Food Microbiology 59 (2016) 7–13

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

# Potential for transfer of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Senftenberg from contaminated food waste derived compost and anaerobic digestate liquid to lettuce plants

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ARTICLE INFO

*Article history:*
Received 21 September 2015
Received in revised form
17 April 2016
Accepted 21 April 2016
Available online 23 April 2016

*Keywords:*
Foodborne pathogens
Plant uptake
Contaminated fertilisers

ABSTRACT

The diversion of food wastes from landfill to sustainable disposal methods, such as composting and anaerobic digestion, has led to an increase in the soil amendment products that are now commercially available and which are derived from both of these processes. The use of such products as soil amendments during the production of ready-to-eat (RTE) crops is increasing worldwide.

The aim of this study was to investigate the potential of three well-recognised bacterial pathogens of importance to public health, namely *Escherichia coli* O157:H7, *Salmonella* Senftenberg and *Listeria monocytogenes*, to become internalised in lettuce plants from peat growing media amended with contaminated food waste derived compost and anaerobic digestion liquid. The results demonstrated both S. Senftenberg and *E. coli* O157:H7 are capable of internalisation at lower inoculation levels, compared to previous studies. The internalisation was visualised through confocal microscopy. Internalisation of *L. monocytogenes* did not occur, however significant levels of *L. monocytogenes* contamination occurred on the non-sterilised plant surface.

Assessing the internalisation potential for each of these pathogens, through the compost and anaerobic digestate matrices, allows for better risk assessment of the use of these products in a horticultural setting.



## 1. Introduction

The occurrence of foodborne illness attributed to ready-to-eat (RTE) vegetables is on the increase worldwide. In Europe it is thought that this is due to increased surveillance of RTE vegetables as potential vehicles for transmission of foodborne illness, and the increase in both the production and supply of RTE vegetables. An increase in consumer demand has led to the globalisation of the RTE vegetable production (Jacxsens et al., 2012). RTE sprouting seeds are increasingly being associated with outbreaks due to the ideal incubation environment for pathogens provided during germination of the seeds. Leafy greens have also been repeatedly implicated in foodborne outbreaks. In Europe fourteen outbreaks specifically linked to leafy greens were documented between 2004 and 2012 (Callejón et al., 2015). In the same period in the United States there were 37 outbreaks specific to leafy greens.

Contamination of leafy greens can occur at any stage of the farm-to-fork chain. This study however is concerned with the pre-harvest environment. In particular, the use of organic fertilisers such as composted food wastes may increase the risk of exposure of leafy greens to bacterial pathogens. Up to now there have been no outbreaks directly linked to contaminated organic fertilisers. However, these potential sources are not routinely tested after application to the land and are generally not available for testing once the outbreak occurs (EFSA, 2014). Although an efficient composting process should eliminate bacterial pathogens present in the waste, the product can become re-contaminated during storage or may contain discrete pockets within the matrix which do not reach required temperatures during composting (Avery et al., 2012). Both scenarios may lead to the survival and growth of

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http://dx.doi.org/10.1016/j.fm.2016.04.006
0740-0020/

pathogens. The practice of burying the composted waste (CW) into the soil weeks before crops are planted may also not be sufficient to eliminate pathogens, as studies have shown enteric pathogens such as *Escherichia coli* O157:H7 and *Salmonella enterica* are capable of surviving for months in the soil environment (Jiang et al., 2002; You et al., 2006; Fremaux et al., 2008). As *Listeria monocytogenes* is an environmental pathogen it can easily survive for long periods in the soil environment (Kim and Jiang, 2010).

Anaerobic digestate liquid (AD) is an example of an organic fertiliser which has the potential to be used in horticultural settings. The use of anaerobic digestion to transform food waste to a fertiliser is increasing worldwide. Currently this product is mainly used on grasslands but its high nutrient content and sustainable production method means additional uses such as soil fertilisers for crops are being investigated (Alburquerque et al., 2012). As with composting the anaerobic digestion process should reduce or eliminate bacterial pathogens. Studies have reported on the identification of both *S. enterica* and *L. monocytogenes* in the final AD product (Bonetta et al., 2011). Other authors have also reported on the survival of bacterial pathogens in soil which has been amended with contaminated AD liquid (Goberna et al., 2011).

If horticultural growth substrates are contaminated with foodborne pathogens there is the potential for subsequent crop contamination to occur. However, previous studies in this area have reported conflicting results. *S. enterica* were shown to internalise into the root structure and to translocate to the edible part of RTE plants (Bernstein et al., 2007; Ge et al., 2012; Gorbatsevich et al., 2013). The results for *E. coli* O157:H7 vary, with some studies showing apparent internalisation (Solomon et al., 2002; Erickson et al., 2010), while others report an inability to internalise into the lettuce plant (Johannessen et al., 2005; Erickson et al., 2014a). By comparison limited research has been done on *L. monocytogenes*, but a recent study by Chitarra et al. (2014) demonstrated the internalisation of lettuce plants at 24 °C. Several factors determine the internalisation ability of these pathogens, such as temperature, moisture content of growing media, soil type and also the stage of growth of the plant and the plant type itself (Ge et al., 2012). The inoculum level has also been shown to affect internalisation ability (Erickson et al., 2014b). Internalisation has mainly been observed at high levels ($Log_{10}$ 6–9 CFU $g^{-1}$) of pathogen inoculation. These bacterial numbers may not reflect actual pathogen numbers normally encountered under field conditions.

