of plant- and soil-associated GFP-expressing *S.* Typhimurium, 180 ml of sterile saline was added to each leaf and stalk sample, and the samples were pummeled in a stomacher for 3 min at normal speed (620 paddle strokes per min). Root samples were washed three times by adding 100 ml of sterile saline and agitation for 15 s in order to wash off soil particles from the roots, after which 90 ml of saline was added, and the samples were pummeled in a stomacher. Soil samples were added with 180 ml of sterile saline and vigorously agitated by hand for 30 s. Serial dilutions (1:10 in saline) were prepared and plated for the enumeration of fluorescent colonies of *S.* Typhimurium on LB agar supplemented with ampicillin. The limit of detection for plating was 2 log CFU/g. Dilutions and plating for each sample were conducted in duplicates.

Surface disinfection of leaves. Samples of parsley leaves were dipped in 80% ethanol for 10 s, followed by immersion in 0.1% $HgCl_2$ (wt/vol) for 10 min (55). Leaves were washed in sterile saline twice and processed for the recovery of internal *S.* Typhimurium by viable counting on LB plates, as described above.

Sample collection and enumeration by qRT-PCR without enrichment. Five leaves (each about 0.1 g) were aseptically collected from each planter and processed for the quantification of leaf-associated *S.* Typhimurium by quantitative real-time PCR (qRT-PCR) as described recently (36) with some modifications. In the laboratory setting of our previous study, we utilized primers targeting the *rrn* gene for the detection of *S.* Typhimurium inoculated on leaves. However, the use of these primers in a greenhouse study occasionally resulted in false-positive detection, possibly due to the presence of indigenous bacteria or soil bacteria with homologous *rrn* sequences. Thus, we decided to modify the protocol and to target the *Salmonella*-specific gene *sirA* (2). Briefly, each individual leaf was immersed in liquid nitrogen for 10 min in an Eppendorf tube. Samples were ground to a fine powder (for 2 min) on liquid nitrogen. Total bacterial DNA was extracted using ZR soil microbe DNA kit (Zymo Research) according to the manufacturer's instructions in a final volume of 50 μl. qRT-PCR analysis was performed using 2 μl of each isolated DNA sample, 175- and 250-nmol/liter concentrations of forward (ACTCGCG TTCAGACAAACTG) and reverse (CGCTATTCGTTCGGTGTA) primers, respectively, targeting the *sirA* gene, and 5 μl of ABsolute QPCR SYBR green mix (ABgene) in a 10-μl total reaction volume. A three-step protocol was used in a Rotor-Gene 3000 (Corbett Research): (i) denaturation (15 min at 95°C), (ii) an amplification and extension program repeated 50 times (1 s at 95°C, 15 s at 57°C, and 20 s at 72°C), and (iii) a melting-curve program of heating from 72 to 99°C, at a heating rate of 1°C per 5 s. The concentration of experimental samples was calculated from the linear regression of a standard curve, obtained by purified DNA (isolated by the same procedure) from bacterial suspensions at known concentrations (5×10^1 to 5×10^5 CFU/ml).

Detection of *S.* Typhimurium by RT-PCR after enrichment. Samples of leaves (10 g) were aseptically collected from each planter and placed in sterile stomacher bags as described above. Portions (50 ml) of buffered peptone water were added, and the samples were incubated at 37°C. After 5 and 20 h of incubation, 1 ml of the enrichment medium was collected. The enriched cultures were pelleted by centrifugation, washed in sterile saline, and repelleted. Total bacterial DNA was extracted using a ZR soil microbe DNA kit, and qRT-PCR analysis was performed as described above.

Statistics. Unless mentioned specifically, all experiments were conducted at least twice. Sampling was conducted in triplicates. The data were analyzed using InStat 3.10 (GraphPad Software, Inc.) by one-way analysis of variance and Tukey-Kramer test. A P value of < 0.05 was accepted as indicating significance.

RESULTS

Recovery of *S.* Typhimurium from parsley and soil following spray irrigation with water containing different concentrations of the pathogen. Day irrigation of parsley plants grown in a greenhouse with water carrying the human pathogen *S.* Typhimurium resulted in the contamination of leaves, stalks, and roots, as well as the soil in which they were grown. *S.* Typhimurium was not detected by plate counting in any of the samples after irrigations with water harboring <4.5 log CFU of the pathogen/ml, but above this concentration the level of water contamination was correlated with the levels of the pathogen's abundance detected in the phyllosphere, the rhizosphere, and the soil (Fig. 1). The contaminated

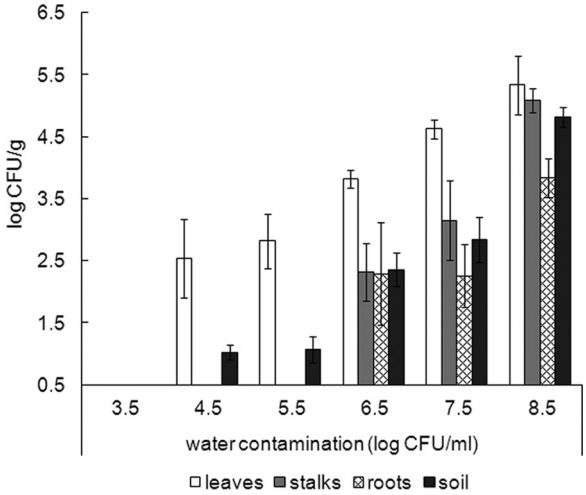


FIG 1 *S.* Typhimurium recovered from parsley phyllosphere, rhizosphere, and soil 48 h after spray irrigations during morning hours (10 to 11 a.m.) with water harboring *S.* Typhimurium at levels of 3.5 to 8.5 log CFU/ml. After recovery of the bacteria, GFP-tagged *S.* Typhimurium was enumerated by plating. The results are the averages of at least two independent repeats analyzed in triplicates. The experiments were conducted in June and July.

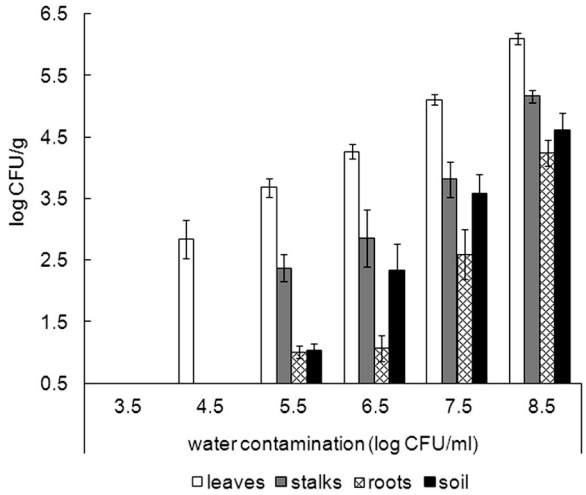


FIG 2 *S.* Typhimurium recovered from parsley phyllosphere, rhizosphere, and soil 48 h following spray irrigation during night hours (10 to 11 PM) with water harboring *S.* Typhimurium at levels of 3.5 to 8.5 log CFU/ml. After recovery of the bacteria, GFP-tagged *S.* Typhimurium was enumerated by plating. The results are the averages of at least two independent repeats analyzed in triplicates. The experiments were conducted in June and July.

water was sprayed directly toward the plant phyllosphere, and consequently the leaves were the most susceptible spheres to contamination. The pathogen was readily detected by plate counting following irrigation with heavily contaminated water (i.e., 6.5 to 8.5 log CFU/ml), while irrigation with water harboring intermediate levels of *S.* Typhimurium (i.e., 4.5 to 5.5 log CFU/ml) resulted in detection of the pathogen only in parsley leaves and in some but not all of the soil samples. At 48 h after irrigation, the levels of the culturable bacteria on the leaves were ca. 2 to 3 log lower than the respective concentration of the pathogen in the irrigation water (Fig. 1). *S.* Typhimurium was not detected in the control planters that were grown and treated in a similar way using *Salmonella*-free water.

Internalization of the pathogen into the leaves. *S.* Typhimurium was also recovered from surface-sterilized leaves, indicating endophytic properties of leaf-associated *S.* Typhimurium. After day irrigation with heavily contaminated water (i.e., water harboring 8.5 log CFU/ml), for example, surface-sterilized leaves harbored 4.5 log CFU/g, which represent ca. 1.5% of the total leaf-associated *S.* Typhimurium (6.3 log CFU/g) recovered from leaves that were not surface sterilized.

Time of irrigation affects the levels of contamination. When irrigation was applied during the night, the contamination of the phyllosphere and rhizosphere exhibited a similar pattern as that found following day irrigation application, with the phyllosphere being the most susceptible sphere to contamination (Fig. 2). Leaves harbored significantly ($P < 0.05$) higher levels of *S.* Typhimurium in comparison to stalks, root, and soil. Interestingly, the leaves harbored significantly ($P < 0.05$) higher levels of the pathogen, 0.5 to 0.9 log CFU/g higher, after irrigations conducted during the night compared to the levels of contamination observed after irrigations during the morning, when irrigation water carried 5.5 to 8.5 log CFU/ml. Similarly to irrigations performed during morning hours, the presence of culturable *S.* Typhimurium was not detected in any of the samples after irrigations conducted during the night with water harboring <4.5 log CFU/ml.

Implementation of qRT-PCR with or without enrichment for detection of *Salmonella* in plants irrigated with low levels of bacteria. In light of the limited ability to detect *Salmonella* by recovery and plating following irrigation with water carrying low levels of the pathogen, we decided to extract DNA from leaves and to implement qRT-PCR for the quantification of leaf-associated *S.* Typhimurium. qRT-PCR proved to be a more sensitive method for bacterial detection (Table 1). *S.* Typhimurium DNA was detected in all of the sampled leaves irrigated with water carrying 4.5 to 5.5 log CFU/ml. Furthermore, the detection of *S.* Typhimurium was feasible even in 60% of leaves irrigated with water carrying low level of 3.5 log CFU/ml. Still, leaf-associated *S.* Typhimurium was undetectable by this method following irrigation with water carrying very low levels of 1.5 to 2.5 log CFU/ml.

In order to further extend our ability to detect *Salmonella* on plants irrigated with water carrying very low levels of the patho-

TABLE 1 Quantification of *S.* Typhimurium on parsley leaves by qRT-PCR

Level of *S.* Typhimurium in water (log CFU/ml)	Frequency[a]	Amt of *S.* Typhimurium on leaves (mean log CFU/g ± SD)[b]
5.5	5/5	5.3 ± 0.3
4.5	5/5	4.3 ± 0.4
3.5	3/5	3.2 ± 0.8[c]
2.5	0/5	ND[d]
1.5	0/5	ND

[a] Calculated as the average number of leaf samples that tested positive for *S.* Typhimurium of the total sampled leaves.
[b] Calculated as the concentration of *S.* Typhimurium on leaves; the mean log CFU/g was quantified by qRT-PCR. Melting-curve analysis of the amplified PCR products confirmed positive amplification of the target gene only in the contaminated leaves and not in the *Salmonella*-free control leaves. The results are the averages of at least two independent determinations. The experiments were conducted in June and July. ND, not detected.
[c] This calculation is based on the positive samples.
[d] Positive after enrichment for 20 h.

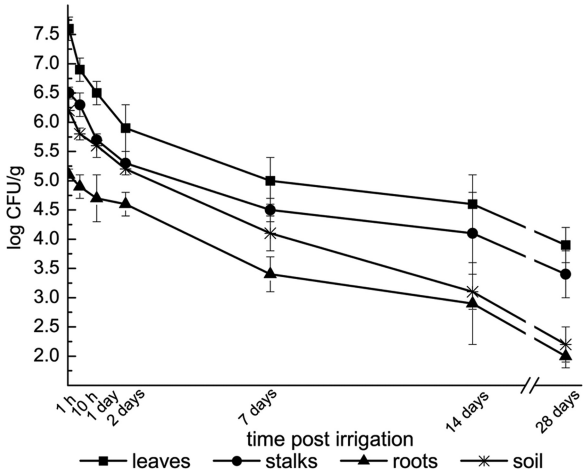
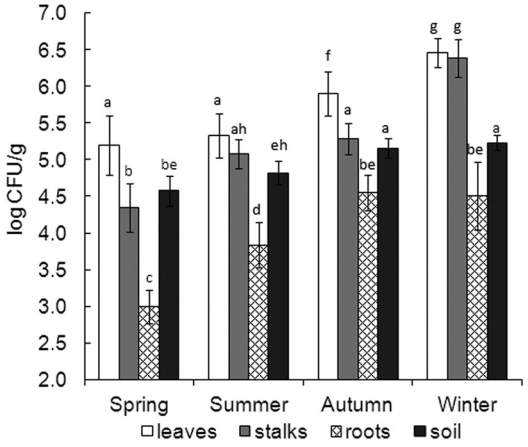


FIG 3 Persistence of *S.* Typhimurium in parsley. Plants were spray irrigated with water harboring 8.5 log CFU/ml. Bacterial abundance was determined at different time points (1 h, 10 h, 1day, 2 days, 7 days, 14 days, and 28 days) postinoculation. After recovery of the bacteria, GFP-tagged *S.* Typhimurium was enumerated by plating. The results are the averages of at least two independent repeats analyzed in triplicates. The experiments were conducted in October and November.



	Spring	Summer	Autumn	Winter
Temp. °C	22.6±5.5	29.2±8.1	22.0±3.2	15.8±3.1
Max. Temp. °C	34.0±4.7	44.6±3.8	27.1±2.0	21.6±1.7
Min. Temp. °C	16.6±1.2	21.1±1.0	17.4±2.1	12.4±1.2
R.H. %	65.5±17.3	65.6±23.9	77.1±17.9	91.8±13.3

FIG 4 Impact of irrigation in different seasons on *S.* Typhimurium contamination levels in parsley in the phyllosphere, rhizosphere, and soil. Plants were spray irrigated with water harboring 8.5 log CFU/ml. After recovery of the bacteria, GFP-tagged *S.* Typhimurium was enumerated by plating. Air temperature (Temp. °C) and relative humidity (R.H. %) were automatically logged, and the averaged values are shown, as well as the average minimum night temperature and maximum day temperature. The results are the average of at least two independent repeats analyzed in triplicates. Columns assigned different letters indicate the statistical significance between bacterial counts ($P < 0.05$).

gen, we decided to add an enrichment step before the PCR for the detection of leaf-associated *S.* Typhimurium. Parsley plants were spray irrigated as described with water carrying 1.5 and 2.5 log CFU/ml and processed by enrichment of leaf samples. After a 5-h enrichment step, all samples tested negative for *Salmonella*. However, after 20 h of enrichment, all leaf samples from plants irrigated with water carrying 2.5 log CFU/ml were found to be positive for *Salmonella*. Melting-curve analysis of the amplified PCR products confirmed the positive amplification of the target gene only in the contaminated leaves and not in the *Salmonella*-free control leaves.

Thus, leaves irrigated with water containing at least 4.5 log CFU/ml were positive for *S.* Typhimurium by both quantification methods: viable counts and qRT-PCR. After irrigation with water containing 3.5 log CFU/g, *S.* Typhimurium was detected on leaves only by qRT-PCR, and after irrigation with water containing 2.5 log CFU/g, *S.* Typhimurium was detected only by RT-PCR following enrichment for 20 h. Leaf-associated *S.* Typhimurium was proven to be undetectable by all three tested methods after irrigation with water carrying 1.5 log CFU/ml.

Short- and long-term persistence of *S.* Typhimurium. Fig. 3 presents the short- and long-term persistence of culturable *S.* Typhimurium (1 h to 28 days) in plant and soil samples after irrigation with contaminated water. The pathogen proved to have the ability to survive in the phyllosphere, in the rhizosphere, and in the soil for at least 28 days after the irrigation challenge. Parsley phyllosphere harbored the highest initial levels of *S.* Typhimurium 1 h after the irrigation challenge, followed by the soil and the rhizosphere. A significant decline of 0.7 log CFU/g of leaves was observed within the first 10 h. The rapid decline continued and, after 24 h, the pathogen counts were 1.1 log CFU/g lower. The levels of *S.* Typhimurium in the rhizosphere and in the soil also declined rapidly, and an ~0.5-log CFU/g reduction was observed within 24 h. Within 48 h, levels of *S.* Typhimurium on leaves declined by ~2 log CFU/g. Pathogen abundance continued to decrease both in parsley and in the soil for the entire period of sampling, but at a lower rate. At 1 and 2 weeks after plant contamination, the phyllosphere still harbored *S.* Typhimurium at a level of 4 to 5 log CFU/g, and 4 weeks into the experiment the parsley leaves and stalks still harbored high counts of 3.9 and 3.4 log CFU/g, respectively (0.02 and 0.08% of the counts at the first hour, respectively). *S.* Typhimurium counts in the rhizosphere and in soil reached levels of 2.0 and 2.2 log CFU/g (0.08 and 0.01%), respectively, 4 weeks after the irrigation challenge.

