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Effect of Volumetric Water Content and Clover (*Trifolium incarnatum*) on the Survival of *Escherichia coli* O157:H7 in a Soil Matrix

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Abstract Studies aimed at understanding *Escherichia coli* O157:H7 soil survival dynamics are paramount due to their inevitable introduction into the organic vegetable production systems via animal manure-based fertilizer. Therefore, a greenhouse study was conducted to determine the survival of *E. coli* O157:H7 in highly controlled soil matrices subjected to two variable environmental stressors: (1) soil volumetric water content (25 or 45 % VWC), and (2) the growth of clover (planted or unplanted). During the 7-week study, molecular-based qPCR analyses revealed that *E. coli* O157:H7 survival was significantly lower in soils maintained at either near water-holding capacity (45 % VWC) or under clover growth. The significant reduction under clover growth was only observed when *E. coli* populations were determined relative to all bacteria, indicating the need to further study the competition between *E. coli* O157:H7 and the total bacterial community in organic soils. Given the significant effect of clover on *E. coli* O157:H7 survival under different moisture conditions in this greenhouse-based study, this work highlights the antimicrobial potential of clover exudates in arable soils, and future work should concentrate on their specific mechanisms of inhibition; ultimately leading to the development of crop rotations/production systems to improve pre-harvest food safety and security in minimally processed, ready-to-eat and organic production systems.

VWC	Volumetric water content
qPCR	Quantitative real-time polymerase chain reaction
EC/TB	*E. coli* O157:H7/total bacteria

Introduction

Due to changing dietary habits that include higher quantities of fresh fruits and vegetables, there has been an increased demand for minimally processed, ready-to-eat produce [18, 29], including an 20 % annual increase in organically grown produce [15]. Inherent in these production systems, especially organic systems, is the use of manures or animal wastes as crop nutrient and soil organic matter sources. The livestock/poultry that produce this organic fertilizer are widely considered to be the source of zoonotic pathogens, including *Escherichia coli* O157:H7, into the "farm-to-fork" production continuum [16, 17, 36]. *Escherichia coli* O157:H7 is classified as an enterohemorrhagic *E. coli* that can cause severe gastrointestinal infections in humans, and this serotype alone annually results in over 100,000 infections and 60 deaths within the United States [10]. The application of manures/animal wastes to arable lands has resulted in human infections [1, 24], and soils can provide a matrix for the transfer of manure-borne zoonotic pathogens from organic wastes to crops [22, 34, 35, 51].

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Under field conditions, culturable *E. coli* O157:H7 have been recovered from 30 to over 230 days following inoculation [22, 32, 36, 47, 51], demonstrating the persistence of this enteropathogen in the seemingly inhospitable soil environment. With recent major *E. coli* O157:H7 outbreaks involving minimally processed vegetables [9, 11], the need to understand the various environmental parameters that significantly influence *E. coli* O157:H7 survival in these ready-to-eat and organic vegetable cropping systems is paramount. To retain soil structure and nutrients and prevent invasive weed growth, organic vegetable production systems utilize a variety of cover crops (i.e., green manures), with numerous clover species being routinely included into these cropping systems. While the use of different crops and cropping systems can significantly affect the survival of *E. coli* O157:H7 [34, 35], no studies have specifically looked at the effect of clover, even though several clover species produce phenolic compounds with antibacterial properties [5, 20, 44, 60].

Of all of the competing physio-chemical soil characteristics that affect *E. coli* O157:H7 survival, soil moisture is one of the most dominant parameters. Studies have shown that *E. coli* O157:H7 survival increases at higher moisture levels [23, 45, 57] and higher (i.e. more positive) water potentials [26, 59]. In these studies, either native field or reactively maintained microcosm (samples taken, moisture determined, and then the soils were re-hydrated to a pre-defined level) moisture levels were compared to *E. coli* O157:H7 survival. Proactively maintaining soil moisture levels by continually measuring volumetric water content (VWC) and adding small volumes of water in real time [46] represents an improvement for assessing the effect of a constant moisture level on *E. coli* O157:H7 survival in soils. While this type of technology has been used in the horticulture/floriculture fields, it can be easily adapted to greenhouse-scale, pot-based studies of pathogen survival/inactivation.

In determining the effect of VWC and clover growth on *E. coli* O157:H7 survival, the accurate detection of this enteropathogen from soils is essential. Traditional culture-based assays (direct/selective plating, substrate utilization) are considered the "gold standard" in microbial food safety and security. Unfortunately these methods are time-consuming; presumptive results take at least 24–48 h depending on the pathogen of interest. Recently, much more rapid (~2–4 h) molecular-based analyses based on quantitative real-time PCR (qPCR) have become available to accurately detect and quantify pathogens from a variety of environmental settings. Recent studies have shown that qPCR-based detection methods for *E. coli* are strongly correlated to culture-based plate assays in water or wastewater settings [33, 42, 48, 61], but very few have looked at these correlations in the more complex soil setting [54]. Therefore, in the context of this study, it is important to determine the efficacy of using qPCR-based assays targeting the *uidA* gene to characterize the survival of *E. coli* O157:H7 in soils. This accurate quantification directly relates to the main goal of this controlled greenhouse-based study: to assess the effect of [1] different soil moistures (maintained in real time) and [2] clover growth on the survival of *E. coli* O157:H7 within a defined soil matrix.

Materials and Methods

Bacterial Strain, Culture Conditions, and Preparation of Inocula

Avirulent *E. coli* O157:H7 strain B6914, without the *stx*1 and *stx2* genes were used in this experiment (obtained from J. Karns of the USDA-ARS in Beltsville MD). This *E. coli* strain also includes an inserted stable plasmid that contains both ampicillin resistance and green fluorescent protein (GFP) genes, which enable for the specific identification of this strain from native microbial flora [22]. Pure cultures of this strain were maintained in Luria Broth (LB; Difco Laboratories, Detroit MI) containing 50 mg ampicillin per mL (LB-A) and 10 % glycerol until needed.

