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Permalink
https://escholarship.org/uc/item/0fc45144

Journal
Frontiers in Microbiology, 7(FEB)

ISSN
1664-302X

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Publication Date
2016

DOI
10.3389/fmicb.2016.00223

Peer reviewed
RNA-Based Detection Does not Accurately Enumerate Living *Escherichia coli* O157:H7 Cells on Plants

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The capacity to distinguish between living and dead cells is an important, but often unrealized, attribute of rapid detection methods for foodborne pathogens. In this study, the numbers of enterohemorrhagic *Escherichia coli* O157:H7 after inoculation onto Romaine lettuce plants and on plastic (abiotic) surfaces were measured over time by culturing, and quantitative PCR (qPCR), propidium monoazide (PMA)-qPCR, and reverse transcriptase (RT)-qPCR targeting *E. coli* O157:H7 *gapA*, *rfbE*, *eae*, and *lpfA* genes and gene transcripts. On Romaine lettuce plants incubated at low relative humidity, *E. coli* O157:H7 cell numbers declined 10^7-fold within 96 h according to culture-based assessments. In contrast, there were no reductions in *E. coli* levels according to qPCR and only 100- and 1000-fold lower numbers per leaf by RT-qPCR and PMA-qPCR, respectively. Similar results were obtained upon exposure of *E. coli* O157:H7 to desiccation conditions on a sterile plastic surface. Subsequent investigation of mixtures of living and dead *E. coli* O157:H7 cells strongly indicated that PMA-qPCR detection was subject to false-positive enumerations of viable targets when in the presence of 100-fold higher numbers of dead cells. RT-qPCR measurements of killed *E. coli* O157:H7 as well as for RNaseA-treated *E. coli* RNA confirmed that transcripts from dead cells and highly degraded RNA were also amplified by RT-qPCR. These findings show that neither PMA-qPCR nor RT-qPCR provide accurate estimates of bacterial viability in environments where growth and survival is limited.

**Keywords:** phyllosphere, foodborne pathogen, EHEC, RT-qPCR, PMA-qPCR, detection, fresh produce, viability

**INTRODUCTION**

Enterohemorrhagic *Escherichia coli* serotype O157:H7 is an organism that causes diseases ranging from self-limiting diarrhea to life-threatening hemolytic uremic syndrome (HUS) and is one of the most important foodborne pathogens worldwide (Pennington, 2010). According to the United States Centers for Disease Control and Prevention, *E. coli* O157:H7 causes 63,153 cases of infection per year in the US alone (Scallan et al., 2011). In recent years, leafy greens have been increasingly associated with *E. coli* O157:H7 outbreaks (Doyle and Erickson, 2008). Although *E. coli* O157:H7 does not survive well or grow to high numbers on intact plants in the field, very low numbers of
viable cells are potential sources of infection (Tuttle et al., 1999). Thus, sensitive and specific *E. coli* O157:H7 detection methods are required to effectively prevent foodborne outbreaks and sporadic infections resulting from consumption of fresh produce. Traditional culture methods for detection of *E. coli* O157:H7 employ enrichment followed by isolation on selective and differential media such as sorbitol-MacConkey or CHROMagar O157 (Feng et al., 2011). Culture-based approaches are expensive, lab intensive, and time consuming, and might potentially result in false negatives due to the presence of viable cells unable to form colonies on standard laboratory culture media. Alternatively, culture-independent molecular detection methods such as PCR can significantly reduce detection times and increase specificity. PCR, in particular, is a widely applied method that is increasingly used for detection of foodborne pathogens. However, a shortcoming of this method is that DNA is typically not rapidly degraded in intact cells and therefore standard PCR and quantitative PCR approaches are not able to distinguish between living and dead bacteria (Lauri and Mariani, 2009). One approach used to discriminate between viable and dead cells is the inclusion of propidium monoazide (PMA) prior to DNA extraction and PCR amplification of pathogen-specific target genes (Nocker et al., 2007). PMA penetrates into bacteria with compromised cell membranes and binds genomic DNA (Nocker et al., 2007). Exposure of the bacteria to light activates the azide group resulting in DNA modification and renders the DNA recalcitrant to PCR amplification. However, the degree of membrane permeability can vary and high numbers of dead cells may interfere with PMA-PCR quantification of the viable cell fraction, as demonstrated by our recent work as well as by others (Li and Chen, 2013; Moyne et al., 2013; Pacholewicz et al., 2013; Barbau-Piednoir et al., 2014).