Impact of seasonality on contamination. Application of contaminated irrigation water in different seasons affected the level of *S.* Typhimurium counts in the phyllosphere and the rhizosphere (Fig. 4). *S.* Typhimurium counts on parsley leaves and stalks were 1.3 and 2.1 log CFU/g higher during the winter compared to irrigation conducted during the spring, the seasons with the highest and the lowest recorded contamination levels, respectively ($P < 0.05$). Contamination of the rhizosphere varied significantly with up to 1.6 log CFU/g difference between the seasons. Similarly to the phyllosphere, the lowest level of *S.* Typhimurium in the rhizosphere was recorded during the spring.

DISCUSSION

Sprinkler/spray irrigation is the main irrigation regime of fruits and vegetables in countries such as the United States and the United Kingdom, in which over half of total irrigated acreage is

subjected to overhead irrigation (56, 58). It is anticipated that spray irrigation results in a great frequency of contamination of greens due to direct contact of the leaves with the pathogen source (10, 22, 56). On the other hand, the ability of enteric bacteria to survive in the hostile environments on the phyllosphere is questionable, because stress conditions on plant surfaces can restrict their survival (11, 42, 61). In the present study, when spray irrigation of parsley was performed with contaminated water, *S.* Typhimurium was detected in the phyllosphere, the rhizosphere, and the soil in which the plants were grown. The conditions on and around parsley plants were not optimal for *Salmonella*, since the quantity of *Salmonella* declined over time. Still a portion of the bacteria persisted and was recovered from the rhizosphere, the phyllosphere, and the soil even at the end of our sampling period (28 days). Several field studies have reported a decline in populations of enteric pathogens over time, but comparison of the reduction rates of *Salmonella* to reported reduction of other pathogens reveals that *Escherichia coli* and *Listeria innocua* decreased more rapidly (23, 25, 27, 45).

S. Typhimurium was not only recovered from leaves but also from surface-sterilized leaves. This may support the hypothesis that *Salmonella* cells invade the inner tissues of leaves (30, 38) or attach to sites that are inaccessible to sterilization treatments (13, 49). Our results are in agreement with reports about a small population of internalized *E. coil* O157:H7 in lettuce leaves after application through spray irrigation (25). It was suggested that *Salmonella* migrates from the surface of leaves to the inner tissues via open stomata (30, 38). Interestingly, the rates of internalization in these experiments, which were conducted in the lab with harvested leaves of parsley (1.9%), were very similar to the rates of internalization into leaves in growing plants in our experiment (1.5%). Nevertheless, internalization to the inner tissues of leaves is not the main strategy for persistence of *Salmonella* since, according to our results, the majority of the cells persisted as epiphytes.

Limited availability of good-quality water increases the need for the use of low-quality water with high microbial loads, including raw or partially treated wastewater, for irrigation of crops even in developed countries (26, 34, 41, 56, 58). The U.S. Environmental Protection Agency (EPA) observed that 40% of streams, 45% of lakes, and 50% of estuaries in the United States were not clean enough. Microbial contamination was also widespread in groundwater, especially in shallow aquifers, where mixing with surface water can occur (59, 60). *Salmonella* occurrence in various water bodies was reported worldwide with frequency of positive samples ranging from 3 to 100% (41), underlining the potential health risk associated with application for irrigation. We showed that the persistence of *S.* Typhimurium on the plant was dependent on its initial levels in irrigation water. Based on this, the quality of water applied in the production environment is linked to the potential for contamination. It was already shown that heavily contaminated water applied for irrigation resulted in extensive level of pathogens recovered from the plants (23, 25, 27, 33, 54). By applying sensitive methods (qRT-PCR with or without enrichment), we were able to show that even irrigation with water containing as little as ~300 CFU/ml results in contamination and persistence of the pathogen in the plants for at least 48 h. To the best of our knowledge, quantification of pathogens from plants that were exposed to such a low *Salmonella* contamination through irrigation has not been described before. Quantification of naturally occurring *Salmonella* in environmental waters is seldom performed, but the existing reports reveal levels of up to 10^4 CFU/ml (41). Values of 300 CFU/ml are still higher than the levels of naturally occurring *Salmonella* in most investigated samples of water. However, it should be emphasized that *Salmonella* cells usually persist in water in a viable but nonculturable (VNC) state, meaning that the real viable counts might be 100- to 1,000-fold higher compared to counts obtained by culture-based methods (21).

In light of the observations that irrigation with water containing 300 CFU/ml results in contaminated leaves and in light of the reports that in many cases the gap between the last water application and harvest may be less than 24 h (58), the existence of *Salmonella* in irrigation water may lead to the harvesting of contaminated foods. Indeed, several outbreaks of salmonellosis from fresh produce were linked to contaminated irrigation water, including an outbreak of *S.* Newport in tomatoes and of *S.* Saintpaul in chili peppers (14, 31). Contaminated fresh produce can constitute a potential health risk even if contaminated with low levels of *Salmonella*, since the ingestion of as few as 10 to 100 cells has resulted in outbreaks associated with herbs, spices, and sprouts. In addition, there is a possibility of bacterial growth during the storage of cut produce. For instance, an outbreak of salmonellosis in Germany was traced to paprika and paprika-powdered potato chips. The estimated infective dose was as low as 4 to 45 salmonellae (40). Similarly, the infective dose of *S.* Newport on alfalfa sprouts was estimated to be <460 CFU (1).

Soil is considered a comparatively less hostile environment, although competition with native microorganisms can restrict the survival of pathogen populations (35). *S.* Weltevreden was detected in soil throughout a 28-day sampling period with only slight reduction in bacterial levels (4). In our study, culturable *S.* Typhimurium was present in soil samples even after irrigation challenge with water carrying as little as 4.5 log CFU/ml, but its levels declined over time. The quantity of *Salmonella* in the soil around the plants was always lower than its quantity on the leaves, probably because most water volume (with the bacteria in it) was retained in the phyllosphere. We presume that most bacteria arrived to the soil directly through drops of contaminated water. The rhizosphere has also been reported to serve as a reservoir for human pathogens (9). In our study, when contamination was mostly applied in the phyllosphere, we found bacteria attached to the roots, but their levels were lower compared to the other parts of the plants or to the soil. The decline of *Salmonella* associated with the roots was similar to the decline in the soil, indicating that in the planters used for the present study, the root environment does not provide better or worse conditions for *Salmonella* compared to the soil. Furthermore, whereas it was shown that *S. enterica* serotypes are able to move via chemotaxis toward root exudates in microcapillaries (37), in the conditions of our experiments the levels of bacteria associated with the roots were always similar or even lower than the levels in the soil. When irrigation was conducted by dripping directly to the soil, the presence of *Salmonella* and *E. coli* in the rhizosphere resulted in the contamination of the phyllosphere (39, 55). Here, when most bacteria were applied on the phyllosphere, the bacteria were also found in the rhizosphere, but further research is needed in order to understand whether the bacteria arrive to the roots through the soil or through the phyllosphere, since we have shown that ca. 1.5% of the bacteria are probably endophytic.

The physiology of the plant changes during the day and during

the year, and it was hypothesized that these variations, together with variations in environmental conditions (such as sunlight, temperature, etc.) could influence the survival of *Salmonella* on the plants. Indeed, the levels of the pathogen recovered from the leaves were higher after the application of contaminated water during night hours. We suggest that variations in plant physiology that lead to different contents and concentrations of secondary metabolites in the infected tissues or impact the production of signals of plant immune response may play a role in the short-term adaptation of *Salmonella* in the leaf. Indeed, it was recently shown that the extent to which methyl salicylate is required for signaling systemic acquired resistance in plants, a defense response which is activated throughout a plant after local infection, is dependent on exposure to light during infection (44). In addition, it was shown that during photosynthesis, parsley cells may produce toxic oxygen species. These toxic oxygen species migrate outside the phytodetritus and potentially could affect the attached bacteria (47). With respect to field applications, our results indicate that irrigation of parsley during morning hours may have lower potential for contamination.

Presence of *S.* Typhimurium in the plant environment was subjected to changes throughout the seasons with the highest levels recorded during winter and the lowest levels during spring. The levels of *S.* Typhimurium in soil remained relatively constant between the seasons, while the levels of the pathogen in the phyllosphere and the rhizosphere varied considerably. A similar profile was reported for *E. coli* sprayed on lettuce (27). Interestingly, similar results (longer survival in the winter) were observed with *Vibrio cholerae* on stored parsley (29). This may suggest interactions of the pathogens with the plant or with the microflora. The quantity and diversity of microbial communities are subjected to seasonal changes (45, 52). Thus, indigenous bacteria may affect survival of *S.* Typhimurium, as shown for *E. coli* O157:H7 on lettuce (19). An increase in the logged relative humidity (RH) between spring and winter corresponded with the increase in *Salmonella* counts. Likewise, higher levels of *Listeria* spp. were recorded on artificially inoculated parsley leaves under high RH conditions (23). Lower counts of *S.* Typhimurium under low RH may be explained by the stress induced by limited available water, in spite the relative tolerance to dry conditions (12). Furthermore, low RH may induce a VNC state and result in lower recovery levels, as reported for *L. monocytogenes* on parsley leaves (24). Higher levels of persistence of enteric pathogens may lead to greater potential for food-borne illness outbreaks linked to fresh produce. Indeed, analysis of several high-profile outbreaks associated with fresh produce reveals that 17 of 25 outbreaks occurred during the autumn and winter (61), as opposed to the fact that most cases of human salmonellosis (originated from all types of foods) occur in summer (46).

Conclusions. We have shown that spray irrigation of parsley with water containing a minimum of 300 CFU of *S.* Typhimurium/ml results in the persistence of detectable *Salmonella* on the leaves of parsley at least 48 h after the irrigation. The extent of contamination was affected mainly by the quantity of *Salmonella* in irrigation water and the time period between irrigation and harvest, seeing that the population levels of *S.* Typhimurium steadily declined during the field study. We furthermore showed that irrigation during the night versus during the day, and in the winter versus the other seasons, results in higher levels of pathogen on the phyllosphere. If parsley, which is used either as a dried spice or a fresh herb, is contaminated with pathogens such as *Salmonella* through exposure to polluted water, such pathogens might enter the food chain in a wide geographic distribution. Understanding the mechanisms underlying the seasonality of *Salmonella* will enable the implementation of effective irrigation and control strategies. Our results strengthen the paradigm that *Salmonella* is able to persist in soil and crops after application of contaminated water or compost for prolonged periods (7, 33, 39, 62), thus posing both health and environmental risks.

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Effect of Repeated Irrigation with Water Containing Varying Levels of Total Organic Carbon on the Persistence of *Escherichia coli* O157:H7 on Baby Spinach†

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ABSTRACT

The California lettuce and leafy greens industry has adopted the Leafy Greens Marketing Agreement (LGMA), which allows for 126 most-probable-number (MPN) *Escherichia coli* per 100 ml in irrigation water. Repeat irrigation of baby spinach plants with water containing *E. coli* O157:H7 and different levels of total organic carbon (TOC) was used to determine the epiphytic survival of *E. coli* O157:H7. Three irrigation treatments (0 ppm of TOC, 12 or 15 ppm of TOC, and 120 or 150 ppm of TOC) were prepared with bovine manure containing *E. coli* O157:H7 at either low (0 to 1 log CFU/100 ml) or high (5 to 6 log CFU/100 ml) populations, and sprayed onto baby spinach plants in growth chambers by using a fine-mist airbrush. MPN and direct plating techniques were used to determine the *E. coli* O157:H7 populations on the aerial plant tissue. Plants irrigated with high *E. coli* O157:H7 populations, regardless of TOC levels, showed a 3-log reduction within the first 24 h. Low levels of *E. coli* O157:H7 were observed for up to 16 days on all TOC treatments, ranging from 76.4 MPN per plant (day 1) to 0.40 MPN per plant (day 16). No viable cells were detected on spinach tissue 24 h after irrigation with water containing fewer than 126 CFU/100 ml *E. coli* O157:H7. Under growth chamber conditions in this study, *E. coli* O157:H7 populations in irrigation water that complies with the LGMA standards will not persist for more than 24 h when applied onto foliar surfaces of spinach plants.

Foodborne outbreaks continue to be associated with fresh produce at an alarming frequency, with 713 outbreaks occurring between 1990 and 2005 *(27)*. Almost one-fourth (24.5%) of all foodborne illness outbreaks from 1996 to 2005 were associated with produce *(11),* including leafy greens *(28, 33),* melons *(20),* tomatoes *(10, 22),* and sprouts *(7)*. In 2007, 14% of all outbreaks that were attributed to a single food commodity were linked to leafy greens, making leafy greens the produce commodity most likely associated with foodborne pathogen contamination *(8)*. As foodborne outbreaks associated with produce continue to occur, all aspects of the farm-to-fork spectrum are under close scrutiny for areas where contamination could occur.

Irrigation water became a recognized source for the preharvest contamination of produce because of its association with outbreaks of *Escherichia coli* O157:H7 on leafy greens. Environmental sampling after the 2006 shredded-lettuce outbreak in the United States traced the source of contamination to irrigation water tainted with dairy effluent from a herd adjacent to the implicated lettuce field *(5)*. Several guidance documents have since been developed to address the microbiological safety of irrigation water, including the Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens *(6)*. The current guidelines set by the LGMA state that irrigation water used for foliar applications may contain ≤126 most probable number (MPN)/100 ml of *E. coli* (rolling geometric mean of five samples), and any single sample may not exceed 235 MPN/100 ml.

Several studies have investigated the persistence of *E. coli* O157:H7 in the phyllosphere of baby spinach plants after foliar irrigation with contaminated water or spot-inoculation with contaminated manure slurry *(9, 15, 18, 21)*. None of these studies, however, involved application of *E. coli* populations in irrigation water containing fewer than 126 MPN/100 ml onto the plant surfaces. Previous studies have not addressed specific parameters regarding irrigation water quality, which could affect the bacterial pathogen populations in the water. Total organic carbon (TOC) content is one indicator of water quality, which measures organic molecules present in water. These molecules (amino acids, sugars, and fatty acids), even when present at very low levels, may be used as nutrient sources by all epiphytic microbiological populations, which might include *E. coli (14)*. Environmental survey data has shown that the TOC

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concentration for on-farm irrigation water would likely be between 5 and 10 ppm *(12, 19, 23)*. It is possible that higher TOC levels could occur after a rain event, caused by runoff from wildlife droppings, stockpiles of manure, or incompletely composted manure from neighboring fields *(25, 30, 31)*.

Baby spinach crops are fast growing, cool-weather crops that are generally harvested between 30 and 45 days after sowing. Leaf maturity may have a role in the epiphytic persistence of *E. coli* O157:H7 on leafy green commodities, as greater population sizes of *E. coli* O157:H7 have been found on younger (inner) lettuce leaves than on older (outer) lettuce leaves *(3)*. While baby spinach leaves do not exhibit the same dense, spiral growth pattern as some lettuce cultivars exhibit, previous research has found that the younger baby spinach leaves might be more susceptible to surface contamination because of physiological and microbiological community differences on the plant surfaces *(24)*.

Spinach plants are sometimes irrigated frequently during dry seasons *(4)*. Therefore, we included repeated irrigation events (two times per week) with a water source containing dilute dairy manure to evaluate the persistence of enterohemorrhagic *E. coli* (EHEC) on the foliar surfaces of spinach plants. We hypothesized that the biweekly introduction of moisture and the accumulation of TOC on the plant surfaces via irrigation water would sustain an epiphytic *E. coli* O157:H7 population. Our study examined TOC levels, as adjusted with diluted dairy manure, in irrigation water to determine its effect on the persistence of *E. coli* O157:H7 on spinach leaves.

MATERIALS AND METHODS

Preparation of irrigation solutions. Fresh bovine manure was obtained from a U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS), Beltsville Area Research Center (BARC) Holstein dairy herd that was not exposed to antibiotics. The dairy solids were suspended in a 1:5 ratio of solids:deionized water, and stirred for 6 h at 25°C. Particulate matter was removed via three sequential centrifugation (Allegra 25 R, Beckman Coulter, Fullerton, CA) events at 10,700 × *g* for 20 min, and the supernatant was then sterilized at 121°C for 60 min. The manure extract was analyzed for TOC (Phoenix 8000, Teledyne Tekmar, Mason, OH), ammonia, and nitrate content (QuickChem 8000 FIA Lachat, Fort Collins, CO), and used to prepare three irrigation stock solutions (2 liters each). These solutions were prepared by diluting extracts in sterile deionized water to obtain three experimental TOC concentrations: treatments A, 0 ppm; B, 12 or 15 ppm; and C, 120 or 150 ppm. Treatment A (sterile, deionized water) was used as the control irrigation solution. All sterile irrigation stock solutions were adjusted to pH 7.0 with either 10 N NaOH or 33% HCl and stored at 4°C. Measurements taken throughout refrigerated storage indicated that TOC of solutions were unchanged throughout the duration of the experiment.