When required, a loopful (~1 mL) of frozen culture was streaked onto LB-A agar plates and incubated overnight at 37 °C under aerobic conditions. After incubation, growth of this strain was confirmed via the excitation of the inserted GFP gene product using a hand-held UV light (Blak-Ray UVL-56, Ultra-violet Products Inc., San Gabriel, CA), and only isolated, fluorescing colonies were chosen for further culturing. A single isolated colony was inoculated into 100 mL of LB-A and incubated for 12–16 h at 37 °C under aerobic conditions with agitation (18–200 rpm). The culture was harvested, transferred to a 250-mL sterile Nalgene centrifuge bottle (Fisher Scientific, Pittsburgh, PA), and three times pelleted by centrifugation at 5000×*g* for 20 min at 4 °C. Pellets were washed and re-suspended in filter-sterilized 0.1× phosphate buffered solution (PBS; Lonza, Vierviers, Belgium). Final cell pellets were re-suspended in 100 mL of 0.1× PBS to achieve a final concentration of $\sim 1 \times 10^9$ cells mL^{-1}. Cultures were inoculated into the soil mixture to achieve an initial concentration of 10^8 CFU g^{-1} soil (dry weight), which was confirmed by both plating on LB-A plates and quantitative real-time PCR (qPCR).

Experimental Set-up, Inoculation of the Soil Mixture, and Sampling Protocol

A soil matrix was constructed that consisted of 1 part topsoil mix (Owen J Folsom, Inc, Old Town, ME) and 3

parts peat-based substrate (Fafard 1P mix; Conrad Fafard, Inc, Agawam, MA). This soil matrix was used not to emulate field conditions, but to minimize any watering issues inherent in field soils (i.e., heterogeneous packing, poor drainage, heterogeneous weed seed persistence) that would have interfered with accurate and repeatable irrigation and nutrient control and uniform rooting conditions. Also, to avoid the heterogeneous addition of nutrients, microflora, chemicals, etc., to these highly controlled soil matrices, *E. coli* O157:H7 was added directly to the soil matrix rather than spiked into manure amended to the soil matrix (more similar to field conditions).

Initial moisture content was determined and soil moisture sensors were calibrated in this matrix. Physio-chemical analysis of the starting soil mixture can be seen in Table 1. Within a controlled greenhouse environment, four treatments (each containing eight replicates) were used to compare the effect of volumetric moisture content (VWC) and the presence of clover (*Trifolium incarnatum*; a commonly used organic cover crop) on the survival of *E. coli* O157:H7 in our soil matrix. Treatments were distributed in a 2 × 2 factorial design, being separated into either low or high VWC (25 and 45 % w w^{-1}, respectively), and within each VWC level there were planted and unplanted treatments.

For each replicate, sterilized (washed in 10 % bleach solution and triple-rinsed in distilled water) 15-cm pots contained 928 g soil (wet weight at ~20 % VWC) to occupy a volume of 1,518 mL. Sterilized plastic pans were placed under the pots to catch any water lost from the soil during the experiment. Prior to filling, soil substrate was mixed inside a plastic bag with the required amount of nano-pure water to achieve the treatment setpoint (125 and 350 mL pot^{-1} for low and high VWC treatments, respectively), controlled release fertilizer (14N–14P–14K at a rate of 3.56 kg m^{-3} or 5.5 g pot^{-1}), and inocula [100 mL of the *E. coli* cell suspensions was added to achieve a starting *E. coli* O157:H7 concentration of ~10^8 CFU g^{-1} (dry weight)]. Fertilizer application rate was low due to a lack of leaching of irrigation water during the experiment. Soils were manually homogenized within the plastic bags and added to the pre-sterilized pots, after which clover ("Cardinal Red", Aroostook Milling, Houlton, ME) was planted at a rate of 20 seeds pot^{-1}, which was an equivalent seeding rate of 17.5 lbs $acre^{-1}$ (19.6 kg ha^{-1}; matches local industry standards for clover as a cover crop). Seed was distributed uniformly over the surface of a filled pot, and then an additional 1 cm of soil mixture was placed on top, covering the seeds. A capacitance sensor (model EC-5, Decagon Devices, Pullman, WA) was inserted in one pot per treatment. The sensor measured VWC of that pot and activated the irrigation system when the VWC was lower than the pre-defined setpoint for that treatment. Finally, 1-g fine vermiculite was spread over the surface to assist in moisture retention during germination. As a no *E. coli* O157:H7 control, additional replicate pots ($n = 3$) for each treatment were created using only sterile nano-pure water to achieve the low and high VWC levels (225 and 450 mL, respectively).

Environmental parameters (temperature, relative humidity, solar radiation) were measured hourly within the greenhouse (Table 2) to determine any environmental sources of variability with a datalogger and multiplexer system (models CR10X and AM416; Campbell Scientific, Logan UT). Weekly soil samples (10 g) were aseptically collected from each replicate pot ($n = 5$ for inoculated experimental and $n = 3$ for uninoculated control) by pooling five randomly selected 2 g core samples from each pot. Soil samples were refrigerated immediately after collection and were analyzed with 24–48 h of collection. Gravimetric water content of the soil mixtures throughout the experiment were determined by drying the samples overnight at 65 °C and calculating the difference between the wet and dried weights of the soil. In rare cases when water was found in the drip pans below the pots, the water was collected aseptically and the water samples were vacuum filtered using a 0.20-μm membrane filter (Barnstead/Thermolyne, Dubuque, IA). These filters were stored at −23 °C until analysis.