Bacterial transcripts are regarded to have short half-lives and high turn-over rates (Richards et al., 2008). This property has been crucial to the development of targeted and global gene expression analyses as a means to identify and compare physiologically relevant cellular responses. Another application is to use mRNA as indicator for viability. It has been assumed that because bacterial transcripts are sensitive to degradation by intra- and extra-cellular RNases, mRNA levels should rapidly decline after death. Therefore, unlike DNA-based detection, mRNA would only be limited to the viable and active cells within the population. Based on this assumption, reverse-transcriptase quantitative PCR (RT-qPCR) assays have been developed to detect a variety of foodborne pathogens such as enterohemorrhagic *E. coli*, *Listeria monocytogenes*, *Salmonella*, *Vibrio*, and *Campylobacter* species (Klein and Juneja, 1997; Sheridan et al., 1998; Szabo and Mackey, 1999; McIngvale et al., 2002; Yaron and Matthews, 2002; de Wet et al., 2008; D’Souza et al., 2009; Miller et al., 2010; Techathuvanan et al., 2010; Kurakawa et al., 2012; Zhou et al., 2014). However, while some reports have concluded that mRNA disappears quickly after cell death (McIngvale et al., 2002), other findings suggest that transcripts can persist for extended lengths of time (e.g., Sung et al., 2005; Xiao et al., 2012). Therefore, detailed investigations of transcript-based methods to enumerate viable bacterial cell numbers are urgently needed.

The aim of this study was to apply and compare RT-qPCR, PMA-qPCR, qPCR and culturing methods for detection of viable *E. coli* O157:H7. These methods were investigated for the pathogen on lettuce, following exposure to desiccation conditions on a sterile plastic surface, after different cell inactivation treatments, and for purified RNA exposed directly to ribonuclease.

**MATERIALS AND METHODS**

**Bacterial Strain and Lettuce Growth Conditions**

Rifampicin-resistant *E. coli* serotype O157:H7 strain ATCC 700728 (lacking stx1 and stx2) was used in previous field trials (Moyne et al., 2013; Williams et al., 2013). The strain was routinely grown in Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB) under agitation (250 rpm) at 37°C. Cells were inactivated by incubation in 70% isopropanol or 92°C for 10 min, and death was confirmed by examining for growth on TSA plates incubated at 37°C for at least 48 h. For selective growth of ATCC 700728, rifampicin (Rif; Gold Biotechnology, St. Louis, MO, USA) was added to the TSA at a final concentration of 50 µg/ml.

Seeds of Romaine lettuce (*Lactuca sativa*) cv. Braveheart were grown in Sunshine mix potting soil (Sun Gro Horticulture Distribution, Bellevue, WA, USA) with a light density of 230 µmol m−2 s−2 and 60% relative humidity (RH) in an environmental chamber (PGR15, Conviron, Pembina, ND). Day (12 h) and night time (12 h) temperatures were set at 23 and 18°C, respectively. One day before *E. coli* inoculation, 4-week old lettuce plants were moved to a growth chamber in the lab (Percival, Geneva Scientific LLC, Fontana, WI, USA) maintained as described above except with a RH of 30%.