Preparation of *E. coli* O157:H7 inoculum. Three human clinical *E. coli* O157:H7 isolates, epidemiologically linked to produce outbreaks, were used to inoculate the irrigation solutions: RM4406 (lettuce outbreak), RM4407 (spinach outbreak), and 5279 (bagged-vegetables outbreak), kindly provided by Robert Mandrell (USDA-ARS, Albany, CA). All strains were adapted to grow in the presence of nalidixic acid (50 μg/ml), as described previously *(26)*. Strains were inoculated from frozen stocks onto MacConkey agar (BD, Franklin Lakes, NJ) supplemented with 50 μg/ml nalidixic acid (MACN; Sigma, St. Louis, MO) and incubated at 37°C for 24 h. All *E. coli* O157:H7 strains were inoculated into 25-ml aliquots of irrigation treatment C (120 or 150 ppm of TOC), and incubated at 37°C for 48 h with agitation (150 rpm). The night prior to each irrigation event where *E. coli* O157:H7 was to be applied to spinach plants, the population of each *E. coli* O157:H7 strain was individually determined by spiral plating (WASP2, Don Whitley Scientific, Frederick, MD) onto MACN plates, which were incubated at 37°C for 24 h. Each liquid manure culture was maintained on ice at 4°C overnight to prevent further growth. Appropriate volumes of each strain (as determined by plate count data) were added to irrigation solutions as prepared above to yield the experimental *E. coli* O157:H7 populations (≤126 CFU/100 ml for the low inocula and 5 to 6 log CFU/100 ml for the high inocula).

Spinach plant cultivation. The spinach (*Spinacia oleracea*) cultivar Avenger, a hybrid semi-Savoy cultivar that is resistant to downy mildew lines 1 through 7 and used in the Central Valley in California, was donated by Seminis, Inc. (Oxnard, CA). Seeds were soaked in 10% commercial sodium hypochlorite solution for 10 min, and then washed three times for 5 min each in sterile deionized water. Seeds were sown in sterile 164-ml cone-shaped plastic vessels (Cone-tainers, model SC10, Stuewe and Sons, Inc., Tangent, OR) filled with 125 g of fine, sandy loam (Keyport-Matawan) soil obtained from the USDA-ARS BARC north farm. Soil was steam pasteurized (85°C for 15 h) to eliminate potential phytopathogens and invertebrates. Cone-tainers with spinach plants were placed into custom trays (RL98, Stuewe and Sons), with one tray for each irrigation treatment to prevent cross-contamination. Plants were maintained in an environmentally controlled growth chamber (CMP 4030, Conviron, Winnipeg, Manitoba, Canada) set to 70% humidity and light intensity of 450 microeinstein units/m^2/s for a 14-h photoperiod at 18°C and 10-h dark period at 13°C. All plants were fertilized and irrigated once per week with diluted (1.32 g/liter) Jack's Classic All Purpose 20-20-20 fertilizer (J. R. Peters, Inc., Allentown, PA), which was applied via pipette to the soil, without contacting spinach leaves.

Irrigation of baby spinach plants. The irrigation treatments began when all plants had developed two true leaves (approximately 14 days after sowing). For the three irrigation solutions (treatments A, B, and C), 200 ml of each irrigation solution was prepared by adding appropriate volumes of each *E. coli* O157:H7 culture (as prepared above) to achieve a low or high population of *E. coli* O157:H7 inocula. During each irrigation event, individual spinach plants were transferred to a biological safety hood and sprayed using a hand-held airbrush (model 200, Badger Air Brush Company, Franklin Park, IL) connected to an air compressor (model 180-10, Badger Air Brush Co.). This technique deposited a fine mist of each irrigation solution onto the entire foliar surface of each plant, including the stem, adaxial, and abaxial surfaces of each leaf (Fig. 1). Each plant was sprayed until total coverage was achieved, without runoff, which varied between 15 and 25 s, depending on the amount of tissue, resulting in the application of 1.5 to 3 ml of the irrigation treatment onto the foliar surface of each plant. Twice a week, each irrigation solution (treatments A, B, and C) was sprayed on all plants within each treatment block. Depending on the day of harvest, the plants were exposed to at least 1 irrigation event (day 0 harvest) and up to 5 irrigation events for plants exposed to the low *E. coli* O157:H7 inoculum, and 10 irrigation events for plants exposed to the high *E. coli* O157:H7

FIGURE 1. *Leaf tissue of a 14-day-old baby spinach plant after an irrigation event with hand-held airbrush.*

inoculum. Plants were harvested daily for microbiological analyses. If 2 consecutive days of foliar microbial analyses of baby spinach plants were negative for *E. coli* O157:H7, then plants would receive an additional inoculated irrigation treatment at the next scheduled event. If these analyses revealed the presence of *E. coli* O157:H7, then the next scheduled irrigation event would contain the appropriate irrigation treatment, but without the addition of the *E. coli* O157:H7 inoculum.

Microbiological analyses. At each harvest, randomly selected plants were excised with sterile scissors, 5 mm above the soil surface. The entire aerial tissue from each plant was placed into a sterile 80-ml stomacher bag (Secure T, Fisher Scientific) containing 40 ml of mEHEC broth (BioControl, Bellevue, WA) supplemented with 50 μg/ml of nalidixic acid. Samples were then homogenized in a Stomacher 80 (Seward, Ltd., Basingstoke, UK) for 2 min at 230 rpm, and then sonicated for 30 s (Astrason ultrasonic cleaner, Plainview, NY). A three-tube MPN was performed on each sample homogenate with 10, 1, and 0.1 ml of the homogenate added to each of three tubes, containing 0, 9, and 9.9 ml, respectively, of sterile mEHEC broth supplemented with 50 μg/ml nalidixic acid. The tubes were incubated for 24 h at 37°C, before 10 μl of each tube was isolated on MACN for identification of positive tubes containing *E. coli* O157:H7 populations. The freeware MPN calculator (VB6 version, www.i2workout.com/mcuriale/mpn/index.html) was used to calculate the final MPN per gram for each sample. Each MPN was performed in triplicate, and the results were expressed as the mean MPN per plant.

The MPN assays for the plants exposed to the low *E. coli* O157:H7 populations in irrigation treatments were based on a single plant through the first three inoculation events. Beginning with irrigation event 4, three plants were harvested per MPN analysis, which increased the detection sensitivity from 1.2 MPN per plant to 0.4 MPN per plant. The plants receiving high *E. coli* O157:H7 population inocula were analyzed, with one plant per MPN assay. Additionally, remaining homogenate was spiral plated (100 μl, in triplicate) onto MACN plates.

Liquid from irrigation treatments containing low and high populations of *E. coli* O157:H7 was collected after each spray event and analyzed with filtration (low inocula) or spiral plating (high inocula). For the low inoculum, 25 ml of liquid was vacuum filtered through each of four sterile, hydrophobic grid membrane filter monitors (56 mm/100 ml, 0.45 μm; BioPath, Inc., West Palm Beach, FL). Membranes were removed aseptically from containers, placed onto MACN plates, and incubated for 24 h at 37°C, after which colonies were visualized and counted on the surface of the membrane.

Growth of *E. coli* O157:H7 in irrigation solutions. Each irrigation solution (150 ml), as described above to contain TOC concentrations of 0, 12, or 120 ppm, was transferred into each of three separate 250-ml Pyrex screw-cap bottles (Fisher Scientific). Each bottle was inoculated with the three-strain *E. coli* O157:H7 inoculum at populations of either 0 or 4 log CFU/ml. Solutions were incubated without agitation, with the caps loosened, and maintained under the same growth chamber temperatures as were baby spinach plants (14 h at 18°C, 10 h at 13°C) in a low-temperature, illuminated programmable incubator (model 818, Thermo Electron Corp., Waltham MA). *E. coli* O157:H7 populations in irrigation solutions were quantified daily for up to 44 days by making appropriate serial dilutions and spiral plating, in duplicate, onto MACN plates, which were incubated at 37°C for 18 h.

RESULTS

Microbiological and nutrient content of irrigation treatments. The *E. coli* O157:H7 populations that were applied to spinach plants in inoculated irrigation events are shown in Tables 1 and 2. For irrigation events involving the low inocula, populations of *E. coli* O157:H7 ranged from 5 to 71 CFU/100 ml. For irrigation events involving high inocula, populations of *E. coli* O157:H7 ranged from 5.13 to 6.48 log CFU/100 ml. Treatment A contained 0 ppm of TOC, 0 ppm of nitrate, and 0 ppm of ammonia; treatment B contained 12 or 15 ppm of TOC, 0.58 ppm of ammonia, and 0.02 ppm of nitrate; and treatment C contained 120 or 150 ppm of TOC, 5.42 ppm of ammonia, and 0.11 ppm of nitrate.

Irrigation with low *E. coli* populations (≤126 CFU/100 ml). Spinach plants reached the two-leaf stage at approximately 17 days, when the biweekly foliar irrigation events commenced (Table 1). Epiphytic *E. coli* O157:H7 populations were all below the MPN detection limit (1.2 MPN per plant) for the plants harvested immediately after irrigation events 1, 2, and 3, on days of analysis 0, 2 and 7, respectively. *E. coli* O157:H7 populations on spinach plants were also below detection limits (<1.2 MPN per plant) for subsequent days of analysis for each of these respective irrigation events. Very low populations of *E. coli* O157:H7 were recovered from plant tissues harvested from the first

TABLE 1. *Low populations of* Escherichia coli *O157:H7 in irrigation solutions containing varying TOC concentrations and on spinach plants 0, 1, and 2 days after repeated irrigation events*

Irrigation event[a]	Day of analysis[b]	Plant age (days)[c]	Treatment: A (0 ppm of TOC)		B (15 ppm of TOC)		C (150 ppm of TOC)	
			CFU/100 ml	MPN/plant	CFU/100 ml	MPN/plant	CFU/100 ml	MPN/plant
1	0	17	5	<1.20	56	<1.20	15	<1.20
	1	18		<1.20		<1.20		<1.20
	2	19		<1.20		<1.20		<1.20
2	2	19	31	<1.20	6	<1.20	55	<1.20
	3	20		<1.20		<1.20		<1.20
	4	21		<1.20		<1.20		<1.20
3	7	24	37	<1.20	36	<1.20	31	<1.20
	8	25		<1.20		<1.20		<1.20
	9	26		<1.20		<1.20		<1.20
4	9	26	22	3.74	60	4.13	50	49.2
	10	27		<0.40		<0.40		<0.40
	11	28		<0.40		<0.40		<0.40
5	16	33	11	0.32	31	0.16	71	0.41
	17	34		<0.40		<0.40		<0.40
	18	35		<0.40		<0.40		<0.40

[a] Irrigation events containing low *E. coli* O157:H7 (≤126 CFU/100 ml) inocula were applied in irrigation events 1 through 5.
[b] Spinach plants were irrigated with low populations of *E. coli* O157:H7 on days 0, 2, 7, 9, and 16.
[c] Plant age is indicated from the time of germination of spinach seedlings.

day of irrigation events 4 and 5 (sampling days 9 and 16, respectively). *E. coli* O157:H7 detection on spinach plants was facilitated by increasing the MPN sensitivity to <0.4 MPN per plant by using a composite sample of three plants for each MPN assay, which were performed in triplicate. No viable *E. coli* O157:H7 cells, however, were recovered 1 or 2 days after irrigation events 4 and 5 (Table 1).

On all days of analysis where *E. coli* O157:H7 populations were below detection limits for MPN analyses, the remaining liquid from each plant sample homogenate (approximately 7 ml) was enriched for 48 h in mEHEC broth and plated onto MACN. All enrichments were negative for the presence of *E. coli* O157:H7.

Irrigation with high *E. coli* O157:H7 populations (5 to 6 log CFU/100 ml). Spinach plants reached the two-leaf stage at approximately 13 days, when the biweekly foliar irrigation events commenced (Table 2). The initial irrigation event (event 1) contained 5.49 log CFU/100 ml for treatment A (0 ppm of TOC), 5.88 log CFU/100 ml for treatment B (12 ppm of TOC), and 6.48 log CFU/100 ml for treatment C (120 ppm of TOC), as shown in Table 2. Recovered populations of *E. coli* O157:H7 on the plant tissues harvested immediately after irrigation on day 0 for irrigation event 1 were all above the maximum limit (too numerous to count, >440 MPN per plant) for the MPN assay. Calculations based on the irrigation population density and amount of volume that was applied to the plants suggested that populations were ≥4 log MPN per plant. *E. coli* O157:H7 populations declined within 24 h to 34 MPN per plant for treatment A, 76 MPN per plant for treatment B, and 76 MPN per plant for treatment C. Low populations of *E. coli* O157:H7 were recovered sporadically in both treatments A and C, until the 15th day of analysis (28-day-old plants), where means of 0.5 and 67 MPN per plant were recovered, respectively. Populations of *E. coli* O157:H7 were detected on plants in treatment B until the 16th day of analysis (29-day-old plants), where 0.48 MPN per plant were recovered. Plants were harvested for the next 3 consecutive days; the results were consistently below MPN detection limits (<1.20 MPN per plant). All remaining liquid from the plant homogenates were enriched but remained negative for the presence of *E. coli* O157:H7.

The second application of *E. coli* O157:H7 population occurred during irrigation event 7, on 34-day-old spinach plants, where the irrigation water contained 5.13 log CFU/100 ml (treatment A), 5.47 log CFU/100 ml (treatment B), and 5.54 log CFU/100 ml (treatment C) (Table 2). Recovered populations immediately after irrigation were 1,467, 2,467, and 3,133 MPN per plant for respective treatments A, B, and C. *E. coli* O157:H7 populations declined by over 3 log MPN per plant after 1 day, with 0.48, 1.23, and <1.20 MPN per plant for treatments A, B, and C, respectively. *E. coli* O157:H7 persisted for 2 days after irrigation event 7 (harvest day 23) for treatments A and C, when 1.23 and 2 MPN per plant were recovered, respectively.

The third and final application of irrigation water containing *E. coli* O157:H7 occurred during event 9, on 41-day-old spinach plants, where 5.54 log CFU/100 ml (treatment A), 6.01 log CFU/100 ml (treatment B), and 6.13 CFU/100 ml (treatment C) were applied onto the foliar surfaces of each plant. Recovered populations of *E. coli* O157:H7 immediately after irrigation on day of analysis 28 were 2,467, 5,267, and 6,000 MPN per plant for treatments A, B and C, respectively. *E. coli* O157:H7 populations declined within 24 h (day of analysis 29) after application to

TABLE 2. *High populations of* Escherichia coli *O157:H7 in irrigation solutions containing varying TOC concentrations and on spinach plants 0, 1, and 2 days after repeated irrigation events*

			Treatment:					
			A (0 ppm of TOC)		B (12 ppm of TOC)		C (120 ppm of TOC)	
Irrigation event[a]	Day of analysis[b]	Plant age[c]	log CFU/100 ml	MPN/plant	log CFU/100 ml	MPN/plant	log CFU/100 ml	MPN/plant
1	0	13	5.49	TNTC[d]	5.88	TNTC	6.48	TNTC
	1	14		34.21		76.4		76.4
	2	15		3.07		1.71		15.87
2	2	15		6.13		0.96		1.71
	3	16		3.55		2		10
	4	17		0.48		3.07		61.33
	7	20		1.71		<1.20		<1.20
3	7	20		403		3.23		25.47
	8	21		1.23		<1.20		2
	9	22		3.07		<1.20		0.48
4	9	22		<1.20		<1.20		0.48
	10	23		<1.20		<1.20		<1.20
	11	24		<1.20		32		<1.20
	14	27		3.07		<1.20		2
5	14	27		10		<1.20		<1.20
	15	28		0.48		<1.20		66.67
	16	29		<1.20		3.07		<1.20
6	16	29		<1.20		0.48		<1.20
	17	30		<1.20		<1.20		<1.20
	18	31		<1.20		<1.20		<1.20
	21	34		<1.20		<1.20		<1.20
7	21	34	5.13	1,467	5.47	2,467	5.54	3,133
	22	35		0.48		1.23		<1.20
8	23	36		1.23		<1.20		2
	24	37		<1.20		<1.20		<1.20
	25	38		<1.20		<1.20		<1.20
9	28	41	5.54	2,467	6.01	5,267	6.13	6,000
	29	42		2.69		0.48		0.48
	30	43		0.48		0.48		<1.20
10	30	43		<1.20		12.4		<1.20
	31	44		<1.20		<1.20		0.48
	32	45		<1.20		<1.20		<1.20
	35	48		<1.20		<1.20		<1.20

[a] Irrigation events containing high populations of *E. coli* O157:H7 (5 to 6 log CFU/100 ml) inocula were applied in irrigation events 1, 7, and 9.
[b] Spinach plants were irrigated with high populations of *E. coli* O157:H7 on day 0, 21, and 28.
[c] Plant age is indicated from the time of germination of spinach seedlings.
[d] TNTC, too numerous to count.