Table 1 Physiological characteristics of the soil matrix

Parameter	Unit	Value[a]
Total solids	%	70.0
Volatile solids	%	14.4
Total carbon	%	6.3
Total nitrogen	%	0.17
C:N ratio		37.1
NH_4-N	mg kg^{-1}	38.60
NO_3-N	mg kg^{-1}	51.40
Total potassium	%	0.13
Total phosphorus	%	0.07
pH		6.0
Bulk density	g cm^{-3}	0.26
Conductivity	mmho cm^{-1}	1.0

A soil matrix consisted of 1:3 ratio of topsoil to peat-based substrate

Analysis performed by the Soil Analytical Laboratory at the University of Maine, Orono, using standard methods

[a] Values reported on a dry weight (d.w.) basis

Irrigation System

Substrates were irrigated using an automated irrigation system similar to that described by Nemali and van Iersel [46]. This system uses capacitance sensors (EC-5, Decagon Devices, Pullman, WA) to measure the VWC (θ = volume

Table 2 Average environmental parameters within the greenhouse throughout the 7-week study

Environmental parameter	Unit	Value[a]
Day length	h	13–14[b]
Day temperature	°C	17.5 (1.32)
Night temperature	°C	26.4 (4.90)
Relative humidity	%	33.3 (8.85)
Daily light integral	mol m^{-2} day^{-1}	16.6 (6.96)
Average instantaneous light	µmol m^{-2} s^{-1}	341[c]

[a] Mean (SD) of hourly measurements over the entire 7-week study

[b] Day length increased during the study

[c] Assuming an average day length of 13.5 h

of water ÷ volume of soil) of substrates every 60 s. The datalogger then calculated θ using a substrate specific calibration equation (θ = voltage × 1.7647 − 0.4745, $r^2 = 0.95$). The datalogger sent a signal to a relay driver (SDM-CD16AC; Campbell Scientific, Logan, UT) to open a solenoid valve (2.5 cm, 24 VAC solenoid valves; Nelson Turf, Peoria, IL) if θ was below the θ set point. When solenoid valves opened, plants were irrigated using pressure compensated drip emitters. The volume of water discharged from each emitter within each line was measured before the experiment at least three times. Emitters were replaced until outputs were uniform within each line for each treatment. Lines emitted 3.0 mL of water during a single irrigation event.

Extraction and Quantification of *E. coli* O157:H7 DNA

Methods for DNA extraction and quantitative real-time PCR (qPCR) analysis of total bacteria (based on 16S rRNA gene [28]) and *E. coli* (based on the *uidA* gene [21]) have been previously described. The primers and probes used, including the original method reference, are shown in Table 3. For each replicate, genomic DNA was extracted from 500 mg of the pooled soil sample using the MP Bio FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH) following the manufacturer's specifications. Quantification and purity of the extracted genomic DNA was assessed using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). All extracts were diluted 1:10 in sterile molecular biology grade water (5 Prime, Inc., Gaithersburg, MD) so that ~10 ng of genomic extract DNA was added to each qPCR reaction. All qPCR assays were run on the Eppendorf realplex 2 S thermal cycler (Eppendorf, Happauge, NY) in a total volume of 25 µL using the PerfeCta® qPCR Supermix (Quanta Biosciences, Gaithersburg, MD) following the published thermocycling conditions and final primer and probe concentrations (Table 2). Total cell concentrations for *E. coli* O157:H7 and total bacteria were calculated by determining the copy number g^{-1} soil (as determined by qPCR) and factoring in the average copy number $cell^{-1}$ of either the *uidA* [21] or 16S rRNA [38] genes (1 and 3.6, respectively).

To confirm the presence of culturable *E. coli* O157:H7 in the different treatments, 45 mL of 0.1× PBS was added to 5 g of each soil sample in a sterile Whirl-Pak bag (Nasco, Ft. Atkinson, WI) and manually homogenized for 2 min. Serial dilutions (1:10) were made in 0.1X PBS, and 0.1 mL of each dilution was plated on LB-A agar plates. Plates were incubated overnight at 37 °C under aerobic conditions, and the *E. coli* O157:H7 colonies that fluoresced under UV light were counted.

Clover Observations/Measurements

Seedling emergence was monitored daily until no new seedlings emerged for a period of 3 days. Thrice a week, photographs were taken to document canopy coverage [39] and analyzed using digital analysis software (Assess 2.0, APS Press, St. Paul, MN). Canopy growth rate was estimated from these images by calculating change in leaf canopy coverage over time. Every other day, a sterilized hand-held soil moisture sensor (model WET-2; Delta-T Devices, Cambridge, UK) was used to spot-check all replicate pots in all treatments. Water was added to or withheld from replicate pots that measured below or above the setpoint, respectively.

Statistical Analyses

Data were statistically analyzed (SAS version 9.2 [58]) by means and standard errors (PROC MEANS), analysis of

Table 3 qPCR primers and probes used in this study

Oligo name	Target (gene)	Sequence (5′–3′)	Insert size (bp)	Reference
UidA784 F	*Escherichia coli* O157:H7	GTG TGA TAT CTA CCC GCT TCG GCA	82	Frahm and Obst (21)
UidA866 R	(*uidA*)	AGA ACG CTT TGT GGT TAA TCA GGA		
UidA807FAM		TCG GCA TCC GGT CAG TGG CAG T		
1055F	Total bacteria	ATG GCT GTC GTC AGC T	337	Harms et al. (28)
1392R	(16S)	ACG GGC GGT GTG TAC		
16STaq1115-BHQ		CAA CGA GCG CAA CCC		

variance (PROC ANOVA), and least significant difference at a 0.05 probability level ($LSD_{0.05}$) for multiple comparisons among means. For two-way ANOVA, Bonferroni post-tests were used to determine significant differences between pair-wise combinations. Correlations were performed using regression analysis (PROC REG). Prior to statistical analyses, all bacterial and *E. coli* O157:H7 cell concentrations were $\log_{10}$-transformed based on tests of normality (PROC UNIVARIATE) of the residuals (residuals = $\text{concentration}_{\text{observed}} - \text{concentration}_{\text{expected}}$) using the Shapiro–Wilk test statistic [13].