**Measurements of *E. coli* O157:H7 on Lettuce**

Living or dead (killed by incubation in 70% isopropanol for 10 min) exponential-phase *E. coli* ATCC700728 cells were collected by centrifugation at 5,000 g for 3 min at 22°C, and the cells were washed twice with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4, at pH 7.0). Romaine lettuce plants were inoculated with 20 10-µl aliquots of either the viable or inactivated *E. coli* ATCC 700728 cell suspensions to result in approximately 108 cells per leaf and then immediately returned to the growth chamber maintained at 30% RH. Leaves were sampled prior to *E. coli* inoculation and then at 0, 3, 24, 48, 72, and 98 h. Each sample consisted of two lettuce leaves that were randomly excised at the stem with sterile forceps and immediately transferred into a Whirl-pak bag (Nasco, Fort Atkinson, WI, USA) containing 100 ml sterile 0.1% peptone (Becton Dickinson, Franklin lanes, NJ, USA). Bacterial cells were dislodged from the lettuce by sonication at room temperature for 7 min in a Branson 8510 Ultrasonicator (Branson Ultrasonics Corporation, Danbury, CT, USA). For *E. coli* ATCC700728 colony enumeration, serial dilutions of lettuce washes were plated onto TSA containing...
µ suspensions were inoculated in 20 10-E. coli E. coli Living or dead (killed by incubation in 70% isopropanol for Surfaces Measurements of E. coli − nitrogen, and then stored at below, and the remaining samples were flash frozen in liquid nitrogen, and then stored at −80°C.

**Measurements of E. coli on Abiotic Surfaces**

Living or dead (killed by incubation in 70% isopropanol for 10 min) exponential-phase E. coli ATCC700728 were collected by centrifugation at 5,000 g for 3 min at 22°C, and the cells were washed twice with PBS as described above. The E. coli suspensions were inoculated in 20 10-µl aliquots onto sterile petri dishes (Fisher Scientific, Pittsburgh, PA, USA) to reach approximately 10⁸ cells and the petri dishes were left open in a biosafety cabinet (Baker Company, Sanford, ME, USA) at the ambient RH of approximately 30%. At multiple times, petri dishes were collected and the E. coli cells washed off using 1 ml of 0.1% peptone for colony enumeration or genomic DNA extraction. For RNA extraction, E. coli cells were suspended directly from the petri dishes with ice-cold, acidic-phenol, ethanol RNA-stabilizing solution as described above. For nucleic acid extractions, the cell suspensions were centrifuged at 12,000 g for 2 min at 4°C, the pellet was washed twice with TE buffer and the remaining samples were flash frozen in liquid nitrogen and stored in −80°C.

**PMA Treatment and DNA Extraction**

A fraction of the cells recovered from Romaine lettuce or petri dishes were exposed to PMA (Biotum, Hayward, CA, USA) as described previously (Moyne et al., 2013). Briefly, cell suspensions were incubated in the dark for 30 min with shaking (500 rpm) in the presence of a final concentration 0.04 mM PMA. The cells were placed on ice and then exposed to a 500 W halogen light at a distance of 20 cm for 3 min prior to DNA extraction. For testing the interference of dead cells in PMA-qPCR detection, 10⁶ exponential-phase E. coli ATCC700728 cells inactivated in 70% isopropanol were mixed with different proportions of viable, exponential-phase cells of that strain in 0.1% peptone water prior to PMA exposure. For DNA extraction, bacteria (with or without exposure to PMA) were lysed by boiling for 5 min in Prepmem solution (Life Technologies, Foster City, CA, USA) and debris was removed by centrifugation at 10,000 g for 2 min.

**RNA Extraction and Digestion**

Bacterial cells were lysed by incubation in 200 µl TE (30 mM Tris-Cl, 1 mM EDTA), containing 0.5 mg proteinase K (Qiagen, Valencia, CA, USA) and 15 mg/ml lysosome (Sigma–Aldrich, St Louis, MO, USA) for 30 min at 25°C. Total RNA was then purified with the RNAeasy mini kit according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA). Remaining DNA was digested with TURBO DNase (Life Technologies) prior to measuring RNA quality on the Agilent 2100 Bioanalyzer system with the Agilent RNA 6000 Nano kit (Agilent, Santa Clara, CA, USA).