2.69, 0.48, and 0.48 MPN per plant for treatments A, B and C, respectively. *E. coli* O157:H7 populations in treatments A and B declined to 0.48 MPN per plant, while those in treatment C declined to below the detection limit. After irrigation event 10 (day of analysis 30), which did not contain *E. coli* O157:H7 inocula, spinach plants irrigated with treatment B had 12.4 MPN *E. coli* O157:H7 per plant, whereas treatments A and C were below detection limits. The plant harvest continued until day 48, after all remaining plants were negative for *E. coli* O157:H7 for at least 2 consecutive days.

***E. coli* O157:H7 growth in irrigation treatments.** Low (0 log CFU/ml) and high (4 log CFU/ml) populations of *E. coli* O157:H7 were inoculated into irrigation solutions containing 0, 12, or 120 ppm of TOC and incubated under the same conditions used to grow the spinach plants. In all trials with low (Fig. 2) or high (Fig. 3) initial starting populations of *E. coli* O157:H7, the populations rapidly increased over several days before reaching approximate maximum population densities of 7 and 8 log CFU/ml, respectively. These high population densities were sustained by the nutrients contained in both TOC irrigation solutions of 12 and 120 ppm for longer than 44 days. Conversely, in all irrigation solutions containing 0 ppm of TOC, the *E. coli* O157:H7 were not recovered after 6 days when starting with a low initial population (Fig. 2) and 13 days for the high initial population (Fig. 3).

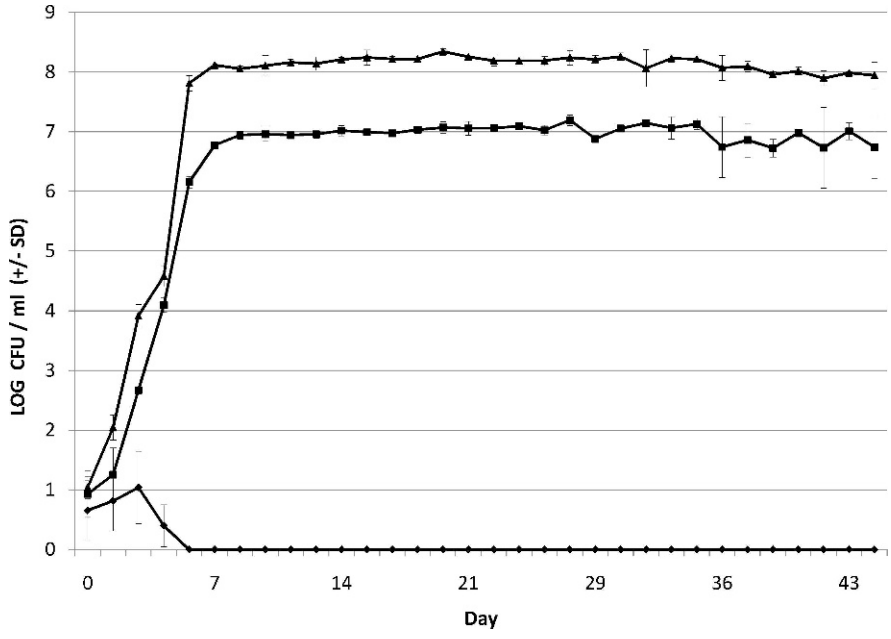

FIGURE 2. *Growth of low initial populations (0 log CFU/ml) of* Escherichia coli *O157:H7 in irrigation treatments containing varying amounts of total organic carbon (TOC): treatment A (0 ppm), treatment B (12 ppm), and treatment C (120 ppm).* ◆*, 0 ppm TOC;* ■*, 12 ppm TOC;* ▲*, 120 ppm TOC.*

DISCUSSION

Irrigation water quality has recently become a contentious issue, as current irrigation water microbiological standards for leafy greens (LGMA) are based on the recreational water standards determined by the U.S. Environmental Protection Agency. These standards do not take into account the fate of human pathogens on foliar surfaces of leafy greens, where they appear to decline rapidly in population size. For example, the microbiological standards for irrigation water intended for leafy greens in the LGMA do not take into account the chemistry regarding irrigation water quality, prevalence, or persistence of foodborne pathogens on foliar surfaces as applied via overhead irrigation. This study replicated on-farm irrigation water by adding organic carbon content, using a dairy manure source, to prepare water representative of various irrigation water sources found in the United States.

Our recovery methods detected low populations of the surface-applied *E. coli* O157:H7. Our methodologies might have underestimated these populations, as some cells may have been internalized through the stomata, the recovery of which has not been evaluated with our methodology. *Salmonella* has recently been shown to exhibit a chemotactic motility response toward the photosynthetic nutrients produced by lettuce plants, resulting in colonization surrounding, and subsequent internalization through, stomata *(13),* but it is unclear whether *E. coli* O157:H7 cells possess this chemotactic ability.

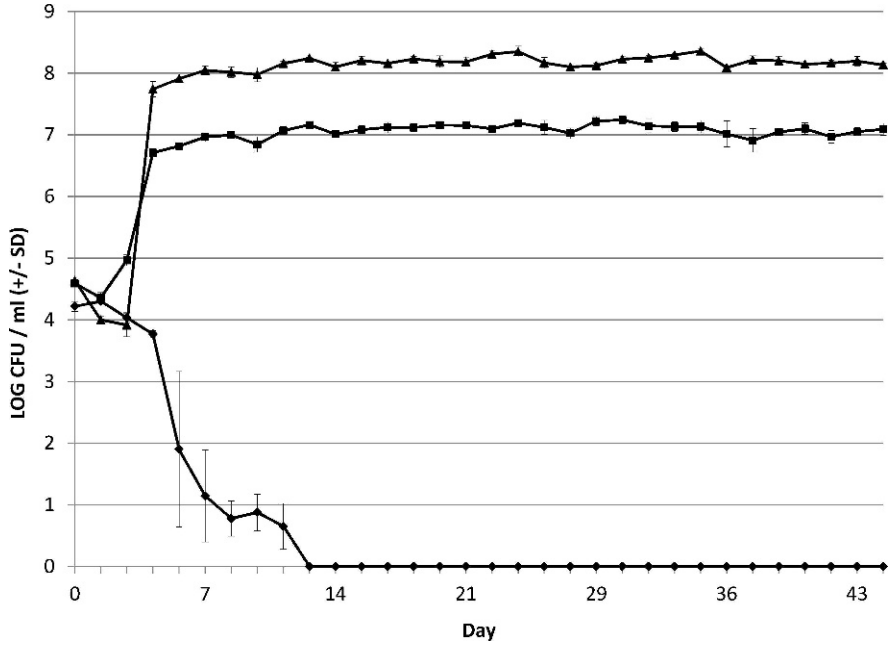

FIGURE 3. *Growth of high initial populations (4 log CFU/ml) of* Escherichia coli *O157:H7 in irrigation treatments containing varying amounts of total organic carbon (TOC): treatment A (0 ppm), treatment B (12 ppm), and treatment C (120 ppm).* ◆*, 0 ppm TOC;* ■*, 12 ppm TOC;* ▲*, 120 ppm TOC.*

As the spinach plants grew, more tissue was present during each subsequent irrigation event and, therefore, older plants should have collected greater *E. coli* O157:H7 populations than the younger plants. For example, older plants containing four or more true leaves had more surface area, which would be more likely to collect larger *E. coli* O157:H7 populations as deposited from the irrigation water than would younger plants containing fewer leaves. However, our results did not support this hypothesis. When young plants (13 days old) were inoculated with high populations of *E. coli* O157:H7, these populations persisted at low levels for 16 days postinoculation. In addition, we observed a temporary (but substantial) population increase in treatments A and C when the plants were 20 days old after irrigation event 3, which did not contain additional *E. coli* O157:H7. In contrast to the inoculated 13-day-old plants, *E. coli* O157:H7 populations that were inoculated onto 34- and 41-day-old plants did not survive for more than 2 and 4 days, respectively. These findings suggest that the spinach plant tissues could support *E. coli* O157:H7 survival at various capacities, depending on the developmental stage (age) of the plant. Pu et al. *(24)* reported significant plant-age effects on the ability of populations of *E. coli* O157:H7 in soil to transfer to the surfaces of baby spinach. They concluded that the epiphytic surfaces of baby spinach tissues were most susceptible to contamination with *E. coli* O157:H7 between 3 and 5 weeks in development. Our findings are in agreement with those of others *(3),* who suggest that baby spinach tissue is more likely to support *E. coli* O157:H7 growth and survival at specific ages.

A previous study determined that *E. coli* O157:H7 persistence on lettuce was not statistically different when using water or cow manure extract as an inoculum carrier *(34).* This study, however, did not adapt *E. coli* O157:H7 strains to growth in the carrier (sterile cow manure), as performed in our study. We also confirmed that *E. coli* O157:H7 cells were able to grow and sustain high population densities in irrigation solutions containing TOC (treatments B and C), under the same environmental conditions in which spinach plants were grown. However, no differences in the survival of *E. coli* O157:H7 on spinach leaves were observed, based on the TOC content in the irrigation solutions. These results indicate that TOC concentrations in the irrigation water at or below 150 ppm, even after repeated irrigation events on the same plants, do not appear to have any effect on the persistence of *E. coli* O157:H7. Repeated irrigation events containing high or low levels of TOC did not confer any enhanced survival to *E. coli* O157:H7 on foliar surfaces. The rapid die off of *E. coli* O157:H7 observed on all surfaces of the spinach leaves indicates that the TOC treatments did not aid in the formation of *E. coli* O157:H7 aggregates on baby spinach tissues, which have been shown to aid microbial survival on foliar surfaces *(17, 32).* In addition, the TOC treatments did not enhance the survival of *E. coli* O157:H7 that were reintroduced through repeated irrigation at later stages of spinach development. The repeated, low-level introduction of *E. coli* O157:H7 populations onto spinach plants did not appear to help the pathogen in overcoming the obstacles necessary for survival, including limited nutrient sources, desiccation, and microbial competition found on foliar surfaces *(2).*

Our overhead-irrigation strategy involved the airbrush application of the treatments to all aerial tissues until saturation was achieved and terminating application just before runoff. However, it is possible that if more irrigation volume were introduced to the foliar surfaces (e.g., with the incorporation of chemical ''spreader-stickers,'' commonly used in commercial horticulture to enhance the ability of liquid to cover and stick to leaf surfaces), the survival of *E. coli* O157:H7 might have been enhanced. In cases where high populations of *E. coli* O157:H7 were applied, and two plant harvests occurred on the same day within the same treatment, the recovered *E. coli* O157:H7 populations were higher in plants harvested immediately after the uninoculated irrigation treatment was applied (Table 2). For example, two plant harvests occurred on the seventh day of analysis where one harvest occurred prior to, and one occurred after, irrigation event 3. Greater populations of *E. coli* O157:H7 were recovered from all treatments immediately after the irrigation event. This effect was most likely the result of the residual *E. coli* O157:H7 population's ability to utilize the brief application (and availability) of moisture. This additional moisture also could have facilitated detection by enhancing the separation of *E. coli* O157:H7 cells from the surface matrix of spinach leaves. Because this effect was seen among all irrigation treatments (including A, 0 ppm of TOC), the surface-applied soluble carbon did not appear to provide any protective or selective advantage to the residual *E. coli* O157:H7 populations in the spinach phyllosphere. In fact, even after the application of 10 irrigation events containing high concentrations of TOC (treatment C, 150 ppm of TOC), the harvested spinach tissues did not exhibit any accumulated carbon or nitrogen effects (data not shown). It is possible that the small volumes of the irrigation solutions introduced onto foliar surfaces were insufficient to influence the overall concentrations of the available carbon sources on foliar surfaces.

The literature is replete with a wide range of reports describing various survival and persistence data of *E. coli* O157:H7 on leafy green commodities. These varying results could be due to the deposition of cells in some places on the leaf surfaces that are more favorable for bacterial survival than are others, i.e. stomata, grooves along the leaf veins, and the base of the trichomes and cell wall junctions *(1, 16).* Textured microenvironments of certain spinach cultivars (e.g., Savoy) have been determined to be more suitable for *E. coli* O157:H7 survival than spinach varieties with smooth surfaces *(15).* Solomon et al. *(29)* determined low levels of *E. coli* O157:H7 persistence on lettuce for 30 days after irrigation with 6 log CFU/100 ml, whereas Erickson et al. *(9)* did not recover residual surface or internalized *E. coli* O157:H7 cells in spinach tissue 6 days after irrigation with 6 log CFU/100 ml. Mootian et al. *(18)* found persistence of *E. coli* O157:H7 on lettuce leaves for at least 30 days postexposure with water containing 3 log CFU/100 ml.

This study was designed to determine the efficacy of the current LGMA irrigation water microbiological stan-

dards. We determined that baby spinach plants do not support the survival of *E. coli* O157:H7 for more than 1 day after overhead irrigation with water that complies with the LGMA standards for *E. coli* content. It should be noted that our results were obtained in a controlled growth chamber environment by using *E. coli* populations that were <126 CFU/100 ml. Further study involving field experiments are encouraged to corroborate these findings. Our results also suggest that the repeated introduction of *E. coli* O157:H7 at low populations in irrigation water does not enhance the survival or persistence of the pathogen on foliar surfaces. Furthermore, the TOC content of irrigation water ≤150 ppm, even with repeated irrigation, does not appear to affect the persistence of *E. coli* O157:H7 on spinach tissues. Further work establishing microbiological standards for irrigation water should consider the fate of specific microbial populations on foliar surfaces.

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Quantification of low and high levels of *Salmonella enterica* serovar Typhimurium on leaves

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ABSTRACT

Precise and rapid quantification of low levels of pathogens associated with fresh produce may be particularly challenging and yet evermore necessary to guarantee microbial safety of the produce and to carry out research on the subject of persistence of the pathogens. Here, microbiological and molecular based methods were examined for their ability to precisely quantify different amounts of *Salmonella enterica* serovar Typhimurium artificially inoculated on parsley leaves. Recovery of *S.* Typhimurium from parsley by mechanical detachment using stomacher, mortar and pestle, vortex, sonicator or homogenizer followed by plating resulted in underestimation with less than 1% recovery when leaves were inoculated with 3.5–6.5 log CFU/g. Lower levels were undetectable by most assayed methods, and only recovery with mortar and pestle or adding of enrichment step resulted in partial detection of 300 CFU/g. Implementation of PCR based methods with/without pre extraction of the DNA from the contaminated leaves resulted in more accurate values of the pathogen (about 20% of the initial inocula) and as low as 300 CFU/g were detected even without an enrichment step. These methods can be applied to study transfer of *Salmonlla* from contaminated water or soil to plants using low and more reasonable levels of contamination.

1. Introduction

The modern life, changes in dietary habits and globalization of the produce industry have increased the consumption of fresh fruits and vegetables and minimally processed salads (Brandl, 2006; Little & Gillespie, 2008). As these kinds of foods are consumed raw with minimal or no processing or treatments aimed to destroy pathogens, their contamination hides potential risks (Berger et al., 2010; Little & Gillespie, 2008). Indeed, during recent decades, fresh fruits and vegetables, and in particular leafy greens, have been increasingly recognized as significant reservoirs of food-borne pathogens (Berger et al., 2010; Brandl, 2006; Doyle & Erickson, 2008). In the US, for instance, the proportion of outbreaks associated with fresh fruits and vegetables, of all reported food-borne outbreaks with an identified food source, has increased from 0.7% in the 1970s to 6% in the 1990s (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004) and to 13% in the 2000s (Doyle & Erickson, 2008). Produce associated outbreaks are also reported in Europe. During 1999 and 2000, for example, fruits and vegetables were the incriminated food at 6%, 10% and 17% of all identified cases of food-borne disease outbreaks in Sweden, in the United Kingdom and in Iceland, respectively (Anonymous, 2003).

Outbreaks were linked to various food-borne pathogens, whereas *Salmonella enterica* is considered one of the most commonly identified etiologic agents of such outbreaks. Between 1973 and 1997 48% of outbreaks from fresh produce with identified sources in the US were caused by *S. enterica* serovars. Furthermore, 49 outbreaks caused by *S. enterica* were linked to the consumption of fresh produce in the US during 1990–2004 (Brandl, 2006), and during 2006–2007 *S. enterica* was the confirmed etiologic agent in 19 out of 110 produce related outbreaks reported to CDC (CDC, 2010). Many of these food-borne outbreaks were multi-state and often caused high numbers of illness (Anonymous, 2003; CDC, 2010). During 2007 in the US two of the three largest reported outbreaks were caused by *Salmonella* (CDC, 2010). In 2001, an FDA survey found that 3.5% of produce items were contaminated with *Salmonella*, furthermore the incidence of *Salmonella* on leafy herbs (parsley, celery, cilantro and culantro) was 6.7% (FDA, 2001).