Results and Discussion

Comparison of *uidA* qPCR to Direct Plate Count Estimates

In terms of microbial food safety and security, traditional culture-based assays are currently considered the "gold standard". Therefore, human pathogen inactivation/survival data obtained from non-traditional methods need to be compared to the culture-based standard. In this study, qPCR-based (targeting the *uidA* gene) *E. coli* O157:H7 estimates (Fig. 1, closed symbols) closely matched those obtained using the traditional culture-based direct plate counts (Fig. 1, open symbols) for the first month, but became progressively higher towards the end of the study for both the 25 and 45 % treatments (Fig. 1a, b, respectively). This difference between qPCR and plate count estimates was more pronounced in the 25 % VWC treatments, where qPCR estimates were 2.2–2.3 logs higher by the end of the study, as compared to being only 1.2–1.6 logs higher in the 45 % VWC treatments. Even with these divergent estimates near the end of the study, molecularly derived concentrations of *E. coli* O157:H7 were significantly correlated ($R^2 = 0.8382$–0.9773) to the culture-based concentrations for all four treatments, although only the 45 % VWC treatments were very highly significant ($\alpha = 0.001$; Table 4).

Similar patterns between qPCR and direct plate counts were recently observed by Rogers et al [54] when comparing estimates targeting one of the virulence genes of *E. coli* O157:H7 (*stx1*) and direct plate counts from manure-amended soils; especially in regards to the increasingly larger difference between qPCR and direct plate counts after 3–4 weeks of the study. The slope and *y*-intercept of the regression between *stx1* and CFU g^{-1} soil in their study (0.586 and 3.60, respectively) fall within the range of slopes (0.4962–0.7791) and *y*-intercepts (1.833–4.084) obtained from the four treatments when the *uidA* gene was targeted (Table 4). It can be argued that the reason for these observed differences between qPCR and plate count

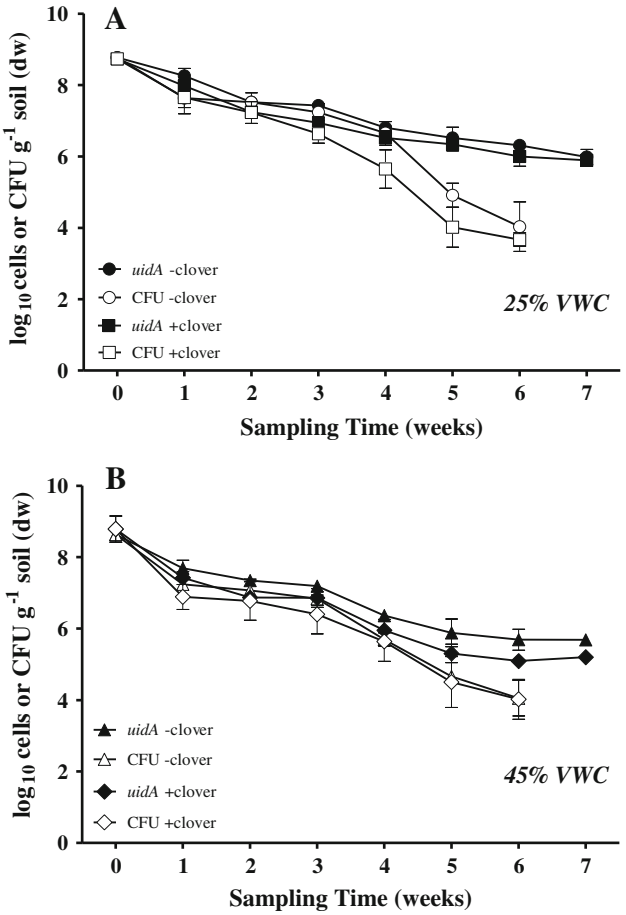

Fig. 1 Comparison of $\log_{10}$-transformed *E. coli* O157:H7 concentrations as determined by qPCR amplification of *uidA* gene (closed symbols) and direct plate counts (*open symbols*) in soil matrices maintained at **a** 25 % VWC or **b** 45 % VWC. *Error bars* indicate the standard deviation of duplicate analyses of triplicate samples

estimates for *E. coli* O157:H7 is the fact that qPCR analyses do not require cells to be active, uninjured, or alive for detection, only the presence of the appropriate DNA targets. Therefore, over time, when the *E. coli* cells die off, the DNA from the dead cells will be picked up by qPCR, but not by the direct plate counts. While extracellular DNA can be a significant portion of the total DNA in the soil at a given time [43, 52], studies have shown that approximately 70 % of extracellular fungal DNA added to soil microcosms was degraded within 12 h [4] and 92 % degraded by 4 days [31]. Given this evidence, along with the relatively large bacterial populations throughout the study ($\geq 3.0 \times 10^8$ cell g^{-1}), the likelihood that the observed difference between qPCR and direct plate count estimates is due to the amplification of extracellular DNA (thus artificially increasing these qPCR estimates) is low.

There is also the potential for *E. coli* to enter a viable but non-culturable state (VNBC) under sub-optimal conditions [14, 57, 62], which may be occurring in the later part of this

Table 4 Correlation between *E. coli* O157:H7 concentrations as determined by selective plate counts and qPCR

Treatment	Regression equation	R^2	*P* value
25 % −clover	$\log_{10}$ (qPCR copies g^{-1}) = 0.5002 $\log_{10}$ (CFU g^{-1}) + 4.084	0.8382	0.0038
25 % +clover	$\log_{10}$ (qPCR copies g^{-1}) = 0.4952 $\log_{10}$ (CFU g^{-1}) + 3.995	0.8718	0.0021
45 % −clover	$\log_{10}$ (qPCR copies g^{-1}) = 0.6560 $\log_{10}$ (CFU g^{-1}) + 2.819	0.9765	<0.0001
45 % +clover	$\log_{10}$ (qPCR copies g^{-1}) = 0.7791 $\log_{10}$ (CFU g^{-1}) + 1.833	0.9773	<0.0001

study. Recent studies on zoonotic pathogens including *E. coli, Campylobacter jejuni,* and *Salmonella* spp. have demonstrated the potential for the qPCR-based estimates to be ≥ 1 log greater than culture-based estimates from soils [25, 54, 55] and VNBC cells have been implicated as the source of these observed differences. The highly significant correlations between the qPCR and direct plate count data in this study (Table 4) indicate that VNBC (as well as dead cells and extracellular DNA) are not strongly affecting the qPCR estimates for *E. coli* O157:H7. It should be noted, however, that the 25 % VWC treatments (less optimal environment) exhibited lower R^2 values than the 45 % VWC treatments. Therefore, VNBC cells may be playing a role in the larger difference between the qPCR and direct plate count *E. coli* O157:H7 estimates in the 25 % VWC treatments. Considering VNBC cells can still pose a human health risk resulting in illness [12, 49, 50], the efficacy of using qPCR-based methods as a conservative indicator of *E. coli* O157:H7 survival in soil matrices is evident.