RNA digests were performed by incubating 400 ng high-quality (RIN:2.0) E. coli ATCC700728 RNA in 0.1 ng of RNase A (0.1 µg/ml; Life Technologies) and nuclease-free water (Life Technologies) at 37°C for different lengths of time. To inhibit RNase activity, 40 U of RNase inhibitor (Life Technologies) was added at the indicated time points.

**Reverse Transcriptase (RT)-qPCR**

Reverse transcription was performed using RETROscript® Reverse Transcriptase as indicated by the RETROscript kit with included random decamers (Life Technologies). To confirm the absence of genomic DNA, negative control reactions contained RNA and all reagents except for the reverse transcriptase (no-RT controls).

To quantify DNA and cDNA, qPCR was performed on an ABI 7500 Fast Real-time PCR system (Life Technologies) using 0.20 µM forward and reverse primers (Table 1), 1X Ssofast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), and 2 µl gDNA or cDNA template. Cycling conditions included an initial activation step at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension temperatures at 60°C for 30 s. Melting curves were then performed by increasing the temperature from 60 to 95°C at

| Target | Primer sequence | Size (bp) | Reference |
|-------|----------------|----------|-----------|
| lptA  | 5′-CACCGTTAAGAGCGACCAGGG-3′  
|       | 5′-GAGAGTGGATACCACCG-3′  
|       | 5′-TCTGTGGATAGATATAAAATTITG-3′  
|       | 5′-AGAATTGCGATACCACCG-3′  
| rfbE  | 5′-TCAAAGGACACTATTTCCAGAAGTTG-3′  
|       | 5′-CGATACTTATACCAAAAGCACCCTCG-3′  
| gapA  | 5′-TAGGGGGCCAGATTATTATTCGCTG-3′  
|       | 5′-AACCAATGCTCTATACCA-3′  | 165 | Sharma et al., 2010 |
|       | 105 | Sharma, 2006 |
|       | 129 | Sharma, 2006 |
|       | 143 | This study |
0.2°C/10 s and recording the fluorescence. Threshold cycle (C) 
vales were automatically generated by the 7500 Fast Real-Time 
PCR software. All (RT)-qPCR assays were performed in triplicate. 
PCR amplification efficiency for each of the primer pairs was 
similar to the values reported previously ( Sharma, 2006; Sharma 
et al., 2010) and at 91% for the gapA primers developed here. 
Negative controls for PCR and RT-qPCR consisted of reactions 
lacking template DNA. No-RT controls were also used for all RT-
qPCR assays and were confirmed to either lack any detectable 
amplification (C over 40) or exhibited a C that was at least 
five cycles higher than the sample cDNA template. To quantify 
the number of E. coli ATCC 700728 cells, standard curves were 
included for each (RT)-qPCR run by amplifying a 5 log_{10} dilution 
of either gDNA or cDNA (ratio of 23S rRNA to 16S rRNA = 1.8) 
from a known number of viable, exponential-phase E. coli ATCC 
700728 cells. For all genes, the lower limits of detection by qPCR 
were between 100 and 1000 cells and for RT-qPCR between 200 
and 500 cells.

Statistical Analysis
Microbial data [colony forming units (CFU) or estimated cells 
per leaf or plate] were log transformed before examining 
with JMP Pro 12 software (SAS Institute Inc., Cary, NC, 
USA). Because the data did not follow a normal distribution, 
the nonparametric Kruskal–Wallis test was used with a 5% 
significance level. If a significant effect was found, pair-wise 
comparisons were performed using the Steel-Dwass test. For 
comparing the different methods used to estimate the number 
of live and dead cells, data obtained from all amplicons 
and time points were combined. For comparing the four 
amplicons used to quantify the cell numbers, data for each 
time point were combined according to the method of 
quantification.