Contamination of raw fruits and vegetables by human pathogenic microorganisms may occur along the food production chain "from farm to fork" (Burnett & Beuchat, 2000). *Salmonella* is able to colonize plants via contaminated soil, manure or irrigation water

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(Franz et al., 2007; Islam et al., 2004; Lapidot & Yaron, 2009). Under appropriate conditions and time such as highly contaminated sprouts grown at high humidity conditions in a growth chamber, the pathogens population can increase and even grow to populations exceeding 10^7 CFU/g (Gandhi, Golding, Yaron, & Matthews, 2001).

Ensuring microbial safety of fresh produce requires accurate and efficient recovery, detection and enumeration methods. The optimal procedure for retrieving pathogenic microorganisms from fruits and vegetables may differ. Washing, blending, homogenizing, stomaching, grinding and sonication are commonly implemented to process samples prior to plating or enrichment (Anonymous, 2009; Buesing & Gessner, 2002; Donegan, Matyac, Seidler, & Porteous, 1991). With growing efforts to improve qualitative and quantitative detection of enteric pathogens, researchers have implemented PCR based methods (Bhagwat, 2003; Gonzalez-Escalona et al., 2009; Miller, Draughon, & D'Souza, 2010; Shearer, Strapp, & Joerger, 2001). These approaches require the extraction of DNA/RNA from the attached bacteria with or without preliminary detachment steps. The efficient separation of bacteria from the leaf tissue (for plate counting or nucleic acids purification) raises a conflict between using procedures harsh enough to detach all adhered bacteria and the risk of plant cell disruption, which may release compounds that inhibit bacterial growth or nucleic acids amplification. Therefore, conditions must be carefully adjusted so that cell disruption is kept to a minimum while detachment efficiencies are maximized. Most enumeration methods allow the detection of high levels of contamination. Lowering the detection threshold is of particular importance for pathogens which characterized with low infectious doses and due to the possibility of bacterial growth during storage (Berran, Brackett, & Beuchat, 1989; Beuchat & Brackett, 1990). For that reason most standard detection methods (microbial counts and PCR based methods) include an enrichment step, which increases the sensitivity, but limits the results to qualitative rather than quantitative detection.

The lack of methods for quantification of low levels of pathogens associated with fresh produce makes it difficult to study routes of contamination in the field, to assess the effectiveness of treatment with sanitizers or to study plant–pathogen interactions. To solve the problem many researchers used very high concentrations of pathogens (above 10^5 CFU/g) to determine how the pathogen transfers from contaminated water/soil/manure to the plants (Islam et al., 2004; Lapidot & Yaron, 2009; Solomon, Yaron, & Matthews, 2002) or to compare the effectiveness of different sanitizers (Shirron et al., 2009). The high levels of contamination applied in these experiments are usually not realistic in terms of contamination levels that possibly occur in the environment or during processing. Other researchers applied an enrichment step or microscopic analysis to determine the transfer and persistence of the pathogens without quantification of the exact numbers (Mootian, Wu, & Matthews, 2009; Solomon et al., 2002). Thus there is a pressing need for the evaluation of implemented procedures for accurate enumeration of low levels of pathogens on produce in order to ensure food safety and facilitate *in-planta* studies.

The aim of this research was to examine and to improve the ability of different, commonly implemented, microbiological and molecular based methods to detect, recover and precisely quantify *S.* Typhimurium bacteria artificially inoculated on parsley leaves and to establish minimal detection thresholds.

2. Materials and methods

2.1. Bacterial strains

Salmonella enterica serovar Typhimurium ATCC 14028 (*S.* Typhimurium) was employed for contamination. Electrocompetent *Salmonella* cells were transformed with pGFP plasmid (Clontech, Palo Alto, CA) by electroporation using a MicroPulser™ electroporator (Biorad Laboratories), to obtain Green Fluorescent Protein (GFP)-labeled cells. Transformants were selected by plating onto Luria-Bertani (LB) agar plates supplemented with ampicillin (100 μg/ml), and stored at −80 °C in Luria-Bertani supplemented with glycerol 20 g/100 g. The stability of GFP expression by *S.* Typhimurium ATCC 14028 on parsley leaves had been investigated and described in the past (Lapidot & Yaron, 2009).

2.2. Preparation of bacterial suspension and inoculation

Overnight cultures of *S.* Typhimurium grown in LB broth supplemented with ampicillin (100 μg/ml) were diluted 1:100 in fresh LB with ampicillin and incubated at 37 °C until the cells reached an optical density at 600 nm of ~0.4. Cells were harvested by centrifugation (4600 g for 10 min at 4 °C), washed in saline and resuspended in saline to reach a final concentration of $\sim 10^7$ CFU/ml. The cultures were serially diluted in saline to prepare bacterial suspensions at concentrations ranging from $\sim 10^2$ to 10^6 CFU/ml. For each experiment samples of parsley leaves, each weighing 20 g, 1 g or a single leaf (weighed about 0.1 g) were surface inoculated by applying 20, 1 or 0.1 ml, respectively, of bacterial suspension to reach final concentrations of $\sim 10^2$ to 10^6 CFU/g. Surface inoculation was performed by spotting the suspension on to the leaves' adaxial surface. Six to ten spots in a total of 0.1 ml were applied on each leaf and spread by sterile tip to cover the leaf's surface. Inoculated samples were kept 45 min under aseptic conditions to dry the liquids, and then processed in one of the methods described below.

2.3. Bacterial detachment and recovery for plate counting

Following inoculation bacteria were recovered by one of the following methods:

2.3.1. Pummeling in a stomacher

Each 1 g sample was immersed in 40 ml sterile saline or saline supplemented with Tween 20 (0.5, 5 and 50 ml/L). Each sample was pummeled in a stomacher (IUL instruments, Spain) for 3 min at normal speed (620 paddle strokes per min) to release the attached bacteria. The extracts were filtered through 3 layers of sterile gauze and centrifuged (6000 g for 15 min at 4 °C). The pellet was resuspended in 1 ml sterile saline.

2.3.2. Mortar and pestle

Each 1 g sample was manually crushed by mortar and pestle for 2 min in 20 ml sterile saline to release the attached bacteria. The extracts were filtered through 3 layers of sterile gauze and centrifuged (6000 g for 15 min at 4 °C). The pellet was resuspended in 1 ml sterile saline.

2.3.3. Vortexing

Each 1 g sample was added to 40 ml sterile saline in a 50 ml conical tube and vigorously vortexed for 1 min. The liquid was collected and centrifuged (6000 g for 15 min at 4 °C) and the pellet was resuspended in 1 ml sterile saline.

2.3.4. Homogenization

Each single leaf sample (0.1 g) was immersed in 0.9 ml saline in a 2 ml sterile test tube and processed by homogenization for 30 s at 14,000 rpm (Polytron, Kinematica, Switzerland). The homogenized sample was collected.

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2.3.5. Ultrasonication bath

Each 1 g sample was added to 20 ml sterile saline in a sterile blender bag (100 ml) and sonicated in an ultrasonic bath for 16 min at 30 kHz, 300 W (TPC-40, Telsonic, Switzerland). The liquid was collected.

2.3.6. Ultrasonication probe

Each 1 g sample was added to 20 ml sterile saline in a 50 ml conical tube and sonicated by an ultrasonic probe for 90 s at 20 kHz, 750 W, 20% amplitude on ice (VCX750, Sonics & Materials Inc., U.S.A.). The liquid was collected.

Control samples (bacteria in saline without parsley leaves) indicated that there was no bacterial lysis due to homogenization or ultrasonication under these conditions. Following detachment and recovery samples were serially diluted 1:10 in sterile saline and plated on LB agar supplemented with ampicillin (100 μg/ml) for enumeration of green fluorescent colonies.

2.4. Detection of Salmonella following enrichment

Salmonella detection was carried out according to Israeli Standard 885-7 - "Microbiological test methods for foodstuffs: Detection and identification of Salmonellae", which is very similar to international standard procedures. Briefly, samples of contaminated leaves (20 g or 1 g) were immersed in buffered peptone water (180 ml or 9 ml, respectively), processed in a stomacher and incubated for 24 h at 37 °C. One ml and 0.1 ml of each enriched sample was added with 9 ml Tetrathionate Brilliant Green Broth (Difco) and 10 ml Rappaport-Vassiliadis Broth (Difco), respectively, and incubated for 24 h at 37 °C and 42 °C, respectively. Following enrichment, loop-fulls were streaked on Brilliant Green Agar plates (Difco) and incubated for 24 h at 37 °C. From each plate at least 3 typical *Salmonella* colonies were confirmed as *Salmonella* on Triple Sugar Iron Agar slants (Difco) and on Lysine Iron Agar slants (Difco).

2.5. Enumeration by real time qPCR following lysis by heat treatment

Five samples of single parsley leaves, each weighing approximately 0.1 g, were surface inoculated with 0.1 ml of bacterial suspension. Additional leaves samples were not inoculated by *Salmonella* and served as negative control samples. Following contamination, each leaf was immersed in 1 ml of double distilled water in an eppendorf test tube and subjected to heat treatment at 100 °C for 30 min. Following the treatment the supernatant was collected. Real Time qPCR was performed using 2 μl of supernatant containing bacterial cells, 100 and 250 nmol/L concentrations of Forward (CGGGGAGGAAGGTGTTGTG) and Reverse (GAGCCCGGGGATTTCACATC) primers respectively, and 5 μl ABsolute™ QPCR SYBR Green Mix (ABgene) in 10 μl total reaction volume. This couple of primers was designed specifically for the 16S rRNA gene of *S.* Typhimurium (Fey et al., 2004; Lee et al., 2009), but can also detect other *Salmonella* serovars such as *S.* Paratyphi. A recent BLAST search showed that except for *Salmonella* the ribosome sequence of very few strains has homology to both primers. However the expected PCR products of these strains differ in size and sequence from that of *Salmonella* and thus are expected to show a different melting curve. Only three bacteria give expected product which is similar to the *Salmonella* PCR product: *Leclercia adecarboxylata*, *Enterobacter ludwigii* and *Enterobacter cloacae*. All three are opportunistic pathogens in human and *E. cloacae* was also isolated from fruits and vegetables. A three-step protocol was used in Rotor-Gene 3000 (Corbett Research): (i) denaturation (15 min at 95 °C); (ii) an amplification and extension program repeated 50 times (1 s at 95 °C, 15 s at 65 °C and 20 s at 72 °C); and (iii) a melting curve program of heating from 72 to 99 °C, at a heating rate of 1 °C per 5 s. The concentration of experimental samples was calculated from the linear regression of a standard curve, obtained by heat treated bacterial suspensions at known concentrations (10^1–10^6 CFU/ml).

2.6. Enumeration by real time qPCR following DNA extraction

Following contamination, all samples as well as non-inoculated leaves (*Salmonella*-free control samples) were individually placed in an eppendorf test tube and immersed in liquid nitrogen for 10 min. Samples were grinded to a fine powder (for 2 min) by a sterile QuadLoop on liquid nitrogen. Total bacterial DNA was extracted using different methods. The best results were obtained when DNA was extracted with ZR Soil Microbe DNA kit™ (ZYMO RESEARCH) according to the manufacturer's instructions in a final volume of 50 μl of DNA prep. Real-Time qPCR analysis was performed as described above using 2 μl of each isolated DNA. The concentration of experimental samples was calculated from the linear regression of a standard curve, obtained by purified DNA (isolated by the same kit) from bacterial suspensions at known concentrations (10^2–10^6 CFU/ml).

2.7. Staining and imaging of contaminated leaves by laser scanning confocal microscopy

In order to quantify bacteria that survived the inoculation process, Laser Scanning Confocal Microscopy (LSCM) was utilized and the ratio of viable vs. dead bacteria on the leaf surface was determined by staining. Parsley leaves, each weighing 0.1 g, were placed in sterile Petri dish and surface inoculated with 0.1 ml of GFP-expressing bacterial suspension by spotting as described above. Samples were stained with Propidium Iodide (PI) - red fluorescent nucleic acid stain- for the differentiation of viable and dead bacteria. Freshly inoculated leaves were added with 200 μl of staining solution (0.2 μmol/L PI in sterile saline) directly on to the leaves' surface, so that the surface was entirely covered by the staining solution. The Petri dish was covered and incubated for 15 min at room temperature, protected from light. Leaves were very gently rinsed with 10 ml sterile saline to remove all excess stain and placed on a microscope glass slide. Leaves were imaged using Zeiss LSM 510 META Confocal Microscope (Carl Zeiss, Germany). The entire procedure beginning from PI stock dilution through imaging was protected from light and carried out the same day. Green fluorescent bacteria were counted as viable whereas red fluorescent bacteria were counted as dead. A total of 6 independent experiments were conducted and for each stained leaf at least 10 images were taken randomly for counting. *Salmonella*-free parsley leaves served as control samples.

2.8. Statistics

All experiments were conducted at least three independent times in duplicates. Data was analyzed in JMP 4.0.4. (SAS Institute Inc.) by one-way analysis of variance; means were analyzed using Student's *t*-test. P value of <0.05 was accepted as indicating significance.

3. Results

3.1. Recovery of S. Typhimurium from parsley leaves

Accurate enumeration of bacteria adhered to fresh produce using a protocol which is based on plating on selective medium requires their detachment from the plant tissue prior to plating.

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This research focused on several commonly used methods and compared their ability to detach and recover *S.* Typhimurium from parsley leaves. Leaves were inoculated with known numbers of bacteria and following the detachment process the recovered bacteria were plate counted. Results show that no method completely recovered the pathogen (Table 1). Following stomacher processing, for example, the cultivable *S.* Typhimurium bacteria represented less than 1% of the initial pathogen population inoculated on parsley leaves. This low recovery was obtained with all initial concentrations between 3.5 and 6.5 log CFU/g. In addition, contamination with 2.5 log CFU/g was not detected. The addition of surfactant Tween 20 at concentrations up to 50 ml/L did not facilitate the detachment and recovery of *S.* Typhimurium from parsley and did not result in higher bacterial counts. Recovery with mortar and pestle was slightly but not significantly higher than recovery with stomacher. Moreover, lower abundance of the pathogen, as little as $\sim 10^2$ CFU/g was detected in some (but not all) the samples. Like the detachment with stomacher, mortar and pestle also recovered only ~1% of the adhered cells. Vortexing method has proven to be limited in its ability to recover the bacteria and was not efficient at contamination levels below 5 log CFU/g (Table 1). Similarly, sonication and homogenization of parsley leaves did not result in notable detachment of *S.* Typhimurium from parsley even with the high contamination levels.

3.2. Enrichment of S. Typhimurium from parsley leaves

Detection of *S.* Typhimurium after enrichment followed by plating on the selective medium allowed only qualitative results. Table 2 presents the frequency of detection of *S.* Typhimurium inoculated on 1 g samples of parsley leaves in order to compare these results with the other methods. The sensitivity of the enrichment method was similar to the sensitivity of plate counting after recovery with mortar and pestle. At high concentrations of 4.5–5.5 log CFU/g the pathogen was readily detected. However, at lower concentrations the enrichment method was limited in its ability to detect the pathogen and resulted in false negative, as observed in some samples when leaves were inoculated by 3.5 log CFU/g and below. Inoculated *S.* Typhimurium at levels of 1.5 log CFU/g was not detected following the enrichment.

3.3. Recovery of microbial DNA from parsley leaves

Techniques based on the detection of microbial DNA resulted in more accurate estimation of *S.* Typhimurium on parsley leaves (Table 3). Real Time qPCR on DNA released from lysed bacteria was the most rapid technique. With this method the estimated bacterial abundance was within a range of 0.7–1.2 log CFU/g lower than the initial inoculated concentration. Low bacterial levels of 2.5 log CFU/g proved to be undetectable by this method. Implementation of Real Time qPCR, following the direct extraction of DNA, resulted in the most accurate estimation of *S.* Typhimurium on parsley leaves. This method has proven to be significantly ($P < 0.05$) more accurate when compared to stomacher processing and plating. Implementation of this method resulted in detection of 20–30% of the inoculated cells. Moreover it allowed the detection and estimation of 2.5 log CFU/g. Melting curve analysis of the amplified PCR products confirmed positive amplification of the target gene only in the inoculated leaves (data not shown).

Table 1
Recovery of *S.* Typhimurium from parsley leaves by different detachment methods.[a]

Detachment process	Inoculum (log CFU/g)				
	6.5	5.5	4.5	3.5	2.5
Stomacher	4.2 ± 0.2	3.1 ± 0.2	1.8 ± 0.6	0.6 ± 0.8	ND
Stomacher & Tween 20[b]	3.2 ± 0.5	2.0 ± 0.4	0.6 ± 0.7	ND	NA
Mortar and pestle	4.4 ± 0.6	3.7 ± 0.5	2.3 ± 0.3	1.2 ± 0.8	0.7 ± 0.8
Vortexing	2.3 ± 0.6	1.7 ± 0.6	ND	ND	NA
Homogenization	3.5 ± 0.4	ND	ND	ND	NA
Ultrasonication bath	2.7 ± 0.5	ND	ND	NA	NA
Ultrasonication probe	3.2 ± 0.5	ND	ND	NA	NA

ND – Not Detected, bacteria were below the detection limit (1 log CFU/g for Stomacher, Mortar and pestle and Vortexing methods, 2 log CFU/g for Homogenization and 2.3 log CFU/g for Ultasonication).
NA – Not Analyzed.
[a] Mean log CFU/g recovered by plating after detachment process ±SD.
[b] Results with 5 ml/L Tween 20 are shown. Higher or lower concentrations of Tween 20 gave lower recovery.