Effect of Volumetric Water Content and Clover on *E. coli* O157:H7 Survival

The construction of the container and rooting substrate was carefully considered in the design of this experiment. Utilizing straight field soil in container studies is fraught with watering issues including non-uniform packing, poor drainage, lack of homogeneity in microflora, and weed seed persistence, all of which could lead to difficulty in implementing accurate, repeatable irrigation and nutrition control. By combining well-formed compost with field soil and peat, and pre-mixing controlled release fertilizer throughout the substrates, we obtained uniform rooting conditions for the clover, a highly controllable substrate for irrigation and fertility, and an adequate inoculation target for applying our target bacteria. Adding *E. coli* O157:H7 directly to the soil matrix, rather than within manure amendments, was chosen to avoid potential variably introduction of a variety of factors (biological, chemical, nutrient) in the different treatments and replicates that could not be adequately accounted for. While not a representative of a manure-amended soil or a certified organic soil typically under clover rotations, this highly controlled soil matrix provided a good research environment to test the hypotheses of water availability and the presence of clover on the survival of *E. coli* O157:H7.

Before the *E. coli* O157:H7 survival data can be discussed in relation to soil moisture, it is important to note the moisture conditions of the soil matrix used in this study. The water holding capacity of the soil matrix was ~47–49 % VWC (data not shown). Therefore, the 45 % VWC treatments were extremely wet, and near water holding capacity conditions. Conversely, the 25 % VWC treatments appeared dry at the soil surface, but retained sufficient moisture to form macro-aggregates when handled.

The combination of two different soil moistures (25 and 45 % VWC) and the presence or absence of clover within the soil matrix resulted in significantly different survival in the *E. coli* O157:H7 and total bacteria populations (Fig. 2a, b, respectively). For most of the study, *E. coli* O157:H7 survived at significantly lower concentrations in the 45 % VWC treatments as compared to 25 % VWC (Table 5), with survival being highest in the 25 % unplanted treatment (Fig. 2a, open bar) and lowest in the 45 % planted treatment (Fig. 2a, hatched bar) for the entire study. The growth of clover, a commonly used cover crop, significantly reduced the survival of *E. coli*, but only in the 45 % VWC treatments, whereas no inactivation effect of clover was observed in the drier 25 % VWC treatments (Table 5).

Unlike *E. coli* O157:H7 populations, total bacterial populations (as estimated by amplification of the 16S rRNA gene) were generally higher in the 45 % VWC treatments, with estimates remaining significantly lower in the 25 % unplanted treatment by week 4 (Fig. 2b). In fact, the greatest response in the total bacterial population was between the unplanted and planted soils maintained at 25 % VWC (no effect found for *E. coli*), where significant reductions in the total bacterial communities occurred by week 2 (Table 6). There was no statistical difference in total bacterial populations under clover growth regardless of soil moisture (Table 5) or under 45 % VWC whether planted or unplanted (Table 6).

Considering the overall differential responses to the *E. coli* O157:H7 and total bacterial populations to both soil moisture and clover growth, the response of *E. coli* relative to the total bacterial community was also established (EC/TB = $\log_{10}$*E. coli*/$\log_{10}$16S) to determine the survival of *E. coli* in the context of the survival of the bacterial

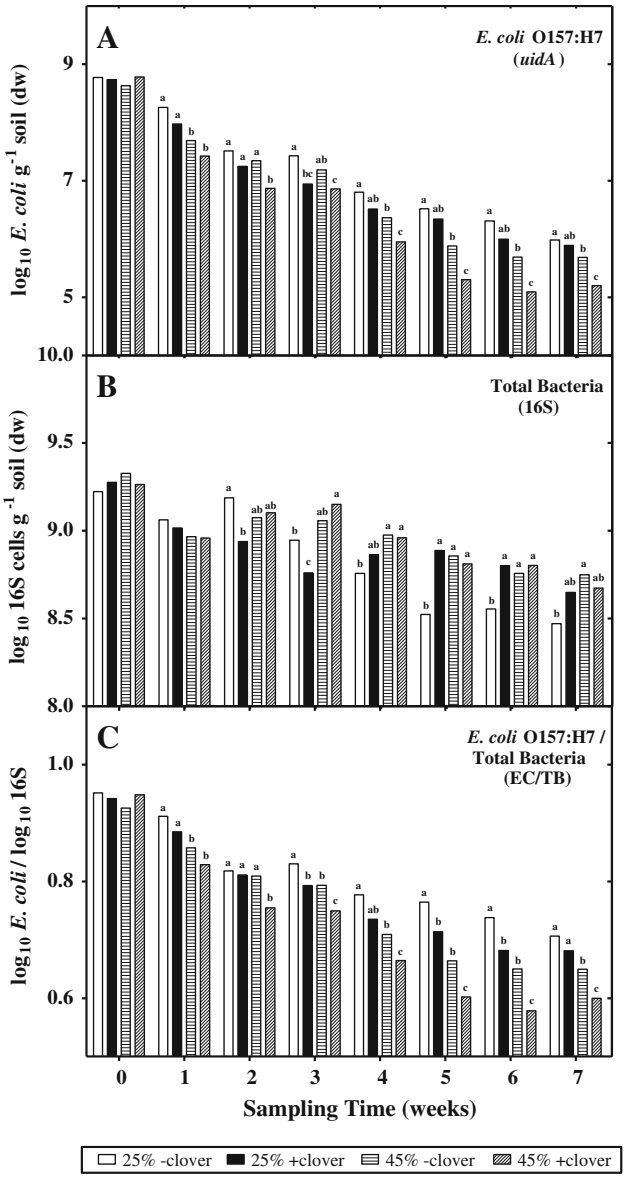

Fig. 2 Effect of volumetric water content and clover growth on qPCR-derived $\log_{10}$-transformed concentrations of **a** *E. coli* O157:H7 using the *uidA* gene, **b** total bacteria using the 16S rRNA gene, as well as **c** the relative change in the *E. coli* O157:H7 population to the total bacteria (EC/TB). *Bars* represent the mean value for duplicate analyses of triplicate samples, and the *letters above the bar* indicate significantly different groups during a sampling time based on least significant difference at a 0.05 probability level for multiple comparisons among means ($LSD_{0.05}$) from the ANOVA analysis of these datasets