RESULTS
Comparison of Transcript-Based
Enumeration of E. coli O157:H7 on 
Romaine Lettuce to Culture 
and DNA-Based Methods
Escherichia coli O157:H7 strain ATCC700728 was inoculated 
on-4-week old Romaine lettuce plants in small aliquots (10 µl) 
to avoid aerosolization as previously described (Moyne et al., 
2013). The plants were then incubated at 30% RH and near 
ambient temperatures (18 and 23°C) in an environmental 
chamber. Estimates of viable cells according to growth on TSA 
showed that the numbers of E. coli ATCC 700728 decreased 
sharply after application onto the plants from 8.8 log_{10} colony 
forming units (CFU) per leaf to 2.8 log_{10} CFU per leaf within the 
first 48 h (Figure 1). By 96 h, culturable E. coli decreased to only 
1.8 log_{10} CFU per leaf (Figure 1).

In contrast to culture-based estimates, qPCR enumeration 
of E. coli ATCC 700728 showed that the total number of 
(living and dead) E. coli cells on the Romaine lettuce did not 
change from inoculum levels (Figure 1). These findings did not 
differ between any of the housekeeping [gapA (glyceraldehyde-
3-phosphate dehydrogenase) and virulence [eae ( intimin), 
lpfE (long polar fimbriae), and rfbE (O-antigen transporter)] genes 
tested and estimates of total cell numbers were similar for all 
target genes at all time points (Kruskal–Wallis, P = 0.5502). 
To attempt to quantify only the viable fraction of E. coli cells 
on lettuce, plant leaf washes were also incubated in PMA prior 
to DNA extraction and qPCR. One day (24 h) after E. coli 
inoculation, cell quantities measured by PMA-qPCR were similar 
to those estimated by cultivating on laboratory culture medium 
(Steel-Dwass, P = 0.7838) and significantly lower than estimates 
according to qPCR alone (Steel-Dwass, P = 0.0002; Figure 1).

However, when measured again 1 day later and at all subsequent 
time points, the three methods yielded different results. Notably, 
PMA-qPCR indicated only a 10-fold decline in E. coli cell numbers 
whereas culture-based estimates were 1000-fold lower 
and these differences did not change for the remainder of the 
4 days experiment.

Lastly, transcript quantification was used as an approach to 
estimate viable E. coli O157:H7 on lettuce. Transcript levels 
for each of the four target genes, gapA, eae, lpfE, and rfbE, 
did not change according to RT-qPCR from inoculum levels 
during the first 24 h after inoculation of E. coli ATCC 700728 
into Romaine lettuce (Figure 1). To this regard, there were no 
significant differences between the numbers of cells estimated 
by RT-qPCR and qPCR at that time (Steel-Dwass, P = 0.9998). 
One day later, RT-qPCR suggested that E. coli cell numbers 
were reduced by 100-fold to approximately 10^7 cells per leaf; 
a value significantly lower than detected according to qPCR 
(Steel-Dwass, P = 0.0002). Estimates of cell quantities did not 
decline further for the remainder of the study. This finding was 
not likely due to differences in gene expression because E. coli 
cell enumerations were equal for all four target genes tested.
Because estimates of viable cells according to PMA-qPCR were significantly higher than those detected by CFU enumeration starting 48 h after inoculation of E. coli ATCC 700728 on lettuce, we investigated whether a high number of dead cells, such as the 10⁶ fold reduction in living cells estimated by culturing, might interfere with the accuracy of PMA-qPCR. To test this possibility, we mixed different proportions of isopropanol-treated (dead) E. coli ATCC700728 cells with exponential-phase E. coli ATCC700728 in 10-fold cell increments and then exposed the cells to PMA followed by DNA extraction and qPCR. When the ratio of living to dead cells was equivalent [log (ratio of dead to living cells) = 0], PMA-qPCR was accurate and there was no difference between viable numbers estimated by CFU enumeration and PMA-qPCR (Figure 3). Similarly, when the numbers of dead cells were 10-fold higher than living cells, PMA-qPCR was similarly in agreement with the culture-based assessment (Figure 3). However, PMA-qPCR on mixtures of 100-fold more dead cells than living cells [log (ratio of dead to living cells) = 2], this technique overestimated the number of viable cells in the mixture (Figure 3). Similarly, when only 10⁶ dead cells were exposed to PMA treatment, PMA-qPCR resulted in estimates of approximately 10⁴ viable cells. Hence, it is likely that the high number of viable E. coli ATCC 700728 estimated on lettuce and petri dishes by PMA-qPCR actually consisted of dead cells