Table 2
Frequency of detection of *S.* Typhimurium inoculated on parsley leaves after enrichment.

	Inoculum (log CFU/g)				
	5.5	4.5	3.5	2.5	1.5
Frequency[a]	6/6	6/6	4/6	2/6	0/6

[a] Number of samples that tested positive for *S.* Typhimurium following the enrichment process, out of the total inoculated samples.

3.4. Survival of Salmonella on the leaves

Estimation of *S.* Typhimurium by detachment and plating for viable counts allowed the detection of less than 1% of the inoculated cells, while DNA based enumeration detected more than 20% of the cells. This raised the question whether the bacteria are viable on leaves after the inoculation. To answer this question we inoculated the leaves and stained the bacteria with PI. Enumeration of viable *S.* Typhimurium in 6 different experiments, as presented in Table 4, indicated that approximately 40% (from 31% to 50%) of artificially inoculated bacteria in the leaf are viable.

4. Discussion

Detection of pathogens in samples of fresh ready to eat foods such as leafy greens is required for two main purposes – to identify contaminated foods prior to distribution in order to increase the safety of the consumers, and to carry out research on the subject of transfer and persistence of the pathogens in/on the plants. The current international standards for *Salmonella* is lack of bacteria in 20 or 25 g. Thus detection of the pathogen is sufficient for routine tests by the food industry (positive/negative results), but for research activities it is crucial not only to detect the pathogen, but also to accurately quantify its levels. A rapid test for detection of low levels in fresh produce is urgently needed, since as opposed to foods from animals such as poultry and meat that harbor high levels of enteric bacteria, fruits, vegetables and leafy greens usually harbor low levels of contamination. A widely accepted notion that

Table 3
Quantification of *S.* Typhimurium on parsley leaves by real-time qPCR based methods.[a]

Extraction method	Inoculum (log CFU/g)				
	6.5	5.5	4.5	3.5	2.5
Heat lysed bacteria[b]	5.8 ± 0.8	4.8 ± 0.8	3.3 ± 0.4	2.4 ± 0.9	ND
Extracted DNA[c]	6.0 ± 1.3	4.8 ± 1.0	3.9 ± 1.7	2.8 ± 1.2	0.6 ± 1.2

ND – Not Detected.
[a] Mean log CFU/g ±SD as quantified by Real Time PCR.
[b] Limit of detection 1.5 log CFU/g.
[c] Limit of detection 0.1 log CFU/g. Controls of heat lysed and extracted DNA samples from non-spiked parsley samples were performed. Limit of detection for each extraction method was determined by the *Salmonella*-free control samples. Melting curve analysis of the amplified PCR products confirmed positive amplification of the target gene only in the inoculated leaves.

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Table 4
Counts of viable vs. non-viable *S.* Typhimurium on spiked parsley leaves.[a]

	Total cells	Non-viable	Viable	% Viable
Ex1	94 ± 27	47 ± 19	47 ± 20	50 ± 26
Ex2	99 ± 26	54 ± 20	45 ± 16	45 ± 20
Ex3	77 ± 23	39 ± 14	38 ± 19	49 ± 28
Ex4	81 ± 20	54 ± 17	27 ± 11	33 ± 16
Ex5	112 ± 20	63 ± 16	49 ± 12	44 ± 13
Ex6	61 ± 14	42 ± 13	19 ± 5	31 ± 11
Average[b]	87 ± 9	50 ± 7	37 ± 6	42 ± 8

[a] Numbers of viable (green fluorescent) and dead (red fluorescent) *S.* Typhimurium cells harboring pGFP plasmid that were inoculated on parsley leaf surface and stained with PI. A total of 6 independent assays were conducted and at each stained leaf random 10 areas were analyzed.

[b] Average counts of *S.* Typhimurium observed on spiked leaves. The non-viable microflora of *Salmonella*-free control samples was characterized with amorphic clusters and the cells that were similar in shape and size to *Salmonella* represented less than 5% of the counts observed on spiked leaves.

the ingestion of $\geq 10^5$ *Salmonella* cells is required for clinical symptoms in humans, based on studies involving healthy volunteers (Blaser & Newman, 1982; Kothary & Babu, 2001), has been challenged by investigations of food-borne outbreaks of Salmonellosis. These investigations led to the conclusion that the infective dose is serovar dependent. In large outbreaks infective dose was often low and the ingestion of as little as 10 to 100 cells could result in illness (Blaser & Newman, 1982). For instance, a nationwide outbreak of Salmonellosis in Germany, which led to about 1000 cases, was traced to paprika and paprika-powdered potato chips. The estimated infective dose was as low as 4–45 Salmonellae (Lehmacher, Bockemuhl, & Aleksic, 1995). Similarly, the infective dose of *S.* Newport on alfalfa sprouts was estimated to be less than 460 CFU (Aabo & Baggesen, 1997). The lack of methods used to quantitatively detect low levels of pathogens associated with fresh produce makes the study of routes of contamination in the field with reasonable numbers of the pathogens in soil, fertilizers or in water unfeasible. Thus the aim of this study was to examine and improve procedures for accurate enumeration of low levels of pathogens on produce.

A critical step in enumerating pathogens on fresh produce using the standard plate counting methods is the release of the surface-attached cells into the buffer (Miller, Davidson, & D'Souza, 2010). It was suggested in the past that recovery of pathogens by mechanical detachment may not result in complete detachment of the viable bacterial population from the surface due to the hydrophobic nature of the produce surface together with its roughness (Iturriaga, Escartin, Beuchat, & Martinez-Peniche, 2003). Indeed recovery of *S.* Typhimurium from parsley leaves by mechanical detachment followed by plating did not allow the precise detection and enumeration of the population of *S.* Typhimurium. According to our study the maximal recovery was 2 log lower than the initial levels of inocula. Furthermore, the efficiency of recovery and enumeration varied substantially in the methods examined. Although very low, recovery of 1% is slightly better than previous reports about recovery of *Salmonella* from vegetables. For example, the maximal recovery of *S.* Typhimurium from inoculated lettuce, peppers and tomatoes by direct plating was more than 3 log lower than the initial levels of inocula (Miller, Davidson et al., 2010; Miller, Draughon et al., 2010). Studies have reported on variations in the efficiencies of methods for recovery and enumeration of bacteria applied to the phyllosphere. In leaf litter, for instance, about 2.5 log difference between grinding manually by mortar and pestle and sonication, the most and least efficient methods, respectively, was described (Maamri, 2000). In other experiments stomacher blending allowed the greatest recovery of microorganisms from leaf surface (Donegan et al., 1991). Further studies, on the other hand, reported that processing by stomacher resulted in significantly lower bacterial counts in comparison to ultasonication and tissue homogenization of leaf litter samples (Buesing & Gessner, 2002). Both ultrasonication and homogenization methods, according to our results, were limited in their ability to recover *S.* Typhimurium from parsley leaves. It was found that addition of Tween 20 improves recovery of *Salmonella* from surface of lettuce (Miller, Davidson et al., 2010), but here the addition of Tween 20 did not improve the recovery efficiency. However it should be mentioned that our recovery of viable *Salmonella* from parsley without Tween 20 was 0.8 log higher than the highest recovery in the study cited above for *Salmonella* from lettuce with Tween 20 added.

Treatment with mortar and pestle was the only method that gave positive results in portion of the samples inoculated with 300 CFU/g. Based on these results, implementation of standard recovery methods for the detection of enteric pathogens in fresh produce may result in underestimation of the contamination levels. Furthermore, in cases of low abundance of pathogens on leafy greens, such methods may not detect the presence of the pathogens. This may explain why routine testing of fresh produce or investigation of potential sources of outbreaks usually does not recognize contaminations. Failure to accurately detect pathogenic microorganisms may have implications on food safety and research.

Another standard option to qualitatively detect low levels of *S.* Typhimurium on leaves can be achieved following enrichment. However, again, detection of low levels of *S.* Typhimurium gave false negative results. Actually enrichment increased the detection time but did not improve the sensitivity comparing to recovery with mortar and pestle. False negative results following enrichment of *Salmonella* in other foods and environmental samples spiked with low levels of the pathogen have been previously reported. Inoculation of red salsa with ~20 cells per sample resulted in false negative results following enrichment (Franco, Hsu, & Simonne, 2010). Similarly, inoculation of meat, poultry and dairy products with low levels of *Salmonella* resulted in partial ability of the enrichment method to detect presence of the pathogen (Bennett, Greenwood, Tennant, Banks, & Betts, 1998). Nam et al. reported that inoculation of sterilized fecal samples by *S.* Typhimurium, *Listeria monocytogenes* or *Escherichia coli* O157:H7 at levels of 10–100 CFU/sample resulted in successful detection of the pathogens following enrichment. However, inoculation of unsterilized samples resulted in false negative results (Nam, Murinda, Nguyen, & Oliver, 2004). Samples harboring high numbers of competing bacteria may lead to overgrowth of the background microflora and result in false negative results following enrichment, especially in cases of low levels of the target pathogen (Bhagwat, 2003; Liao & Shollenberger, 2003; Maddox, 2003; Nam et al., 2004; Shearer et al., 2001). For example, high numbers of coliforms can readily out-compete *Salmonella* (Maddox, 2003). Parsley leaves, investigated in the present study, harbor high levels of natural microflora (as many as 7.3 log CFU/g) and an abundant coliform population of 6.6 log CFU/g (Shirron et al., 2009). Thus, low levels of *S.* Typhimurium inoculated on to parsley leaves may have been outcompeted by the natural microflora during the enrichment process which lead to false negative results.

Irreversible attachment to the surface of the leaf and the presence of competing bacteria are probably not the only factors that restrict detection and decrease the bacterial counts. It is possible that a portion of the bacteria die on the leaves (Anonymous, 2009). Images of the stained bacteria showed that about 60% of the inoculated bacteria indeed die on the leaves following application of the inoculum. This observation does not fully explain the low recovery rates (usually less than 1%), but if we add to the calculation injured, stressed or non-cultivable cells we can explain at least 1-log reduction between inoculation and plate counting. This reduction should be considered in studies that include artificial

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contamination. In field studies the situation is even more complicated. Plants are considered as a hostile habitat for enteric pathogens, exposing them to harsh physicochemical conditions. Bacteria in produce may be debilitated by desiccation and harsh acidic environment or by the plant defense systems (Anonymous, 2009; Brandl, 2006; Iniguez et al., 2005). Thus, the possibility that the detached cells might include stressed or injured pathogens should be recognized. These pathogens, thought viable, may not be cultivable and detectable by plating. Furthermore, previous observations showed that *Salmonella* forms aggregates on/in the leaves days after the contamination (Brandl & Mandrell, 2002; Brandl, Miller, Bates, & Mandrell, 2005; Lapidot & Yaron, 2009; Warriner & Namvar, 2010). These aggregates may cause underestimation of the cells on the plants. In our study we eliminated this option by counting the bacteria short time after the inoculation. Indeed we observed very few aggregates in the images obtained by the confocal microscope.

We showed that the PCR based methods gave very accurate values (20–30% of the initial inocula when parsley was inoculated with 3.5–6.5 log CFU/g), and detected low levels of contamination, even without enrichment. However at low levels (2.5 log CFU/g) the precision of the method decreased. Detection of *Salmonella* by PCR based methods in fruits, vegetables and environmental samples had been demonstrated. Detection of *Salmonella* in artificially inoculated tomatoes by PCR resulted in detection limits of 10 and 100 CFU/g on and in tomatoes, respectively, after 6 h of enrichment (Guo, Chen, Beuchat, & Brackett, 2000). Similarly, qualitative detection of *Salmonella* in artificially inoculated fecal samples at concentrations of 10^2 CFU/g was achieved by PCR based methods following 6–72 h enrichment step (Halatsi et al., 2006; Pathmanathan et al., 2003). A lower limit was achieved (~4 CFU/25 g produce) for detection of *Salmonella* species in artificially inoculated cantaloupe, mixed salad, cilantro and alfalfa sprouts by Real Time PCR following 16 h enrichment (Liming & Bhagwat, 2004). Several commercial kits are now available for the detection of *Salmonella* in food matrices by PCR. These kits require an enrichment procedure of 16–48 h prior to the PCR test (Malorny, Huehn, Dieckmann, Kramer, & Helmuth, 2009). Implementation of a commercial kit allowed the detection of 5 CFU/25 g *Salmonella* following enrichment for 16 h in foods (Hein, Flekna, Krassnig, & Wagner, 2006). Cheung et al. evaluated the detection limit of a different commercial system to be 60–6000 CFU/g depending on the type of food, after an enrichment step of 18 h (Cheung, Chan, Wong, Cheung, & Kam, 2004). Assays for the detection frequencies of *Salmonella* by commercial kits from spiked fresh produce and meat samples (3–5 CFU/25 g) resulted in detection of *Salmonella* after enrichment of 18–27 h (Liming & Bhagwat, 2004; Uyttendaele, Vanwildemeersch, & Debevere, 2003). As mentioned, all studies and commercial kits are based on an enrichment step prior to PCR assay of the inoculated samples, and have resulted in only qualitative detection of the pathogen population. PCR based methods that were implemented in this study, allowed the quantitative detection of the target pathogen within as little as 2–4 h, thus providing an advantage in terms of food safety and research, in comparison to enrichment and plating based methods which require 1–5 days. Moreover, we were able to detect accurate values of contamination even without extraction of DNA, by heat treatment of the sample. The high sensitivity was probably achieved because we chose *rrn* gene (16S rRNA) as a target (Fey et al., 2004), unlike other reports, which described PCR targeting of *Salmonella* specific genes (e.g. *invA*) (Hein et al., 2006; Malorny et al., 2009; Miller, Davidson et al., 2010; Perelle et al., 2004). There are seven *rrn* operons in the genome of *Salmonella*, whereas *invA* is a single-copy gene (McClelland et al., 2001). The disadvantage of *rrn* as a target relies on the fact that the bacterial *rrn* genes are conserved. Although we chose *Salmonella* specific primers, it is still possible that other bacteria will be positive too. For that reason it is crucial not only to check the specificity of the primers in updated data bases, but also to analyze the melting curve of the PCR product, when the target microorganisms should be detected in the presence of high numbers of natural microflora.

Molecular techniques such as techniques based on the detection of bacterial DNA, can detect non-cultivable or stressed bacteria in plant tissues, increasing the sensitivity (Lopez et al., 2003). On the other hand a main drawback of DNA based methods relies on their inability to discriminate between live and dead cells. However this drawback is less relevant in field studies aimed to detect mechanisms of transfer of the pathogen from irrigation water/soil/fertilizers/insects and other factors in the environment to the plants. In these cases the detection method is aimed to identify and quantify bacteria that moved to the plants and the fate of the bacteria in the following stages of adaptation to the plant environment is less important. Implementation of detection methods based on real-time reverse transcriptase PCR could solve some of the shortcomings of direct PCR, because they detect only the viable cells. However since bacterial RNA is unstable, it is very difficult to obtain enough RNA from low numbers of cells, and the sensitivity of the method is lower, as can be seen from results published recently (Miller, Davidson et al., 2010). With regards to food safety, and particularly with foods not originated from animals, detection of both viable and non-viable enteric pathogens in produce may indicate improper agricultural practices and prompt interventions in order to prevent further health hazard.

To summarize, we have shown that quantitative evaluation of *Salmonella* on parsley leaves with standard methods results in significant underestimation of the actual bacterial levels with both high and low levels of contamination. On the other hand real-time qPCR based methods are more accurate and can be performed rapidly with no need for an enrichment step. At very low numbers (2.5 log CFU/g) direct plating following detachment with mortar and pestle gave more repeatable results (lower SD values). However this method is time consuming and cannot be used for large numbers of samples. Real-time qPCR based methods can now be used to study transfer of *Salmonella* to the plants from contaminated irrigation water, soil or compost using realistic levels of contamination. Detection after enrichment was also very sensitive at the high inoculation levels, but it is not applicable for accurate quantification. We showed that detection of pathogens on plants can be achieved even without extraction of DNA, making the detection approach rapid and cheap. This may indicate that the PCR based approach can be implemented in order to quantify the presence of pathogenic bacteria, study plant–pathogen interactions, and evaluate the potential for contamination and invasion of the pathogen to fresh produce, especially in cases of low levels of the pathogens.