population as a whole. The EC/TB ratio estimates followed similar patterns to the *E. coli* O157:H7 estimates (Fig. 2c, a, respectively), and in many cases the significance of most pair-wise comparisons between treatments were strengthened using the EC/TB ratio (Tables 5, 6). The most noticeable difference occurred between the unplanted (open bar) and planted (closed bar) treatments maintained at 25 % VWC (Fig. 2c). Whereas the *E. coli* estimates only

Table 5 Effect of volumetric water content (VWC) on *Escherichia coli* O157:H7 and total bacterial populations both with and without clover growth

Week	− Clover (bare soil)						+Clover					
	E. coli O157:H7 (*uidA*)		Total bacteria (16S)		*E. coli* O157:H7/total bacteria		*E. coli* O157:H7 (*uidA*)		Total bacteria (16S)		*E. coli* O157:H7/total bacteria	
	25 % VWC	45 % VWC	25 % VWC	45 % VWC	25 % VWC	45 % VWC	25 % VWC	45 % VWC	25 % VWC	45 % VWC	25 % VWC	45 % VWC
0	8.774 (0.137)	8.630 (0.122)	9.223 (0.139)	9.327 (0.094)	0.973 (0.022)	0.936 (0.015)	8.737 (0.082)	8.785 (0.365)	9.275 (0.079)	9.264 (0.244)	0.936 (0.015)	0.969 (0.037)
1	8.260 (0.211)	7.689*** (0.226)	9.063 (0.131)	8.967 (0.107)	0.919 (0.019)	0.860*** (0.016)	7.978 (0.091)	7.425*** (0.078)	9.016 (0.071)	8.958 (0.082)	0.860 (0.016)	0.830*** (0.005)
2	7.517 (0.264)	7.345 (0.108)	9.188 (0.088)	9.075 (0.072)	0.819 (0.027)	0.810 (0.011)	7.247 (0.040)	6.868* (0.039)	8.938 (0.118)	9.103 (0.196)	0.810 (0.011)	0.755*** (0.018)
3	7.428 (0.080)	7.188 (0.166)	8.947 (0.084)	9.058 (0.121)	0.832 (0.009)	0.794* (0.013)	6.946 (0.077)	6.859 (0.261)	8.760 (0.041)	9.150*** (0.069)	0.794 (0.013)	0.750** (0.029)
4	6.806 (0.172)	6.365** (0.119)	8.758 (0.039)	8.976** (0.127)	0.778 (0.019)	0.709*** (0.013)	6.518 (0.200)	5.952*** (0.306)	8.865 (0.069)	8.961 (0.069)	0.709 (0.013)	0.665** (0.039)
5	6.519 (0.305)	5.881*** (0.389)	8.523 (0.173)	8.856*** (0.078)	0.765 (0.020)	0.664*** (0.041)	6.342 (0.183)	5.303*** (0.259)	8.887 (0.061)	8.812 (0.058)	0.664 (0.041)	0.602*** (0.032)
6	6.312 (0.100)	5.689*** (0.296)	8.555 (0.036)	8.727* (0.073)	0.738 (0.015)	0.650*** (0.038)	6.000 (0.272)	5.091*** (0.161)	8.801 (0.115)	8.802 (0.083)	0.650 (0.038)	0.578*** (0.014)
7	5.985 (0.113)	5.686* (0.149)	8.471 (0.182)	8.750*** (0.119)	0.707 (0.23)	0.650*** (0.009)	5.893 (0.164)	5.202*** (0.107)	8.649 (0.046)	8.674 (0.102)	0.650 (0.009)	0.600*** (0.018)

Values represent mean (SD) of duplicate analyses from triplicate soil samples

Asterisks represent significant differences (*<0.05, **<0.01, ***<0.001) between pair-wise comparisons (according to two-way ANOVA)

Values for *E. coli* O157:H7 and total bacteria reported as $\log_{10}$ cells g^{-1} soil as calculated from qPCR targeting the genes indicated in the parentheses

Table 6 Effect of clover on *Escherichia coli* O157:H7 and total bacterial populations in soils held at 25 and 45 % volumetric water contents