The variation in results highlights the need to further investigate the internalisation potential of each of these pathogens so as to assess the potential risk posed for contamination of RTE crops. Gu et al. (2013) reported that the internalisation of *S.* Typhimurium into tomato seedlings was reduced when organic fertilisers were used. The impact of the feedstocks for both composted waste and AD liquid on the internalisation ability may also play a role, but is rarely considered. To assess the risk posed by the use of these products as a fertiliser for RTE crop production, the potential for foodborne pathogens to internalise RTE crops from growth media amended with contaminated food waste derived compost and AD liquid should be investigated.

The aim of this study was to investigate the potential of three bacterial pathogens, *E. coli* O157:H7, *S. enterica* species and *L. monocytogenes,* to internalise lettuce plants from peat growing media amended with contaminated food waste derived compost and AD liquid. Plant surface sterilisation techniques were applied to ensure any positive samples were the result of internalisation and not the presence of the pathogens on the surface of the plants. Assessing the internalisation potential of each of these pathogens provides valuable information on the potential risk posed and enables the development of appropriate mitigation strategies.

## 2. Materials & methods

### 2.1. Bacterial strains and media

*E. coli* O157:H7 (ATCC 43888), *L. monocytogenes* and *S.* Senftenberg were used throughout this study. The *E. coli* O157:H7 is a non-toxigenic reference strain. *S.* Senftenberg is a compost isolate, and was kindly supplied by the Irish Department of Agriculture, Food and the Marine. Both *E. coli* O157:H7 and *S.* Senftenberg were transformed with the GFP containing plasmid pGFPuv (Clontech), by electrotransformation (Ma et al., 2011). The *L. monocytogenes* strain was kindly supplied by the National University of Ireland, Galway (Utratna et al., 2012). The latter contains an integrated GFP gene; the expression of which is dependent on the exposure of the *L. monocytogenes* to stressful environments. A second non-fluorescent strain of *Listeria, L. monocytogenes* 403T12B, was also used.

All bacterial strains were stored on Protect Beads, at −80 °C (Technical Service Consultants Ltd, UK). Throughout the study strains were maintained on tryptone soya agar (TSA) and over night cultures were grown in tryptone soya broth (TSB) at 37 °C and overnight cultures were washed twice in phosphate buffered saline (PBS), by centrifuging at 8000 rpm for 10 min, prior to use.

All broths and agars used in this study were purchased from Oxoid, (Hampshire, U.K.). Xylose lysine deoxycholate (XLD) agar was used for *S.* Senftenberg recovery. For *E. coli* O157:H7 recovery, sorbitol MacConkey agar with cefixime and tellurite (CT-SMAC) was used. *Listeria* chromogenic agar (CLA), with supplements was used to recover *L. monocytogenes*. Buffered *Listeria* enrichment broth (BLEB) was used for the detection of *L. monocytogenes* by enrichment.

AD samples were taken from a commercial facility and the feedstocks included food waste and animal manures. The pH and total solid content of the AD was 7.92 and 4.67%, respectively. CW samples were also taken from a commercial facility; feed-stocks here included food waste and green waste. The pH, electrical conductivity and organic matter content of the CW were 6.98, 4.12 and 42.85, respectively. The lettuce used throughout this trial was a commercial variety called *Lactuca sativa* var *capitata* (AMICA). The growing media mixed with both the CW and AD was a commercial peat based growing substrate, Jack's Magic peat based compost (Westlands, United Kingdom).

### 2.2. Inoculation of CW waste and AD

One ml of washed overnight cultures of either *E. coli* O157, *S.* Senftenberg and *L. monocytogenes* were inoculated into 60 g (w/w) CW or 10 ml AD. The inoculated CW/AD samples were the added to each pot containing peat growing media to give a final concentration in each 180 g pot of $Log_{10}$ 5 CFU g $(CW/AD)^{-1}$.

### 2.3. Experimental setup

All lettuce plants were grown from seeds for three weeks before transplanting the plugs to individual 9 × 9 $cm^2$ pots. To prepare the pots for these plugs two approaches were used; one for compost and the other for AD. For the compost treatment, 120 g (w/w) peat growing media was weighed. This was mixed with 60 g (w/w) of inoculated CW and placed into a labelled pot. A divet was made in the centre of the pot and the lettuce plug planted. For the AD treatment the 10 ml volume of inoculated AD was mixed using a large tongue depressor with 180 g (w/w) of the growing media (GM). The lettuce plug was planted using the same method as for the CW treatment. Lettuce plants were grown at room temperature under artificial lights. The lighting was set up to reflect day light

conditions (twelve hours of light, twelve hours of dark). Plants were watered on a regular basis as needed. On a weekly basis two plants from each treatment were analysed for the internalisation of the test pathogens. Once the plant had reached maturity, or the pathogen numbers in the GM declined, eight samples from each treatment were analysed.

The temperature of the growth environment was measured using temperature data loggers throughout the trials. Three biological replicates of each treatment were carried out. Within each replicate a total of 120 plants were analysed. This equates to 40 plants *per* pathogen, 20 for the AD treatment and 20 for the CW treatment. In parallel with each replicate control samples were also included numbering ten *per* treatment *per* replicate. The pH and water activity data for each plant was also analysed.

### 2.4. Microbiological analysis

For both *S.* Senftenberg and *E. coli* O157:H7, plant parts selected for microbiological analysis were surface sterilised by dipping in 1% silver nitrate ($AgNO_3$) for 10 s followed by two washing steps of 10 s, in sterilised water (Franz et al., 2007). Both plant roots and leaves were weighed and TSB added to give a 1:10 dilution, then homogenised in a stomacher for 90 s. Both the GM in the pot (GMP) and the GM around the roots (GMR) were analysed. Ten grams of both were weighed and TSB added to give a 1:10 dilution. Both GMP and GMR subsamples were homogenised by shaking on an orbital shaker for 15 min at 200 rpm.