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ORIGINAL ARTICLE

Survival of Pathogenic *Escherichia Coli* on Basil, Lettuce, and Spinach

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Impacts

- Theresults of this study emphasise the need for future studies assessing the survival of environmental and zoonotic *E. coli* isolates compared with known pathogenic *E. coli* isolates on produce crops.
- Thiswork is the first reported survival of *E. coli* O104:H4 on a produce commodity.
- Thepersistence of APEC and *E. coli* O104:H4 were similar on basil plants, indicating that *E. coli* isolates may be adaptable to non-host environments and show increased persistence compared with other clinical, pathogenic *E. coli* isolates.

Keywords:
E. coli; persistence; irrigation; basil; survival; lettuce; spinach; promix; avian pathogenic *E. coli*

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Summary

The contamination of lettuce, spinach and basil with pathogenic *E. coli* has caused numerous illnesses over the past decade. *E. coli* O157:H7, *E. coli* O104:H4 and avian pathogenic *E. coli* (APECstx- and APECstx+) were inoculated on basil plants and in promix substrate using drip and overhead irrigation. When overhead inoculated with 7 log CFU/ml of each strain, *E. coli* populations were significantly ($P = 0.03$) higher on overhead-irrigated plants than on drip-irrigated plants. APEC*stx*-, *E. coli* O104:H4 and APEC*stx*+ populations were recovered on plants at 3.6, 2.3 and 3.1 log CFU/g at 10 dpi (days post-inoculation), respectively. *E. coli* O157:H7 was not detected on basil after 4 dpi. The persistence of *E. coli* O157:H7 and APECstx- were similar when co-inoculated on lettuce and spinach plants. On spinach and lettuce, *E. coli* O157:H7 and APEC populations declined from 5.7 to 6.1 log CFU/g and 4.5 log CFU/g, to undetectable at 3 dpi and 0.6–1.6 log CFU/g at 7 dpi, respectively. The detection of low populations of APEC and *E. coli* O104:H4 strains 10 dpi indicates these strains may be more adapted to environmental conditions than *E. coli* O157:H7. This is the first reported study of *E. coli* O104:H4 on a produce commodity.

Introduction

Fresh fruits and vegetables are increasingly recognised as significant sources of foodborne illness outbreaks, causing about 5–23% of confirmed cases of foodborne illness in many countries, including the United States (Little and Gillespie, 2008; Tyler and Triplett, 2008). Fruit and vegetable products can become contaminated in the pre-harvest environment through a variety of routes including improperly composted manure, contaminated irrigation water, wild and domestic animals and soil (Beuchat, 2002). Leafy greens are most commonly eaten raw without processing steps that would reduce or eliminate pathogenic bacterial populations. Within the last few years, outbreaks associated with fresh produce, including leafy greens, have been linked to the emergence of shiga toxin producing non-O157 *E. coli* serotypes still considered enterohemorrhagic *E. coli* (EHEC). One notable outbreak in Germany in 2011 was caused by *E. coli* O104:H4, and a shiga toxin produce *E. coli* (STEC) strain linked to sprouted contaminated fenugreek seeds that sickened thousands of people in 2011 (Centers for Disease Control, 2011). This particular *E. coli*

isolate is an enteroaggregative E. coli (EAggEC) that also produces shiga toxin, characteristic of EHEC strains; which classified the E. coli O104:H4 isolate as an STEC (Wu et al., 2011). A multistate outbreak of *E. coli* O145 in the United States linked to shredded romaine lettuce from a single processing facility occurred in May of 2010 (Centers for Disease Control, 2012a,b). More recently, a multistate outbreak of *E. coli* O26 linked to raw clover sprouts occurred in February and April of 2012 (Centers for Disease Control, 2012a,b). The emergence of these highly virulent non-O157 STEC species associated with produce outbreaks poses a threat to public health. Little is known about the fitness and survival of these pathogens on leafy greens in comparison with known *E. coli* O157:H7 strains, and whether they endure environmental stresses of pre-harvest environments better than O157-serotypes.

The versatile behaviour of *E. coli* species throughout the environment can be explained in part by the genomic diversity of strains within this species. The genomes of *E. coli* strains can differ by up to 20% (Liu et al., 1999). The virulence gene for shiga toxin production (*stx*) in STEC has been found to be transferable to nonpathogenic *E. coli* strains through transduction, allowing for enhanced virulence of these previously nonpathogenic strains (Yaron et al., 2000; Herold et al., 2004). Until recently, the *E. coli* serotype most commonly associated with foodborne illnesses was thought to be shiga toxin producing *E. coli* O157:H7. In Scallan et al. (2011). reported that annually in the United States, there are approximately 63, 153 domestically acquired illnesses associated with O157 STEC (*E. coli* O157:H7) and approximately 112 752 domestically acquired illnesses associated with non-O157 STEC serotypes. Therefore, the examination of the survival of non-O157 STEC in pre-harvest produce growing environments is needed.

APEC (avian pathogenic *E. coli*) are widespread in poultry and causes colibacillosis in birds raised for meat and eggs, resulting in millions of dollars in losses in the poultry industry (Barnes et al., 2008). APEC can also cause extraintestinal infections in humans. APEC strains are considered members of the extraintestinal *E. coli* (ExPEC), a pathogenic group of *E. coli* that also includes human uropathogenic *E. coli* (UPEC) and *E. coli* causing neonatal meningitis and septicaemia (Johnson and Stell, 2000; Kaper et al., 2004). Mellata et al. (2009) observed that virulence genes associated with plasmids in APEC strains, including pAPEC-1, particularly those involved in iron acquisition, are also prevalent in human ExPEC indicating the zoonotic risk to humans of APEC strains. Poultry consumption has been found to serve as a source of human acquired ExPEC (Johnson et al., 2005). And, while no APEC isolates from poultry have been classified as EHEC or STEC, in a survey of *E. coli* isolates from wild birds, 5% and 25% of isolates were positive for *stx* and *eae*, respectively (Kobayashi et al., 2009). The virulence factor *eae* is a determinant virulence factor in EPEC and EHEC strains. As these virulence factors are responsible for the ability to cause gastrointestinal infections in humans, potential APEC strains which contain these virulence factors could contribute to the burden of foodborne illness currently unattributable to an aetiological agent (Scallan et al., 2011) as well. Understanding the persistence of these strains on herbs, leafy green commodities and in pre-harvest environments may also help determine the risk to public health from both APEC and non-O157 STEC strains.

The increasing evidence of zoonotic transfer of APEC to humans indicates the need for the study of APEC alongside more established pathogenic serotypes of *E. coli* in high-risk food systems such as fresh produce and leafy greens. The purpose of this study was to determine the survival of APEC and pathogenic strains of O157 and non-O157 *E. coli* on leafy greens including basil, lettuce and spinach. The survival of APEC strains and non-O157 *E. coli* strains was assessed individually and as a multi-strain inoculum on plants.

Materials and Methods

Strains and irrigation solution preparation

For basil plants, the survivals of four different *E. coli* types were individually assessed. An inoculum consisting of two APEC isolates lacking the *stx*2 and *eae* virulence genes, an APEC isolate containing the *stx2* and *eae* genes, *E. coli* O104:H4 (German outbreak strain, ATCC TY2482), *E. coli* O157:H7 strain RM-4407-GFP-Nal was described previously (Sharma et al., 2009) and originally isolated from the 2006 US spinach outbreak. The two APEC isolates lacking the *stx*2 and *eae* genes included one *E. coli* O157 serotype (APEC 07-1707); one *E.coli* O8 serotype (APEC 07-1307) and were labelled as APEC*stx*-. A rare APEC isolate containing the *stx2* and *eae* virulence genes is an *E. coli* O13 serotype (APEC 07-5668) and was labelled as APEC*stx*+. All APEC strains were isolated from poultry broilers within the Delmarva (Delaware, Maryland and Virginia) area. All strains used in survival studies on basil plants were previously resistant to 50 μg/ml nalidixic acid (Sigma-Aldrich, St. Louis, MO). Cultures of each isolate were grown in modified EHEC broth (Biocontrol, Bellevue, WA) with 50 μg/ml nalidixic acid (mEHECN) at 37°C while shaking (New Brunswick Scientific, New Brunswick, NJ) at 200 rpm for 24 h. Cultures were then diluted 10-fold into autoclaved dairy manure slurry prior to inoculation to obtain a concentration of approximately 10^6 CFU/ml as confirmed by enumeration on sorbitol MacConkey agar (SMAC) (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 50 μg/ml nalidixic acid (SMACN). The

method used to create the dairy manure slurry was similar to that of Ingram et al. (2011). In brief, fresh manure was collected from the University of Delaware dairy farm (Newark, DE) and centrifuged in 50 ml tubes at 2500 × ***g*** (Beckman Coulter, Fullerton, CA) for 10 min to separate the solid from the liquid portion of the manure. The liquid portion of the manure was autoclaved for sterilization prior to inoculation and was used as the irrigation solution.

For lettuce and spinach plants, the APEC inoculum consisted of different strains than those used with basil. Four APEC strains which did not contain the *stx*2 gene – 07-0717, 07-6098, 05-2848 and 05-2737- were received from the University of Delaware collection and labelled as APEC*stx*-, were used in these studies. Strains 05-2848 and 05-2737 were made resistant to 50 μg/ml nalidixic acid by selecting a spontaneous mutation resistant to nalidixic acid. Strains 07-0717 and 07-6098 were previously resistant to 50 μg/ml nalidixic acid. Strains were struck from frozen stock on to SMACN. *E. coli* O157:H7 strain RM-4407-GFP-Nal (Sharma et al., 2009) was used in these experiments as well. Each of the five strains was inoculated separately in 10 ml of sterile PLE (poultry litter extract) and incubated at 37°C for 48 h. PLE was obtained by obtaining poultry litter from the University of Maryland Eastern Shore (UMES), diluted 1 : 10 in sterile deionized water and then stirred for 5 min before filtering through a double-layer of cheese cloth. The extract was then diluted 1 : 1 with deionized water and sterilized to prepare PLE. After incubation at 37°C in PLE, *E. coli* strains were placed on ice and stored at 4°C for 24 h to stabilize bacterial populations. Populations of individual strains were enumerated on SMACN. Cultures of APEC and *E. coli* O157:H7 strains were then combined in a single inoculum that contained 1.3×10^5 CFU/ml of each APEC strain and 5×10^5 CFU/ml *E. coli* O157:H7 for a total inoculum at 1×10^6 CFU/ml to be applied to spinach or lettuce plants.

Basil cultivation

Thai basil (*Ocimum basilicum)* seeds (Johnny's Selected Seeds, Waterville, ME) were disinfected by soaking in a 10% bleach solution for 2 min when the outer coat of the seed began to turn white. Seeds were then planted in a sterile promix made up of 85% Canadian sphagnum peat moss with perlite, vermiculite, dolomitic and calcitic lime, a wetting agent and Mycorrhizae (Premier Tech Horticulture, Quakertown, PA) and maintained in a Biosafety Level 2 (BSL-2) growth chamber (Percival Scientific, Boon, IA) at the University of Delaware Fischer Greenhouse (Newark, DE). Growth conditions used included 30°C temperature with a 12-hour photoperiod until 2 two leaves were present on each plant (10–14 days). Plants were then transplanted to individual containers containing sterile promix (square containers 4 cm × 3.5 cm × 4.5 cm in dimension; T.O. Plastics, Ontario, Canada). Holes were created in the bottom of each individual container, which was then placed into plastic bins inside the growth chamber. Throughout the experiment, water was poured into the bottom of each plastic container to prevent influence of bacterial movement on plants and promix by direct watering. The growth chamber temperature conditions were monitored daily, temperature was maintained at 30 ± 0.30°C, relative humidity was 44.3 ± 2.08%, and the water activity (A_w) of the promix was maintained at 1.046 ± 0.04.

Spinach and lettuce cultivation

Plants were grown in the Biosafety Level 2 (BSL-2) growth chamber (Conviron 4030, Winnipeg, Canada) at the Environmental Microbial and Food Safety Laboratory USDA ARS facility in Beltsville MD. Both spinach and lettuce plants were grown in sterilized, fine sandy loam (Keyport-Matawan) soil obtained from the ARS Beltsville Agricultural Research Center (BARC) north farm. For spinach, autoclaved soil was placed in sterile 'conetainers' (cone-shaped plastic vessels, model SC10, 164 ml, Stuewe & Sons, Inc. Tangent, OR.), each suitable for growing a single spinach plant. Spinach (*Spinacia oleracea*) cultivar 'Blackhawk' (Seminis, Oxnard, CA) was planted in these conetainers. During experiments, each plant was irrigated once a week with 20 ml of sterile water supplemented with 1.32 g/L Jack's Classic All Purpose 20-20-20 fertilizer (J.R. Peters, Inc., Allentown, PA). For Romaine lettuce (*Lactuca sativa*), cultivar 'Fresh Heart Paragon' (Seminis) was grown in square containers. Both lettuce and spinach were grown under conditions set to 70% relative humidity for a 14-h photoperiod at 20°C and 10-h dark period at 15°C.

Plant and promix inoculation

Basil plants were inoculated when two true leaves were present on the plant (approximately 14-day-old plants). Basil plants were inoculated with each strain individually by either drip or overhead irrigation to simulate a onetime contamination event. For drip irrigation, 3 ml of irrigation solution was inoculated directly onto the promix of plants by pipette with no or minimal splash. For overhead irrigation, plants were sprayed directly with irrigation solution using an air brush (model 200, Badger Air Brush Company, Franklin Park, IL) for 15 s (3 ml). Plant and promix samples were collected on days 0, 1, 4, 7 and 10. Plants were inoculated in a biosafety cabinet prior to being placed in the growth chamber.

Lettuce and spinach plants were inoculated directly on the foliar surface at 30 and 28 days of age, respectively.. For each plant, 100 μl of the inoculum containing a

multi-strain inoculum of APEC*stx*⁻ and *E. coli* O157:H7 was added to each plant by adding 20 droplets (5 μl/droplet) using a micropipette to foliar tissue. Five droplets/leaf were added to each plant. Droplets were allowed to dry for 30 min in growth chambers before harvesting and enumeration.

Recovery and enumeration

Basil plant and promix samples were pooled into 3 plants per sample (approximately 0.46 g/plant) or 3 promix containers per sample (approximately 13.07 g/container). Plants were cut 1 cm from the promix surface with sterile scissors and placed into sterile stomacher bags, while the entire container of promix was placed into stomacher bags. All samples were mixed with 40 ml of mEHEC broth supplemented with 50 mg/ml nalidixic acid (mEHECN) and stomached for 2 min. Enumeration was performed by standard plate count on SMACN. For lettuce and spinach plants, plants were harvested using sterile scissors to cut the shoot tissue above the promix surface from each spinach or lettuce plant. The average weight of lettuce and spinach plants was 5 and 3 g, respectively. Leaves were then deposited into a sterile stomacher bags, and 40 ml of mEHECN was added to each bag. Samples were then stomached (Bagmixer, Interscience, St. Nom, France) for 2 min. The resulting homogenate was then either plated on SMACN (Day 0) or used for MPN (Most Probable Number) determination (remaining days). A three-tube MPN assay was performed by adding 10, 1, or 0.1 ml of homogenate to 0, 9, or 9.9 ml mEHECN and incubated at 37°C for 24 h. A loopful (10 μl) from tubes which displayed turbidity was then struck for isolation SMACN plates. Colonies which did not show sorbitol fermentation (pale colonies) were determined to be *E. coli* O157:H7; colonies which evidenced fermentation of sorbitol were determined to be APEC*stx*-. For each plant, MPN assays were performed in triplicate. On each day of analysis, either 6 spinach plants or 6 lettuce plants were harvested and microbiologically analysed. When MPN assays yielded undetectable numbers of *E. coli*, plant material in stomacher bags was enriched with 40 ml of mEHECN and incubated at 37°C for 24 h. Enriched samples were then streaked for isolation to determine the presence of *E. coli* O157:H7 or APEC.

Statistical analysis

Experiments were performed in duplicate for at least 2 trials per strain with each trial performed at different times. Results are reported as the means and standard deviations as log CFU/g plant or promix. A one-way ANOVA was performed to compare means within the data set, and a linear regression model was generated using JMP 9 software (SAS Institute Inc., Cary, NC). All p values <0.05 were considered significant ($\alpha = 0.05$). For lettuce and spinach MPN determination, the freeware calculator (VB6, www.i2workout.com/mcuriale/mpn/index.html) was used. Results were expressed as MPN/g.