**Detection Limit for Viable Cells by PMA-qPCR**

Because estimates of viable cells according to PMA-qPCR were significantly higher than those detected by CFU enumeration starting 48 h after inoculation of E. coli ATCC 700728 on lettuce, we investigated whether a high number of dead cells, such as the 10⁶ fold reduction in living cells estimated by culturing, might interfere with the accuracy of PMA-qPCR. To test this possibility, we mixed different proportions of isopropanol-treated (dead) E. coli ATCC700728 cells with exponential-phase E. coli ATCC700728 in 10-fold cell increments and then exposed the cells to PMA followed by DNA extraction and qPCR. When the ratio of living to dead cells was equivalent [log (ratio of dead to living cells) = 0], PMA-qPCR was accurate and there was no difference between viable numbers estimated by CFU enumeration and PMA-qPCR (Figure 3). Similarly, when the numbers of dead cells were 10-fold higher than living cells, PMA-qPCR was similarly in agreement with the culture-based assessment (Figure 3). However, PMA-qPCR on mixtures of 100-fold more dead cells than living cells [log (ratio of dead to living cells) = 2], this technique overestimated the number of viable cells in the mixture (Figure 3). Similarly, when only 10⁶ dead cells were exposed to PMA treatment, PMA-qPCR resulted in estimates of approximately 10⁴ viable cells. Hence, it is likely that the high number of viable E. coli ATCC 700728 estimated on lettuce and petri dishes by PMA-qPCR actually consisted of dead cells

**Figure 3** | Interference of dead cells in the detection of viable cells by PMA-qPCR. A total of 6.7 log₁₀ isopropanol-treated (dead) E. coli ATCC 700728 cells were mixed with 0.7 to 6.7 log₁₀ exponential-phase E. coli ATCC 700728 in 10-fold cell increments. The cell mixtures were then exposed to PMA followed by DNA extraction and qPCR. Cell numbers were estimated by targeting gapA (○), eae (■), lpfA (▲), and rfbE (△) genes compared to standard curves constructed using known quantities of E. coli cells. Each point represents the mean ± standard deviation of three independent replicates.
that lacked the capacity to recover to form viable and active populations.

Detection of Dead E. coli and Degraded RNA by RT-qPCR

To test whether mRNA could be detected in dead cells by RT-qPCR, E. coli ATCC 700728 was killed in 70% isopropanol and then inoculated onto either Romaine lettuce or sterile petri dishes. RNA in dead E. coli inoculated onto lettuce plants was highly degraded (ratio of 23S to 16S rRNA = 1.0) and then was further degraded after inoculation onto plants (ratio of 23S to 16S rRNA = 0.0). However, total cell numbers estimated by RT-qPCR only decreased about 100-fold compared with inoculum levels within the first 24 h (Figure 4). At subsequent time points, the estimated cell numbers remained relatively constant for all four target genes (Figure 4). Notably, cell numbers estimated using gapA transcripts as the target were significantly lower than those estimated for rfbE (Steel-Dwass, P = 0.0019) and eae (Steel-Dwass, P = 0.0002). The same trends were also found for E. coli ATCC 700728 inactivated with 70% isopropanol inactivated and incubated on petri dishes (data not shown) and heat killed cells in suspension (Supplementary Figure S3).