Relevant dilutions of each sub sample were carried out and 100 μl plated onto relevant selective agars. Plates were incubated overnight at 37 °C and colonies were further verified using a UV lamp at 254 nm. All sub-samples were also incubated overnight at 37 °C. If no counts could be detected through direct counts, detection was carried out by enrichment.

For *L. monocytogenes,* the same method as above was applied, except that BLEB was used as an enrichment media instead of TSB. Also, during the final sampling day for *L. monocytogenes* seven extra plants for each treatment were analysed without surface sterilisation. The method employed was the same as above, with the $AgNO_3$ wash step omitted.

### 2.5. Microscopy analysis

Sections of each of the lettuce plants from both the leaves and the roots were fixed using 10% formalin and stored in dark conditions for further analysis. GFP-labelled pathogens in the leaf and root sections were observed with a Lecia SP5 confocal scanning laser microscope. The tissue sections were scanned for fluorescent bacteria under light with an excitation wavelength of 488 nm and a BA505-525 emission filter.

## 3. Results

### 3.1. Survival of E. coli O157:H7, L. monocytogenes and S. Senftenberg in growing media

*E. coli* O157:H7 could be detected in the growth media throughout the trial period for replicates one and two. In the case of the third replicate however the pathogen numbers dropped to approximately $Log_{10}$ 2 CFU $g^{-1}$ by week four. Fig. 1 shows the survival of *E. coli* O157:H7 in both GMP and GMR for both treatments. All the remaining lettuce plants for this trial were analysed at the point where the pathogen numbers in the growing media were falling below the limit of detection by direct plating (day 50 for replicate 1 and 2, day 28 for replicate 3).

Fig. 2 shows the survival results for *S.* Senftenberg in both the

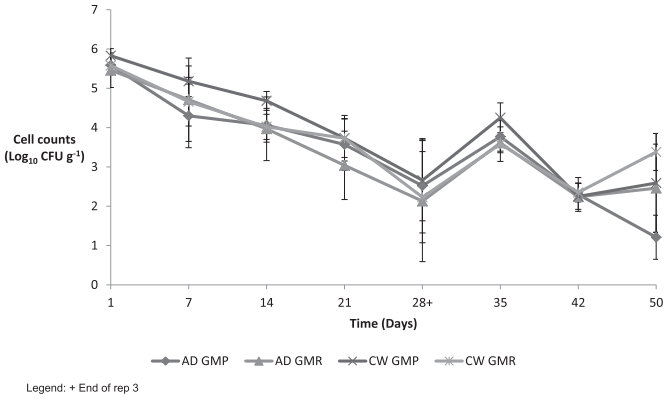


**Fig. 1.** Survival of *E. coli* O157:H7 in the growing media (GMP) and the root soil (GMR) for each treatment (AD/CW) throughout the trial (n = 3).

GMP and GMR for both treatments. *S.* Senftenberg could be detected in the growing media throughout the growing trial for replicates one and two. As with *E. coli* O157:H7, for replicate three the pathogen numbers dropped to approximately $Log_{10}$ 2 CFU $g^{-1}$ by week four. All the remaining lettuce plants for this trial were analysed at this point (day 50 for replicate 1 and 2, day 28 for replicate 3).

For *L. monocytogenes* the trials were stopped before the plants reached full maturity. Fig. 3 provides its survival data in the growing media and the root soil throughout the trials. As can be seen from Fig. 3, the numbers of *L. monocytogenes* detected declined rapidly between days 7–15 for each treatment for all replicates. The results for the second strain of *L. monocytogenes* used demonstrated the same survival trend (data not shown).

### 3.2. Internalisation of E. coli O157:H7 and S. Senftenberg in lettuce plants

No positive internalisation results were observed for *S.* Senftenberg or *E. coli* O157:H7 using direct plating. All positive results were obtained only after enrichment of the samples for 24 h in TSB. Table 1 provides a summary of the internalisation results for both *E. coli* O157:H7 and *S.* Senftenberg. In each case there was variation in the internalisation rates between the replicates.

For *E. coli* O157:H7, the lettuce grown in both AD and CW showed internalisation on day seven of the trial in the third replicate. On day 14 only one root sample from the AD treatment was positive. Both treatments were detected as being positive on both

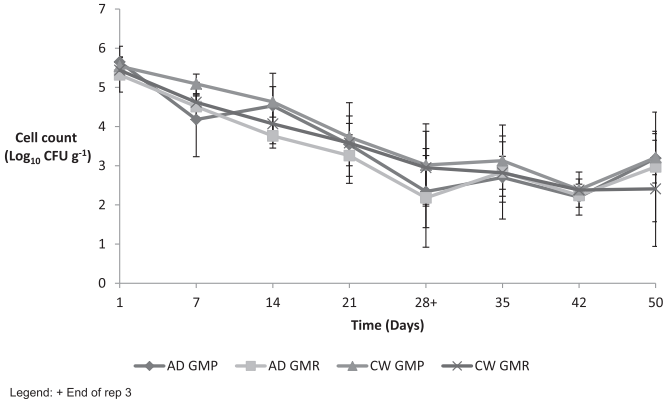


**Fig. 2.** Survival of *S.* Senftenberg in the growing media (GMP) and the root soil (GMR) for each treatment (AD/CW) throughout the trial (n = 3).