Week	25 % VWC						45 % VWC					
	E. coli O157:H7 (*uidA*)		Total bacteria (16S)		*E. coli* O157:H7/total bacteria		*E. coli* O157:H7 (*uidA*)		Total bacteria (16S)		*E. coli* O157:H7/total bacteria	
	−Clover	+Clover	−Clover	+Clover	−Clover	+Clover	−Clover	+Clover	−Clover	+Clover	−Clover	+Clover
0	8.774 (0.137)	8.737 (0.082)	9.223 (0.139)	9.275 (0.079)	0.973 (0.022)	0.957 (0.005)	8.630 (0.122)	8.785 (0.365)	9.327 (0.094)	9.264 (0.244)	0.936 (0.015)	0.969 (0.037)
1	8.260 (0.211)	7.978 (0.091)	9.063 (0.131)	9.016 (0.071)	0.919 (0.019)	0.889 (0.013)	7.689 (0.226)	7.425 (0.078)	8.967 (0.107)	8.958 (0.082)	0.860 (0.016)	0.830 (0.005)
2	7.517 (0.264)	7.247 (0.040)	9.188 (0.088)	8.938*** (0.118)	0.819 (0.027)	0.812 (0.011)	7.345 (0.108)	6.868*** (0.039)	9.075 (0.072)	9.103 (0.196)	0.810 (0.011)	0.755*** (0.018)
3	7.428 (0.080)	6.946*** (0.077)	8.947 (0.084)	8.760* (0.041)	0.832 (0.009)	0.794* (0.009)	7.188 (0.166)	6.859* (0.261)	9.058 (0.121)	9.150 (0.069)	0.794 (0.013)	0.750** (0.029)
4	6.806 (0.172)	6.518 (0.200)	8.758 (0.039)	8.865 (0.069)	0.778 (0.019)	0.736** (0.025)	6.365 (0.119)	5.952** (0.306)	8.976 (0.127)	8.961 (0.069)	0.709 (0.013)	0.665** (0.039)
5	6.519 (0.305)	6.342 (0.183)	8.523 (0.173)	8.887*** (0.061)	0.765 (0.020)	0.718*** (0.020)	5.881 (0.389)	5.303*** (0.259)	8.856 (0.078)	8.812 (0.058)	0.664 (0.041)	0.602*** (0.032)
6	6.312 (0.100)	6.000 (0.272)	8.555 (0.036)	8.801** (0.115)	0.738 (0.015)	0.682*** (0.025)	5.689 (0.296)	5.091*** (0.161)	8.727 (0.073)	8.802 (0.083)	0.650 (0.038)	0.578*** (0.014)
7	5.985 (0.213)	5.893 (0.164)	8.471 (0.182)	8.649* (0.046)	0.717 (0.023)	0.681* (0.016)	5.686 (0.149)	5.202*** (0.107)	8.750 (0.119)	8.674 (0.102)	0.650 (0.009)	0.600*** (0.018)

Values represent mean (SD) of duplicate analyses from triplicate soil samples

Asterisks represent significant differences (*<0.05, **<0.01, ***<0.001) between pair-wise comparisons (according to two-way ANOVA)

Values for *E. coli* O157:H7 and total bacteria reported as $\log_{10}$ cells g^{-1} soil as calculated from qPCR targeting the genes indicated in the parentheses

showed significantly lower estimates in the planted treatment at week 3, EC/TB ratios for the clover treatment were significantly lower from week 3 till the end of the study (Table 6). Therefore, the inhibitory effect of clover on *E. coli* O157:H7 survival in the drier soil can only be determined when the differences in the total bacterial populations between these two treatments are taken into account. This highlights the need to not only quantify *E. coli* O157:H7 populations but also to understand their relative contribution to the overall bacterial population to determine the survivability, and potential human health risk, of *E. coli* O157:H7 in soils.

The influence of soil moisture on the survival of *E. coli* O157:H7 was not unexpected; most studies of the survival of *E. coli* O157:H7 in soils have shown *E. coli* survival to be directly proportional to soil moisture [23, 45, 57, 59]. While the total bacterial populations in this study were higher in the 45 % VWC treatments, survival of *E. coli* O157:H7 was consistently higher in the drier 25 % VWC treatments (Tables 5, 6). The soil matrix used in this study contained a significant amount of peat-based substrate, which may have had a two-fold effect on the increased survival of *E. coli* in the 25 % VWC treatments. First, the peat would result in an increased level of organic matter within the soil matrix, and *E. coli* survival has been shown to be enhanced in soils with high amounts of organic matter [19, 37, 57]. In addition, peat is also known to increase soil water-holding capacity [6, 53], allowing for sufficient water to be available to *E. coli* O157:H7 cells in microsites at the lower VWC treatments.

While these two factors should affect both the 25 and 45 % VWC treatments, competitive pressure from both bacteria and fungi [7, 36, 41] at higher moisture levels could result in the lower survival of *E. coli* O157:H7 of the 45 % VWC treatments. It should also be noted that the gravimetric water contents for these soils averaged 0.32 ± 0.02 and 0.47 ± 0.03 g H_2O g^{-1} soil, respectively. When studying the effect of soil moisture and manure addition on *E. coli* O157:H7 concentrations in cattle feedlot soils, Berry and Miller [3] found that at soil water contents around 0.25 g H_2O g^{-1} soil with minimal manure addition (5 %) resulted in aerobic environmental conditions that favored the persistence of *E. coli* O157:H7 in this environment, whereas at soil water contents above 0.43 g H_2O g^{-1} soil resulted in a fermentative environment which inhibited the growth of *E. coli* O157:H7, with a slow population decline. Soils needed to be as dry as 0.11 g H_2O g^{-1} soil for a severe decline in *E. coli* O157:H7 survivability. Taken in the context of this work, the 25 % VWC treatments may have represented a more aerobic environment conducive to *E. coli* O157:H7 survival, whereas a fermentative environment resulted in the

relative inhibition of the *E. coli* O157:H7 in the near water holding capacity (45 % VWC) treatments.

While clover is a cover crop typically used in organic vegetable cropping systems, there is a paucity of research available that looks at the effect of clover growth on soil-associated human zoonotic pathogens such as *E. coli* O157:H7 that are a major food safety issue in organic vegetable production. Results from this greenhouse-scale study show that clover significantly reduced the survival of *E. coli* O157:H7 at both soil moisture levels, although one needs to consider the EC/TB ratio to observe these differences at the 25 % VWC level (Table 6). Members of the *Trifolium* genus are known to produce a variety of phenolic compounds [20, 60] and these compounds have been shown to have antibacterial properties [5, 44]. In addition, honey derived from clover has been shown to have antibacterial properties against the growth of a variety of non-pathogenic and pathogenic microbes [30, 56], including *E. coli* O157:H7 [2, 27]. The combination of these proven antibacterial properties and the significance of adding clover to the lower survival of *E. coli* O157:H7 in this study highlight the importance of clover as a potential biological control of human pathogens in organic vegetable crops.