Because cell death might result in the destruction of cellular RNases and reduce RNA turnover, we also measured whether a detection limit for E. coli transcripts could be reached upon increasing exposure of purified E. coli ATCC 700728 RNA to active RNases. Exposure to RNase A between 0 and 240 min resulted in significant reductions in intact ribosomal RNA and presumably mRNA transcripts (Figure 5A). By 120 min of exposure to RNase A, the RNA was degraded to the extent that 16S and 23S RNA were no longer detected by capillary electrophoresis (Figure 5A). However, there was only a 0.3 log₁₀ reduction in cell number equivalents according to RT-qPCR on gapA transcripts and only a 10-fold reduction when eae, lpfA, and rfbE transcripts were targeted for detection (Figure 5B).

DISCUSSION

Because bacterial foodborne pathogens encounter a variety of environmental stresses on plants in the field and post-harvest during processing and packaging, it is likely that only a small fraction of cells are able to survive and cause human infection after a contamination event. Therefore, ideal detection methods should only measure viable pathogen numbers (Law et al., 2014). Our findings show that bacterial detection and enumeration on lettuce according to RT-qPCR and PMA-qPCR are not accurate and can detect dead E. coli O157:H7. Hence, applications of these techniques for foodborne pathogens should be used with caution when applied for viable bacteria cell detection and enumeration on fresh produce.

Measuring bacterial gene transcripts by RT-(quantitative) PCR has been used as an indicator of viability for a variety of foodborne pathogens in different foods (e.g., Klein and Juneja, 1997; McIngvale et al., 2002; Miller et al., 2010; Zhou et al., 2014). However, transcript detection is not always correlated with the presence of living and intact cells (Xiao et al., 2012). There are a variety of possible reasons for these different findings. To this regard, longer RNAs are more likely to undergo additional hydrolysis and become undetectable in subsequent cDNA synthesis and qPCR steps. This difference could explain the validation of transcript-detection by standard RT-PCR for longer (>200 nucleotides) products ( Sheridan et al., 1998; Szabo and Mackey, 1999) as opposed to the shorter products typically produced in qPCR assays. Similarly, PMA-qPCR is also more accurate when used to measure longer DNA targets (Moyne et al., 2013). This is possibly consistent with the PMA-qPCR assessments for the four different gene targets measured here. Notably, however, even though there was a 60 bp range in transcript length among the E. coli mRNAs used for RT-qPCR in this study, these size differences were not correlated with estimates of cell viability.

Another possibility for the differences in RT-qPCR viability estimates between studies is the inherent stability of the transcripts tested. For example, 16S RNA was previously found to be more stable than cellular mRNAs ( Sheridan et al., 1998). However, remarkably, we found that the detection of different protein-encoding, E. coli O157:H7 ATCC 700728 transcripts by RT-qPCR was not broadly diminished with extended exposure to ribonuclease. Only a 0.6–1.6 log₁₀ decrease in cell number equivalents was observed according to RT-qPCR on the digested RNA. Although after ribonuclease treatment there were increased levels of gapA mRNA (and hence E. coli cell number equivalents enumerated) compared to the three other transcripts tested, this difference was not consistent with gene-specific, transcript-based measurements of E. coli ATCC 700728 on plants or on petri dishes. These findings
suggest that ribonucleases targeting single-stranded RNAs, such as the RnaseA used here, are not fully effective at digesting mRNA transcripts to lengths smaller than necessary for qPCR detection.