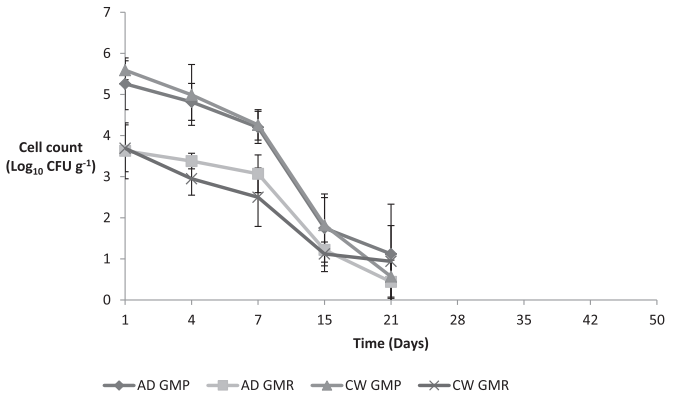


**Fig. 3.** Survival of *L. monocytogenes* in the growing media (GMP) and the root soil (GMR) for each treatment (CW/AD) throughout the trial (n = 3).

days 21 and 28. The data from replicates 1 and 2 showed no internalisation until day 42. By day 50 the plants for these replicates were at full maturity, thus all the remaining plants were analysed at this point. Both treatments contained positive samples on day 50.

The results for *S.* Senftenberg showed a broadly similar trend. There was variation in the internalisation rates between the replicates. For the third replicate, the lettuce grown in CW showed apparent internalisation on days 14 and 21, but not on day 28, the final day of the third replicate. Concerning the AD treatment, positive samples were detected on both days 21 and 28. Data from both replicates 1 and 2 showed no evidence of internalisation until day 35 of the trial. By day 50 the plants for both replicates were at full maturity, thus all the remaining plants were analysed at this point. In the case of both treatments positive samples were detected by day 50.

Table 2 outlines the number of positive plants from each replicate for both *E. coli* O157:H7 and *S.* Senftenberg for each treatment tested. The table also highlights the numbers of plants for which both the root and leaf section were positive.

All control samples for each of the replicates were negative for both *S.* Senftenberg and *E. coli* O157:H7.

### 3.3. Absence of internalisation of *L. monocytogenes* into lettuce plants

There was no internalisation of *L. monocytogenes* into the lettuce plants for either of the two treatments. *L. monocytogenes* numbers declined in the growing media to levels below $Log_{10}$ 2 CFU $g^{-1}$ within the first two weeks of the trial for all replicates. The trial was ended on day 21 for all replicates due to this observation. As the trial finished earlier than planned there was a surplus of plants. In total 30 plants for each replicate were surface sterilised.

Due to the absence of internalisation being observed using surface sterilisation, a selection of plants (7 from each replicate for each treatment) were analysed without surface sterilisation to determine the presence of the pathogen on the exterior of plant. Table 3 shows the results for the non-surface sterilised *L. monocytogenes* treated plants. For the AD treatment nine of 21 (42.9%) plant samples were positive. The CW treatment resulted in three (14.3%) positive samples out of 21 plants tested.

All control samples tested negative for *L. monocytogenes* for all replicates. For the non-surface sterilised samples however, the control samples tested positive for *Listeria* species.

**Table 1**
Total number of positive samples for *E. coli* O157:H7 and *S.* Senftenberg in root (R) and leaf (L) for each treatment (AD/CW) (n = 3).

| Day | Replicate | *E. coli* O157:H7 | | | | *S.* Senftenberg | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | AD | | CW | | AD | | CW | |
| | | L | R | L | R | L | R | L | R |
| 1 | 1, 2, 3 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| 7 | 1, 2 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| | 3 | 2/2 | 1/2 | 1/2 | 1/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| 14 | 1 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 1/2 | 0/2 |
| | 2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| | 3 | 0/2 | 1/2 | 0/2 | 0/2 | 0/2 | 0/2 | 2/2 | 1/2 |
| 21 | 1, 2 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| | 3 | 1/2 | 1/2 | 1/2 | 2/2 | 2/2 | 1/2 | 1/2 | 0/2 |
| 28+ | 1, 2 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| | 3 | 2/10 | 1/10 | 1/10 | 0/10 | 2/8 | 2/8 | 0/8 | 0/8 |
| 35 | 1 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| | 2 | 0/2 | 0/2 | 0/2 | 0/2 | 1/2 | 0/2 | 1/2 | 0/2 |
| 42 | 1 | 1/2 | 0/2 | 1/2 | 1/2 | 1/2 | 1/2 | 0/2 | 0/2 |
| | 2 | 0/2 | 0/2 | 1/2 | 0/2 | 1/2 | 1/2 | 1/2 | 1/2 |
| 50 | 1 | 2/9 | 1/9 | 2/9 | 1/9 | 4/10 | 3/10 | 3/9 | 1/9 |
| | 2 | 1/9 | 2/9 | 1/9 | 0/9 | 1/9 | 2/9 | 2/9 | 1/9 |
| Total positive | 1 | 3/21 | 1/21 | 3/21 | 2/21 | 5/24 | 4/24 | 4/23 | 1/23 |
| | 2 | 1/21 | 2/21 | 2/21 | 0/21 | 3/23 | 3/23 | 4/23 | 2/23 |
| | 3 | 5/18 | 4/18 | 3/18 | 3/18 | 4/16 | 3/16 | 3/16 | 1/16 |
| | 1, 2, 3 | 9/60 | 7/60 | 8/60 | 5/60 | 12/63 | 10/63 | 11/62 | 4/62 |
| % Positives | 1 | 14.3 | 4.8 | 14.3 | 9.5 | 20.8 | 16.7 | 17.4 | 4.3 |
| | 2 | 4.8 | 9.5 | 9.5 | 0 | 13 | 13 | 174 | 8.7 |
| | 3 | 27.8 | 22.2 | 16.7 | 16.7 | 25 | 18.7 | 18.7 | 6.2 |
| | 1, 2, 3 | 15 | 11.7 | 13.3 | 8.3 | 19 | 15.9 | 17.7 | 6.4 |

L refers to leaf samples; R refers to root samples; + refers to the end of rep 3; AD refers to anaerobic digestate treatment; CW refers to composted waste treatment.