Relationship Between Clover Canopy Growth and *E. coli* O157:H7 Survival

The clover was actively growing in this study and only began to reach steady-state conditions towards the end of the study. As plant communities become established, microbial communities associated with the root zones also change [40], which represent a potential source of microbial competition against pathogenic bacteria. The size of root zones are correlated to the size of the developing canopy [8], so as an estimate of the role that this may play in suppressing survivability of *E. coli* O157:H7, projected canopy area was plotted against *E. coli* O157:H7 and total bacterial populations (Fig. 3). As canopy size increased, *E. coli* O157:H7 survival decreased (Fig. 3a). The high moisture treatment resulted in larger canopies by week 5 (when canopies were considered filled and measurements ceased). Those treatments had a greater *E. coli* O157:H7 loss than the low moisture treatments. There was also significant decline in total bacteria populations (Fig. 3b), but the correlation was not as strong. Again, the survival of *E. coli* O157:H7 relative to the total bacterial population (EC/TB; Fig. 3c) was strongly correlated with projected clover leaf growth. Considering that clover canopy growth is directly related to the increase water availability in the high moisture treatment, it cannot be determined whether canopy size or moisture had a greater effect on *E. coli* O157:H7 suppression, but these correlations indicate that

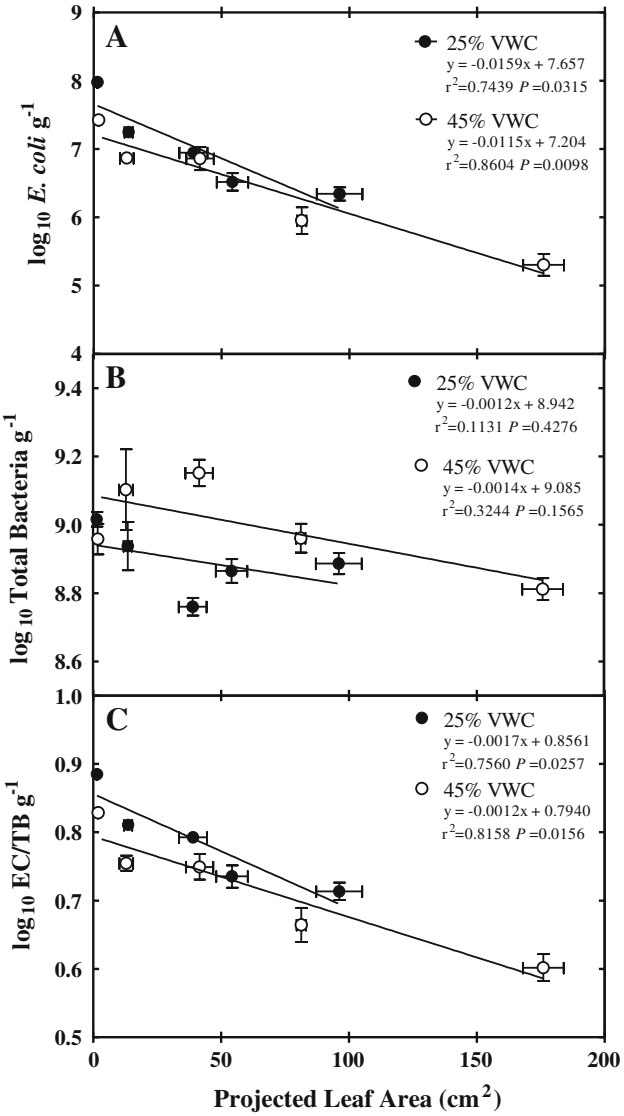

Fig. 3 Relationship between projected canopy area and $\log_{10}$-transformed **a** *E. coli* O157:H7 cells g^{-1} substrate, **b** total bacterial using the 16 s rRNA gene cells g^{-1} substrate, and **c** the relative change in the *E. coli* O157:H7 population to the total bacteria at both high moisture (45 % VWC; *open symbols*) and low moisture (25 % VWC; *closed symbols*). Because root mass was not quantified, the growth of the clover canopies served as a proxy for plant biomass within the root zone. *Error bars* represent ±1 standard error of the mean for both $\log_{10}$-transformed bacteria concentrations and projected canopy area

measurements of clover canopy growth may be predictive of *E. coli* O157:H7 survival within the root zone.

While these relationships between canopy size and bacteria survival are not causative evidence of active *E. coli* O157:H7 suppression, they do suggest a potential role in plant communities influencing the root zone environment in a manner that suppressed *E. coli* O157:H7 survival. Larkin et al. [40] has measured decreases in potato diseases (*Rhizoctonia* spp.), and increases in microorganism diversity and biomarkers for non-pathogenic fungi and *Actinomycetes*, in

particular, in soil treatments associated with disease suppressive crop rotations and organic matter input increases from compost. Measurements of root biomass, rooting density (roots g^{-1} soil), and populations of plant-associated microbial communities beyond stand establishment would help strengthen the relationship between plant establishment and growth and the survival of zoonotic manure-borne pathogens such as *E. coli* O157:H7.

Conclusions

According to the results of this study, qPCR-based estimates are highly correlated to the direct plating "gold standard", which is important for determining the efficacy of using these higher-throughput, faster molecular-based assays in future soil-based studies on the survival of *E. coli* O157:H7. Two important findings resulted from this controlled greenhouse study, with potential broader implications that need to be studied in field-based systems. First, looking at *E. coli* O157:H7 estimates in relation to the survival of the entire bacterial community (EC/TB) should be further investigated as a method to assess *E. coli* survival in soils. As was observed in the lower VWC treatments, *E. coli* O157:H7 can become a significantly larger portion of the overall bacterial community even if their numbers overall are significantly decreased, indirectly concentrating *E. coli* O157:H7 within the soil and potentially resulting in a greater human health risk. Lastly, given the significant effect of clover on *E. coli* O157:H7 survival, future work should focus on the biochemical makeup of clover exudates in agricultural soils and their specific mechanisms of inhibition within the soil. Future testing of these concepts under field-scale conditions could potentially lead to the development of crop rotations/best management practices to accentuate the antibacterial effects of clover towards not only *E. coli* O157:H7, but other important soil-associated zoonotic human pathogens.

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Conflicts of interest The authors declare that there are no conflicts of interest.

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