Another factor that can alter RT-qPCR measurements of bacterial viability is the lethal treatment used for cell inactivation. Prior studies on the development of this method used heat or exposure to solvents (ethanol) to test the capacity of RT-qPCR to measure viable cell numbers. Transcripts were found to be more stable after exposure of bacteria to ethanol as opposed to heat (Sheridan et al., 1998). Herein we found that exposure of E. coli O157:H7 ATCC 700728 to solvent (isopropanol) or heat yielded similarly erroneous estimates of viability with RT-qPCR. We also extended this comparison to E. coli ATCC 70728 exposed to low %RH desiccation stress on a sterile plastic surface and reached similar results. The only notable exception for E. coli O157:H7 ATCC 700728 survival on lettuce was that there was a 100-fold reduction in estimated viable cell numbers by RT-qPCR starting 48 h after application of that strain onto the plants. This reduction might have been due to predation on the dead E. coli cells remaining on the lettuce or other factors that remain to be determined.

Examination of E. coli O157:H7 under low %RH conditions in the laboratory is comparable to the conditions that those foodborne pathogens are exposed to on fresh produce in the field. The phyllosphere, or areal surfaces of plants, is regarded to be a harsh environment for microorganisms because it contains limited quantities of nutrients available for cell growth and is subject to rapid changes in temperature, moisture, ultraviolet radiation (Lindow and Brandl, 2003) that likely occur over diurnal and seasonal scales (Williams et al., 2013). Among these stresses, exposure to low %RH is a major determinant of E. coli survival. On lettuce plants maintained at near 100% RH, E. coli cell numbers increase rapidly and reach population sizes of 10⁹ CFU/g (Cooley et al., 2003). By comparison, inoculation onto plants maintained at low (60%) RH results in rapid declines in culturable cell numbers to levels similar to those detected for E. coli O157:H7 on field lettuce in the Salinas Valley, CA (Moyne et al., 2013). In the present study, the majority of culturable E. coli O157:H7 ATCC 700728 declined within 48 h after inoculation on Romaine lettuce maintained at low (30%) RH. A similar reduction in viable cell numbers was observed according to PMA-qPCR. Although the exposure of the pathogen to desiccation conditions on the abiotic (petri dish) surface resulted in more rapid losses in culturable cell numbers, exposure to both biotic (lettuce) and abiotic (petri dish) surfaces yielded similarly low levels of surviving E. coli cells. In general, a lack of available water results in desiccation stress and induction of a variety of cellular responses including a reduction in capsular layers, increase in salt concentration, reduction in membrane fluidity (Potts, 1994). These changes might also reduce the availability of water required for RNase activity (Malin et al., 1991), and therefore could have contributed to the high estimates of viable cell numbers by RT-qPCR.

To the best of our knowledge, this was the first study to apply RT-qPCR to detect and enumerate viable foodborne pathogen cell numbers on plant surfaces. The qPCR methods developed and applied here, although not high-throughput, are useful because they can be performed more rapidly than culture-based assessments. Of particular interest was the application of RT-qPCR to enumerate viable E. coli O157:H7 cells. Although this approach could be useful for the general detection of E. coli O157:H7, we have unequivocally shown the lack of association between E. coli O157:H7 mRNA transcript abundance and cell
viability. We demonstrated that transcripts of sufficient length for detection by qRT-PCR were present in *E. coli* O157:H7 cells long after death and even after exposure of purified cellular RNA to exogenous ribonuclease. This knowledge is crucial when developing RT-PCR or other RNA related detection methods such as those in development for next-generation bio-sensors (Law et al., 2014). Moreover, RNA stability should be taken into account during experiment design and data interpretation, especially under low-moisture environments such as field-grown plants.

### AUTHOR CONTRIBUTIONS

Conceptualization, MM; Methodology, WJ, A-LM and MM; Investigation, WJ and A-LM; Formal Analysis, WJ; Writing, Reviewing and Editing, WJ, A-LM and MM; Funding Acquisition, MM; Supervision, MM.

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**FUNDING**

This research was supported by a University of California, Davis Research Investments in Science and Engineering (RISE) Grant.

**ACKNOWLEDGMENTS**

We would like to thank Lynn Whang, Eric Tran, and Cindy Ma for their assistance with the lettuce, colony enumerations, and other technical support provided in this study.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00223

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.