**Table 2**
Number of positive samples for each replicate.

| Replicate | *E. coli* O157:H7 | | | | | | *S.* Senftenberg | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AD | | | CW | | | AD | | | CW | | |
| | Leaf | Root | Same plant | Leaf | Root | Same plant | Leaf | Root | Same plant | Leaf | Root | Same plant |
| 1 | 3 | 2 | 1 | 3 | 2 | 2 | 4 | 4 | 2 | 4 | 3 | 1 |
| 2 | 1 | 1 | 0 | 3 | 0 | 0 | 3 | 1 | 1 | 4 | 3 | 2 |
| 3 | 5 | 4 | 2 | 4 | 4 | 2 | 4 | 6 | 3 | 4 | 1 | 1 |

AD refers to anaerobic digestate treatment; CW refers to composted waste treatment.

**Table 3**
Total number of positive non surface sterilised plants for *L. monocytogenes* trial; analysis carried out at day 21 for each replicate (n = 3).

| | | Total non-sterilised positive | | | |
|---|---|---|---|---|---|
| | | L | % | R | % |
| *L. monocytogenes* | AD | 9/21 | 42.9 | 9/21 | 42.9 |
| | CW | 3/21 | 14.2 | 14/21 | 66.7 |

L refers to leaf samples; R refers to root samples.

**Table 4**
Temperature fluctuations between each of the three replicates.

| Pathogen | Temperature (°C) | | | |
|---|---|---|---|---|
| | Replicate | Average | Max | Min |
| *S.* Senftenberg/*E. coli* O157:H7 | 1, 2 | 16.9 | 20.5 | 15 |
| *S.* Senftenberg/*E. coli* O157:H7 | 3 | 25.8 | 27.5 | 21.5 |
| *L. monocytogenes* | 1, 2 | 25.8 | 27.5 | 21.5 |
| *L. monocytogenes* | 3 | 20.7 | 25 | 18 |

### 3.4. Differences in temperature between replicates

Table 4 shows the fluctuations in temperature between each of the independent replicates during this study. The temperature for replicate 3 for *S.* Senftenberg and *E. coli* O157 was on average 8.9 °C higher than replicates 1 and 2.

### 3.5. Visualisation of internalisation of pathogens

Fluorescent microscopy was used to verify the internalisation results. Fig. 4(a) shows internalisation of the *S.* Senftenberg containing the GFP plasmid from AD amended GM, with apparent colony formation within the leaf. Fig. 4(b) shows the presence of *S.* Senftenberg within the root structure, from AD amended GM. However there was no evidence of colonisation. Fig. 5 shows *E. coli* O157:H7 within the root structure of the lettuce, from CW amended GM. *E. coli* O157:H7 could not be visualised in the leaves of any of the positive samples tested.

## 4. Discussion

In this study, internalisation of *S.* Senftenberg and *E. coli* O157:H7 into lettuce was clearly demonstrated from the growth media containing contaminated CW or AD. However, the results show large variation between replicates. For both *S.* Senftenberg and *E. coli* O157:H7, replicates 1 and 2 were carried out in the spring, with one week interval between them. The third replicate however, was carried out during the summer months; there was an increase in temperature in the laboratory during this time. Table 4 showed the fluctuations in temperature between the replicates. A previous study assessing the survival of pathogens in both CW and AD matrices have shown, that an increase in temperature led to a reduction in survival for all three of the pathogens tested, in both AD and CW (unpublished data).

The temperature for replicate 3 in the case of the *S.* Senftenberg and *E. coli* O157:H7 trials and for replicates 1 and 2 of the

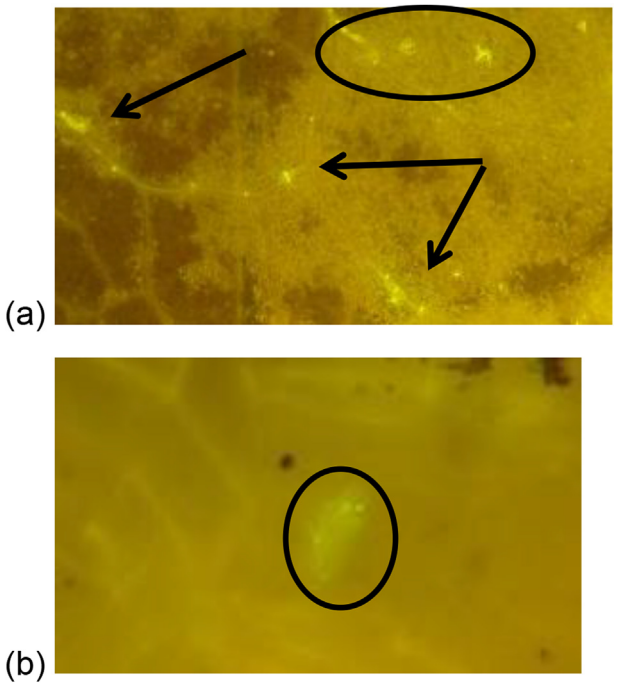


**Fig. 4.** *S.* Senftenberg internalisation in (a) lettuce leaf, (b) lettuce root, black arrows and circle indicates areas of fluorescence.