Results

Individual strain survival on basil plants and promix

Overall, *E. coli* persisted at higher populations for longer periods of time in promix substrate than on plants ($P < 0.0001$). The greatest survival of *E. coli* from promix was for samples drip irrigated with APEC*stx*+ with 4.36 ± 0.04 log CFU/g recovered after 10 days (Fig. 4). Basil plants which were spray irrigated with APEC*stx*- had higher populations (3.60 ± 0.06 log CFU/g) compared with other spray-irrigated *E. coli* (Fig. 1). Recovery of *E. coli* O157:H7 populations was lower than that of other *E. coli* (APEC*stx*-, APEC*stx*+, or *E. coli* O104:H4) from both promix and plants regardless of the irrigation method used; however, differences in survival of *E. coli* populations were statistically insignificant ($P = 0.80$). For this particular isolate, no bacteria were detected after day 4 on plants that were spray irrigated or after day 1 on plants that were drip irrigated (Figs 1 and 2). *E. coli* O157:H7 was recovered from promix at populations of 3.31 ± 0.12 and 2.64 ± 0.65 log CFU/g when overhead and drip irrigated, respectively, on day 10. (Figs 3 and 4).

Simultaneous survival on lettuce and spinach

APEC and *E. coli* O157:H7 were co-inoculated simultaneously on lettuce and spinach plants. Initial populations

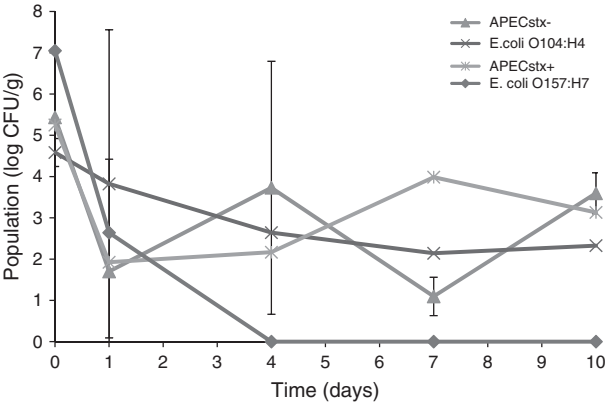

Fig. 1. Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto basil plants by overhead irrigation over 10 days.

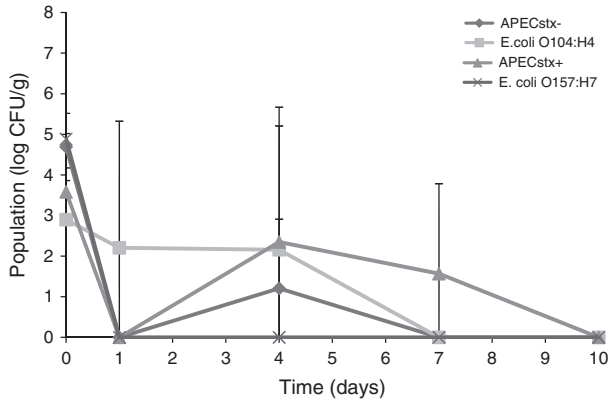

Fig. 2. Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto basil plants by drip irrigation over 10 days.

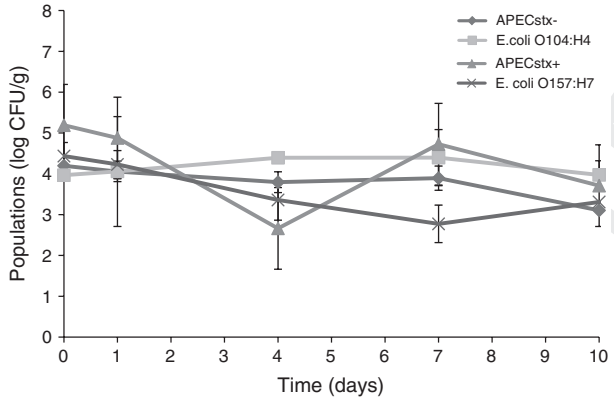

Fig. 3. Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto promix of basil plants by overhead irrigation over 10 days.

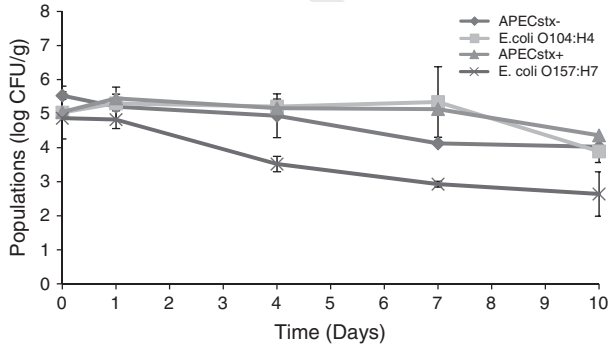

Fig. 4. Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto promix of basil plants by drip irrigation over 10 days.

(day 0) of APEC on spinach and lettuce were 6.1 and 4.5 log CFU/g, respectively. Initial populations (day 0) of *E. coli* O157:H7 on spinach and lettuce were 5.7 and 4.5 log CFU/g, respectively (Figs 5 and 6). Populations of both types of *E. coli* declined rapidly on both commodities by day 1, as APEC populations declined by 4 log MPN/g on spinach and by 2.8 log MPN/g on lettuce. Similar declines were observed with respect to *E. coli* O157:H7 on both leafy green commodities; population declined by 3.9 log MPN/g on spinach and 3.7 log MPN/g on lettuce. *E. coli* populations persisted for shorter durations on spinach compared with lettuce; by day 3, no APEC or *E. coli* O157:H7 were detected by enrichment on spinach plants. On lettuce, however, low levels of APEC strains were detectable by MPN 8 days after inoculation. Both APEC and *E. coli* O157:H7 were detectable by enrichment 14 and 17 days after inoculation. APEC survived at higher levels than *E. coli* O157:H7 on 7 and 8 days post-inoculation on lettuce plants.

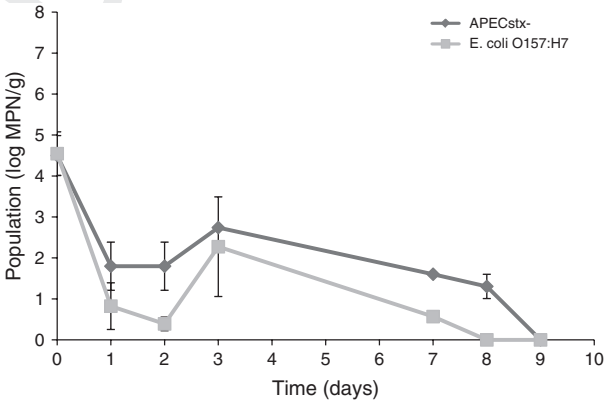

Fig. 5. Survival of a multi-strain inoculum of Avian pathogenic *E. coli* (APEC) without *stx*2 gene (APEC*stx*-) and *E. coli* O157:H7 co-inoculated on spinach plants over 17 days.

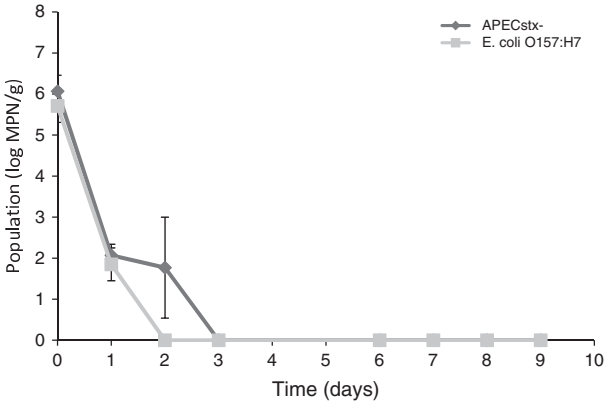

Fig. 6. Survival of a multi-strain inoculum of Avian pathogenic *E. coli* (APEC) without *stx*2 gene (APEC*stx*-) and *E. coli* O157:H7 co-inoculated on lettuce plants over 6 days.

Discussion

Individual survival on basil plants and promix

This study utilises different methodologies to inoculate pathogenic *E. coli* onto three different produce commodities and determines that persistence of *E. coli* may be influenced by use of different plants and bacterial strains. This study also utilised a rare APEC isolate (*E. coli* O13) which contains both *stx* and *eae* genes, classifying it as an EHEC strain. The isolation of an APEC strain with EHEC virulence factors shows importance of evaluating APEC strains on produce for their potential ability to cause human illness. Even among the different inoculum methods and strains used, some overall conclusions can be reached. *E. coli* persisted at significantly ($P = 0.03$) higher populations on plants which were overhead irrigated compared with those which were drip irrigated after 10 days. These results imply that, when irrigation is necessary, basil should be irrigated using a drip irrigation system which prevents direct application of irrigation water onto the foliar surface of plants. However, this study also shows that even when careful application of contaminated water was applied directly to promix, *E.coli* was still detected on plants on day 0. The detection of *E. coli* on plants that were drip irrigated could have been due to unintentional splash from the promix substrate onto plants. Recent studies have demonstrated that splash that occurs during irrigation can propel *E. coli* on promix to heights up to 20 cm and horizontal distances of 25 cm (Monaghan and Hutchison, 2012).

Simultaneous survival on lettuce and spinach

The lack of simultaneous survival of both APEC and *E. coli* O157:H7 strains on spinach plants compared with lettuce was surprising, given that inoculation methods were the same for both leafy green commodities. It is unclear why spinach plants supported a shorter duration of survival than lettuce plants for both APEC and *E. coli* O157:H7 strains. The exact role of epiphytic bacterial populations on enteric bacterial survival on foliar surfaces is unclear (Patel et al., 2010). It is possible that the epiphytic bacteria population on spinach leaves were more antagonistic towards *E. coli* than epiphytes on lettuce (Cooley et al., 2006). However, this interaction could have extended the duration of survival by protecting *E. coli* from ultra-violet stress, and other antagonistic stresses encountered by enteric bacteria on foliar surfaces. Other investigators have shown that bacterial cells introduced to a leaf surface have a better chance of surviving when they are deposited on or in aggregates of other bacteria (Monier and Lindow, 2005). These aggregates are characterised by an exopolysaccharide matrix, which contains a dense population of bacterial cells. The interaction of the APECs and *E. coli* O157:H7 with these aggregates may also partially explain their extended survival on the foliar tissue of lettuce plants; conversely, the potential lack of interaction of these aggregates on spinach surfaces may have also led to the more rapid decline of *E. coli* O157:H7 populations compared with on lettuce surfaces. It is also possible that lower recovery of both APEC and *E. coli* O157:H7 from lettuce plants on day zero compared with spinach plants indicates that *E. coli* attached more tightly to lettuce tissue compared with spinach tissue, and fewer bacteria were able to be dislodged or were agitated from the lettuce tissue during homogenisation, leading to lowered recovered populations detected by MPN. Previous work has shown that *Salmonella* Tennessee exhibited stronger attachment to intact lettuce than to intact cabbage leaves (Patel and Sharma, 2010). In this same work, different serovars (*S.* Tennessee and *S.* Negev) showed different strengths of attachment to the same types of leaves (lettuce). These findings indicate the variations in attachment, which occurred among closely related *Salmonella* serovars, may have also occurred in our study with *E. coli* strains and affected recovery and enumeration from the leafy green tissue. In addition, it should be noted that plants were inoculated during the daytime in a biosafety cabinet, where light intensity may have been different from that of the growth chamber. Because plant stomata respond to changes in light (Zieger, 1983), this light difference may have affected stomatal closure or opening and therefore; entry of the pathogens into the plant. Plants were held within the biosafety cabinet for inoculation for <30 min. These experiments were performed in a controlled environment within a growth chamber inside greenhouse facilities. It should be acknowledged that the results of these experiments may not reflect exact environmental conditions that plants experience in the field, such as animals, insects, wind. However, due to governmental regulations restricting the use of pathogens in field studies, growth chamber studies must be utilised in place of field studies.

Pathogens survive at similar rates on plants individually and simultaneously

In general, APEC survived at populations approximately 1 log MPN/g higher than *E. coli* O157:H7 on lettuce on days 1, 2, 7 and 8. Whether this difference in survival is related to the potential enhanced environmental fitness of APEC strains compared with *E. coli* O157:H7 is unclear. As the APEC inoculum consisted of four strains compared with one strain for the *E. coli* O157:H7 inoculum, it is possible that the strain diversity in the APEC inoculum contained one or more strains which were more persistent than the *E. coli* O157:H7 outbreak strain used in this work. APEC

strain 07-1707 is an *E. coli* O157 serotype and was used in both individual inoculation studies on basil and simultaneous inoculation studies on spinach and lettuce. The potential survival of this strain compared with *E. coli* O157:H7 on all three commodities may indicate that persistence on foliar surfaces is less a function of serotype and more dependent on source of isolation or previous environmental exposure of the strain. Previous studies demonstrated that when co-inoculated on to spinach foliar surfaces, nonpathogenic *E. coli* isolates from produce commodities survived at higher populations for up to 28 days, compared with *E. coli* O157:H7 strains from produce outbreaks, which only survived for 7 days (Patel et al., 2010). Other investigators have shown that a nonpathogenic *E. coli* isolates from soil had significantly higher levels of persistence on spinach plantlets and in potting mix than *E. coli* isolates from irrigation water or Romaine lettuce (Guttierez-Rodriguez et al., 2011). These authors hypothesise that the specific geospatial location of the origin of the isolate may affect its environmental fitness. Our findings may indicate that *E. coli* O157:H7 strains from produce outbreaks may not survive as well in non-host environments (on foliar surfaces, in soilin water) as *E. coli* isolated from environmental sources, where they have had more of an opportunity to adapt to stresses in pre-harvest, leafy green growing environments. APEC are extraintestinal pathogenic *E. coli*; responsible for a variety of infections in humans and domestic animals using diverse virulence factors (Rogers et al., 2010). The high prevalence of faecal carriage of APEC in livestock is thought to lead to dissemination in the environment and foodborne transmission to humans (Rogers et al., 2010; Vincent et al., 2010). These findings again support the hypothesis that although highly pathogenic, *E. coli* O157:H7 outbreak strain may not possess the environmental fitness of other environmentally isolated *E. coli* isolates.

Although different inoculation methods were used for both of these studies, similar patterns in the survival of APEC and *E. coli* on basil, lettuce and spinach were observed. Whether APEC strains were inoculated individually or simultaneously with *E.coli* O157:H7 on plants, APEC persisted at greater populations compared with *E. coli* O157:H7. In addition, on all three plant commodities, *E. coli* O157:H7 declined rapidly initially and could not be detected after 4 days on basil or after 2 days on spinach. This is similar to the rapid decline of *E.coli* O157:H7 observed in previous studies examining inoculated spinach. In that study, more than one isolate of *E. coli* O157:H7 applied to spinach plants with an airbrush declined by 2–3 log MPN/plant between day 0 and day 1 (Ingram et al., 2011). In our study, *E. coli* O157:H7 survived up to 8 days on lettuce when simultaneously inoculated with other APEC strains.

To our knowledge, this was the first study assessing *E. coli* O104:H4 persistence on produce. Interestingly, *E. coli* O104:H4 and APEC populations survived at similar levels on basil plants. These strains may have enhanced environmental fitness compared with the *E. coli* O157:H7 strain used in this study. This addresses the hypotheses that some *E. coli* strains have evolved towards an enhanced fitness in open environments (van Elsas et al., 2011) such as on plants and in soil, and may indicate that these strains have been present in these environments for durations long enough to adapt to these conditions. Much of this hypothesis was applied to the *E. coli* O104:H4 German outbreak strain linked to contaminated fenugreek seeds. A recent study by Safadi et al. (2012) reported that *E. coli* O104:H4 has enhanced abilities to form biofilms *in vivo*, enabling them to survive in a complex host environment. The ability of *E. coli* O104:H4 strain to form biofilms may explain its enhanced persistence on basil compared with *E. coli* O157:H7. However; the *E. coli* O157:H7 isolate used in this study was from the 2006 spinach outbreak that caused over 200 illnesses and 3 deaths in the United States was also found to contain a gene (*norV*, nitric oxide reductase) potentially correlated to increase its ability to cause haemolytic-uraemic syndrome (HUS) and enhance the strain's virulence compared with other *E.coli* O157:H7 isolates (Kulasekara et al., 2009). Similarly, the *E.coli* O104:H4 German outbreak strain caused a higher percentage of HUS cases (19.8%) among victims of this outbreak than any other STEC outbreak previously recorded. (EFSA(European Food Safety Authority), 2011; Safadi et al., 2012). The emergence of these exceptionally virulent pathogenic *E. coli* which show the ability to persist on produce surfaces poses a major threat for public health and consumption of raw produce.

This study demonstrated that avian pathogenic *E. coli* (APEC) isolates survived at higher populations and for longer durations when individually inoculated onto basil plants or co-inoculated on lettuce and spinach plants compared with *E. coli* O157:H7 survival. Duration of survival was observed to be dependent upon the method of irrigation and plant type, which included differences in epiphytic bacteria, leaf structure and topography. On basil, *E. coli* O104:H4 survived similarly to APEC strains for 10 days, indicating enhanced environmental fitness for these strains. The survival of APEC strains on basil and leafy green commodities, and their potential contribution to the burden of foodborne illness worldwide, indicates a potential public health risk associated with contaminated produce. The results of this study emphasise the need for future studies assessing the survival of environmental and zoonotic *E. coli* isolates compared known pathogenic *E. coli* isolates on produce crops.

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