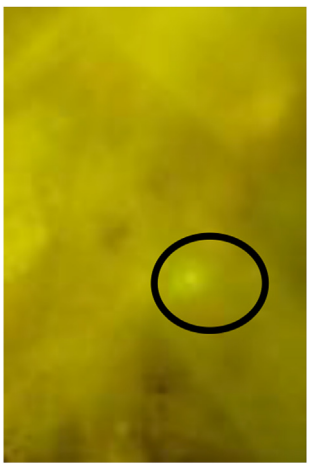

**Fig. 5.** *E. coli* O157:H7 internalisation of the root, black circle indicates areas of fluorescence.

*L. monocytogenes* was between 21 and 27.5 °C. Previous work carried out by this group indicate that pathogen survival times for these pathogens was between 21 and 28 days at 25 °C in these matrices. The third replicate of the *L. monocytogenes* trial was carried out in temperatures of between 18 and 25 °C; again in this case the rapid decline in pathogen numbers may be connected to the temperature. The effect of temperature increase between replicates may have influenced the internalisation ability of the pathogens. The third replicate demonstrated the internalisation of both *E. coli* O157:H7 and *S.* Senftenberg on days 7 and 14, respectively. This was in contrast to both replicates 1 and 2 where internalisation of neither pathogen was observed until days 35 and 42 for *S.* Senftenberg and *E. coli* O157:H7, respectively.

The reduction of pathogen numbers in the growth media due to the increased temperature may have also affected the uptake rate. Studies using a range of pathogen concentrations have only obtained positive results for internalisation at higher starting inoculums of approximately $Log_{10}$ 6, $Log_{10}$ 7 and $Log_{10}$ 8 CFU $g^{-1}$ for both *S. enterica* and *E. coli* O157:H7 (Ge et al., 2012; Erickson et al., 2014b). Interestingly, internalisation was observed during this study when the pathogen numbers were lower. The concentration of *S.* Senftenberg in the GM was $Log_{10}$ 4.63 CFU $g^{-1}$ when internalisation was first observed. *S.* Senftenberg was also positive for internalisation at GM concentrations of $Log_{10}$ 3.19 CFU $g^{-1}$. Similar results were observed for *E. coli* O157:H7; internalisation was first observed at $Log_{10}$ 4.3 CFU $g^{-1}$ and was still observed when *E. coli* O157:H7 concentration in the GM was $Log_{10}$ 1.21 CFU $g^{-1}$.

Upon reviewing the current literature regarding pathogen uptake into leafy greens, external factors have been shown to influence internalisation. One study demonstrated an increase in uptake during the autumn, compared with trials carried out in the spring season (Oliveira et al., 2011). A study by Erickson et al. (2014b) demonstrated that a number of other biotic and abiotic factors affect the ability of *E. coli* O157:H7 to internalise leafy green through the roots. Moisture content of the growth media, as well as plant health and the age of the plant were all factors effecting the internalisation of *E. coli* O157:H7. The variation between the replicates for both *S.* Senftenberg and *E. coli* O157:H7 observed in this study may have been affected by plant health. Franz et al. (2007) observed during a study of internalisation, that plants testing positive for internalisation weighed less than plants without internal contamination.

In total *S.* Senftenberg resulted in a 15.9% internalisation in the leaves and 12.7% uptake in the roots for the AD treatment. CW treatment resulted in a 17.7% uptake in the leaves and 9.7% in the roots. Higher uptake rate in the leaves compared to the roots may be a result of colonisation *via* the stomata and injuries on the leaves (Kroupitski et al., 2009). Methods applied to assess internalisation in RTE plants vary. The current study assessed the ability of selected foodborne pathogens to internalise into four week old seedlings transplanted into contaminated growth media in a laboratory environment. Previous studies using similar methods have reported contradictory results. Gorbatsevich et al. (2013) demonstrated the ability of *S.* Newport to internalise sweet basil through the root system at most time points tested. Similar to the current study positive samples were only detectable by enrichment. Another study carried out on lettuce also showed apparent internalisation through the root system (Bernstein et al., 2007). The study however, did not surface sterilise the leaves, therefore the results are not directly comparable. Ge et al. (2012) employed a similar method, as employed by the current study, to examine the effects of extreme weather conditions (drought and heavy rain) on *S. enterica* internalisation of lettuce. The study showed no internalisation for any of the treatment groups for lettuce.

As with *S. enterica*, the results of previous studies into *E. coli* O157:H7 internalisation vary greatly. A study into both pathogens using a hydroponic growing system showed positive results for both pathogens, with a higher percentage of internalisation for *E. coli* O157:H7 (Franz et al., 2007). Erickson et al. (2010) conducted a field study into internalisation of leafy greens through irrigation water and contaminated compost. The study observed no internalisation for compost and infrequent internalisation with the irrigation water treatment. A number of other studies have also demonstrated the lack of internalisation of *E. coli* O157:H7 into lettuce plants (Erickson et al., 2014a; Zhang et al., 2009). A study by Mootian et al. (2009) however, showed 11% of the lettuce samples grown in contaminated soils were positive for internalised *E. coli* O157:H7 after enrichment. Similar results were observed in the current study with 11.7% of CW treated plants testing positive and 15% of plants from the AD treatment testing positive. As with the results for *S.* Senftenberg the detection rate for the root was less than the leaves with 8.3% and 11.7% for the CW and AD treatments respectively. Several other studies have also been conducted to determine the internalisation ability of both these pathogens. However they cannot be directly compared to the current study as the pathogens were exposed to lettuce seeds rather than to the transplanted seedlings (Jablasone et al., 2005; Kroupitski et al., 2009; Solomon et al., 2002).

The results of the fluorescent microscopy were also variable. For the *S.* Senftenberg samples clear colony formation within the leaf structure was observed. The results for the roots of both *E. coli* and *S.* Senftenberg show apparent fluorescence within the root structure without colony formation. The lack of visual colonisation may be due to the low numbers of the bacteria present in the plants; no positive samples were obtained throughout the trials through direct enumeration. This indicated a low pathogen level present in the samples. Only a small section of the leaves and roots for each sample were stored for fluorescent microscopy analysis, this may also have contributed to the results obtained.

The results for *L. monocytogenes* showed no internalisation for either treatment tested. Previous studies for this pathogen have shown varying results. Chitarra et al. (2014) reported apparent internalisation of *L. monocytogenes* at 24 °C, using 70% ethanol and 0.6% sodium hypochlorite to surface sterilise the samples. This experimental strategy differs from the method applied by this study. Both surface sterilisation methods were assessed by Franz et al. (2007), who found $AgNO_3$ more effective than $NaHClO_3$ and ethanol as a surface steriliser for the samples. This may account for the difference in results. One other study has shown positive results for *L. monocytogenes* in both lettuce seedlings and mature lettuce plants, using a hydroponic growing system (Standing et al., 2013). Other studies have not shown internalization of *Listeria* in plants (Jablasone et al., 2005; Oliveira et al., 2011). The results of the current study did however show the ability of *L. monocytogenes* to survive on the surface of plants. Surface contamination of the pathogen implies that aerosolisation of pathogens from the peat growing media was a likely route of contamination.

The study shows 42.9% and 14.3% of positive samples for AD and CW treatments respectively at the end of the replicates. Comparing both the AD and CW treatments clearly shows a lower number of positive samples from the CW treatment. This may be an effect of the CW matrix itself. A study carried out by Gu et al. (2013) demonstrated a reduction in internal colonisation of tomato plants by *S.* Typhimurium using compost amended soil, compared with non-organic soil amendments.

The results of this study demonstrate the internalisation of both *S.* Senftenberg and *E. coli* O157:H7 in butterhead lettuce. Although the inoculation levels used in this study were lower ($Log_{10}$5 CFU $g^{-1}$) than previous studies reporting internalisation it is important to note that these levels are still higher than would be

expected of natural levels of contamination present in contaminated CW and AD. The study also highlights the influence of external factors such as temperature on the internalisation of foodborne pathogens in lettuce, and the importance of standardising as many factors as possible to ensure reproducibility.

## 5. Conclusions

Research into the area of foodborne bacterial pathogen uptake by plants varies widely. This uncertainty and variation creates challenges for accurately assessing the risk in using organic fertilisers (AD/CW) in horticulture production. The increase in the volume of food waste being transformed in this way further emphasises the need to understand the risks in order to advise growers.

Overall, the results of this study show apparent internalisation for *S.* Senftenberg and *E. coli* O157:H7 in lettuce grown in contaminated food derived CW and AD. *L. monocytogenes* did not show the ability to internalise the lettuce under the experimental conditions tested, but did show the ability to survive on the surface of the lettuce leaves. This research showed the effect of temperature variation between each of the replicates and the resultant decline in pathogen numbers at higher temperatures, for each of three of the pathogens tested.

For both *S.* Senftenberg and *E. coli* O157:H7 there were higher numbers of positives in the roots samples for the AD treatment than the CW. This may indicate possible inhibition by the CW matrix or may be due to experimental variation. The results clearly show the ability of both *S.* Senftenberg and *E. coli* O157:H7 to internalise the lettuce plants. This is of concern for such an RTE crop because internalisation means that washing will not be effective in removing the pathogen prior to consumption. Whilst the inoculum levels used in this study may not represent levels found naturally in the environment, the potential for uptake cannot be overlooked. The study demonstrates that a number of biotic and abiotic factors need to be considered when examining pathogen internalisation to allow accurate comparisons between studies.

## Conflict of interest

No conflict of interest declared.

## Acknowledgements

The authors would like to thank Dr Conor O'Byrne of National University of Ireland, Galway for the *L. monocytogenes* GFP strain and the Irish Department of Agriculture, Food and the Marine for the *S.* Senftenberg isolate. The authors would also like to acknowledge Vincenzo Pennone, Teagasc Food Research Centre Moorepark, for his assistance with the fluorescence microscopy. This study was funded by the Teagasc Walsh Fellowship Scheme.

## References

Alburquerque, J.A., De la Fuente, C., Campoy, M., Carrasco, L., Nájera, I., Baixauli, C., Caravaca, F., Roldán, A., Cegarra, J., Bernal, M.P., 2012. Agricultural use of digestate for horticultural crop production and improvement of soil properties. Eur. J. Agron. 43, 119–128.

Avery, L.M., Booth, P., Campbell, C., Tompkins, D., Hough, R.L., 2012. Prevalence and survival of potential pathogens in source-segregated green waste compost. Sci. Total Environ. 431, 128–138.

Bernstein, N., Sela, S., Neder-Lavon, S., 2007. Assessment of contamination potential of lettuce by *S. enterica* enterica serovar Newport added to the plant growing medium. J. Food Prot. 70, 1717–1722.

Bonetta, S., Ferretti, E., Bonetta, S., Fezia, G., Carraro, E., 2011. Microbiological contamination of digested products from anaerobic co-digestion of bovine manure and agricultural by-products. Lett. Appl. Microbiol. 53, 552–557.

Callejón, R.M., Rodríguez-Naranjo, M.I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M.C., Troncoso, A.M., 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. Foodborne Pathogens Dis. 12, 32–38.

Chitarra, W., Decastelli, L., Garibaldi, A., Gullino, M.L., 2014. Potential uptake of *Escherichia coli* O157:H7 and *Listeria monocytogenes* from growth substrate into leaves of salad plants and basil grown in soil irrigated with contaminated water. Int. J. Food Microbiol. 189, 139–145.

EFSA, 2014. Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*S. enterica* and Norovirus in leafy greens eaten as salads). EFSA J. 12 (3), 3600, 118pp.

Erickson, M.C., Webb, C.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Doyle, M.P., 2014a. Biotic and abiotic variables affecting internalization and fate of *Escherichia coli* O157: H7 isolates in leafy green roots. J. Food Prot. 77, 872–879.

Erickson, M.C., Webb, C.C., Díaz-Pérez, J.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Phatak, S.C., Doyle, M.P., 2014b. Absence of internalization of *Escherichia coli* O157: H7 into germinating tissue of field-grown leafy greens. J. Food Prot. 77, 189–196.

Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J., Ma, L., Doyle, M.P., 2010. Infrequent internalization of *Escherichia coli* O157: H7 into field-grown leafy greens. J. Food Prot. 73, 500–506.

Franz, E., Visser, A.A., Van Diepeningen, A.D., Klerks, M.M., Termorshuizen, A.J., Van Bruggen, A.H.C., 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Food Microbiol. 24, 106–112.

Fremaux, B., Prigent-Combaret, C., Vernozy-Rozand, C., 2008. Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: an updated review. Vet. Microbiol. 132, 1–18.

Ge, C., Lee, C., Lee, J., 2012. The impact of extreme weather events on *S. enterica* internalization in lettuce and green onion. Food Res. Int. 45, 1118–1122.

Goberna, M., Podmirseg, S.M., Waldhuber, S., Knapp, B.A., García, C., Insam, H., 2011. Pathogenic bacteria and mineral N in soils following the land spreading of biogas digestates and fresh manure. Appl. Soil Ecol. 49, 18–25.

Gorbatsevich, E., Pinto, R., Bernstein, N., 2013. Root internalization, transport and in-planta survival of *S. enterica* enterica serovar Newport in sweet basil. Environ. Microbiol. Rep. 5, 151–159.

Gu, G., Cevallos-Cevallos, J.M., Vallad, G.E., Van Bruggen, A.H., 2013. Organically managed soils reduce internal colonization of tomato plants by *Salmonella enterica* serovar Typhimurium. Phytopathology 103, 381–388.

Jablasone, J., Warriner, K., Griffiths, M., 2005. Interactions of *Escherichia coli* O157: H7, *Salmonella* Typhimurium and *Listeria monocytogenes* plants cultivated in a gnotobiotic system. Int. J. Food Microbiol. 99, 7–18.

Jacxsens, L., Uyttendaele, M., Holvoet, K., Kierezieva, K., Luning, P., 2012. The impact of climate change and globalisation on the safety of fresh produce. Int. Food Hyg. 23, 26–27.

Jiang, X., Morgan, J., Doyle, M.P., 2002. Fate of *Escherichia coli* O157: H7 in manure-amended soil. Appl. Environ. Microbiol. 68, 2605–2609.

Johannessen, G.S., Bengtsson, G.B., Heier, B.T., Bredholt, S., Wasteson, Y., Rørvik, L.M., 2005. Potential uptake of *Escherichia coli* O157:H7 from organic manure into crisphead lettuce. Appl. Environ. Microbiol. 71, 2221–2225.

Kim, J., Jiang, X., 2010. The growth potential of *Escherichia coli* O157: H7, *S. enterica* spp. and *Listeria monocytogenes* in dairy manure-based compost in a greenhouse setting under different seasons. J. Appl. Microbiol. 109, 2095–2104.

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., Sela, S., 2009. Internalization of *S. enterica* enterica in leaves is induced by light and involves chemotaxis and penetration through open stomata. Appl. Environ. Microbiol. 75, 6076–6086.

Ma, L., Zhang, G., Doyle, M.P., 2011. Green fluorescent protein labeling of *Listeria, S. enterica*, and *Escherichia coli* O157: H7 for safety-related studies. PLoS One 6, 1–7.

Mootian, G., Wu, W.-H., Matthews, K.R., 2009. Transfer of *Escherichia coli* O157: H7 from soil, water, and manure contaminated with low numbers of the pathogen to lettuce plants. J. Food Prot. 72, 2308–2312.

Oliveira, M., Usall, J., Vias, I., Solsona, C., Abadias, M., 2011. Transfer of *Listeria innocua* from contaminated compost and irrigation water to lettuce leaves. Food Microbiol. 28, 590–596.

Solomon, E.B., Yaron, S., Matthews, K.R., 2002. Transmission of *Escherichia coli* O157: H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. 68, 397–400.

Standing, T.-A., Du Plessis, E., Duvenage, S., Korsten, L., 2013. Internalisation potential of *Escherichia coli* O157: H7, *Listeria monocytogenes, Salmonella enterica* subsp. enterica serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plants. J. Water Health 11, 210–223.

Utratna, M., Cosgrave, E., Baustian, C., Ceredig, R., O'byrne, C., 2012. Development and optimization of an EGFP-based reporter for measuring the general stress response in *Listeria monocytogenes*. Bioengineered 3, 93–103.

You, Y., Rankin, S.C., Aceto, H.W., Benson, C.E., Toth, J.D., Dou, Z., 2006. Survival of *S. enterica* enterica serovar Newport in manure and manure-amended soils. Appl. Environ. Microbiol. 72, 5777–5783.

Zhang, G., Ma, L., Beuchat, L.R., Erickson, M.C., Phelan, V.H., Doyle, M.P., 2009. Lack of internalization of *Escherichia coli* O157: H7 in lettuce (Lactuca sativa L.) after leaf surface and soil inoculation. J. Food Prot. 72, 2028–2037.