Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* and *Staphylococcus aureus* in lettuce seedlings and mature plants

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**ABSTRACT**

The internalisation potential of *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* in lettuce was evaluated using seedlings grown in vermiculite in seedling trays as well as hydroponically grown lettuce. Sterile distilled water was spiked with one of the four human pathogenic bacteria (10^5 CFU/mL) and used to irrigate the plants. The potential for pathogen internalisation was investigated over time using light microscopy, transmission electron microscopy and viable plate counts. Additionally, the identities of the pathogens isolated from internal lettuce plant tissues were confirmed using polymerase chain reaction with pathogen-specific oligonucleotides. Internalisation of each of the human pathogens was evident in both lettuce seedlings and hydroponically grown mature lettuce plants. To our knowledge, this is the first report of *S. aureus* internalisation in lettuce plants. In addition, the levels of background microflora in the lettuce plants were determined by plate counting and the isolates identified using matrix-assisted laser ionisation–time of flight (MALDI–TOF). Background microflora assessments confirmed the absence of the four pathogens evaluated in this study. A low titre of previously described endophytes and soil inhabitants, i.e., *Enterobacter cloacae*, *Enterococcus faecalis*, *Lysinibacillus fusiformis*, *Rhodococcus rhodochrous*, *Staphylococcus epidermidis* and *Staphylococcus hominis* were identified.

**Key words |** foodborne human pathogens, internalisation

**INTRODUCTION**

Dietary guidelines from governmental agencies and nationally recognised health professional organisations in many countries recommend an increase in the consumption of fresh fruit and vegetables (Beuchat 2002). Contrary to food products derived from animals, which are generally cooked, fruit and vegetables are mostly consumed raw. Fresh produce retains much of its natural microflora, including a diversity of bacteria, yeasts and fungi, of which, some may cause spoilage. Most of these natural microfloras represent epiphytic organisms with only a small number being plant pathogens (Beuchat 2002; Lindow & Brandl 2003). Human pathogens including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella Typhimurium* and *Staphylococcus aureus* have been found associated with fresh produce (Takeuchi et al. 2000; Harris et al. 2003; Sharma et al. 2009). Rosenblueth & Martinez-Romero (2006) further demonstrated the endophytic nature of these organisms using ribotyping. It was reported in several papers that these pathogens are able to adapt and survive in the plant environment thereby introducing a potential food safety risk for the consumer (Deering et al. 2011a).

An increase in foodborne disease outbreaks associated with the increased consumption of contaminated fresh produce has been linked with a number of pathogenic bacteria
such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Johnston et al. 2006; Herman et al. 2008). From 1996 to 2005, the incidence of foodborne disease outbreaks linked to the consumption of leafy greens increased by 39% in the United States, while consumption of leafy greens increased by only 9% (Herman et al. 2008). In 2006, the consumption of *E. coli* O157:H7 contaminated fresh spinach caused 204 people to become ill in 26 states in the United States, three died and 31 cases progressed to haemolytic uraemic syndrome which often causes kidney failure (http://www.cdc.gov/ecoli/2006/December/121406.htm). In 2011, a multistate outbreak of *E. coli* O157:H7 in 10 states of the United States caused 60 individuals to become ill (http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuces/120711/index.html). A foodborne disease outbreak in the European Union in May 2011 was caused by *E. coli* O104:H4, a more virulent verocytotoxin-producing strain than *E. coli* O157:H7. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (http://www.bbc.co.uk/news/world-europe-13683270). The recent Listeriosis outbreak during 2011 was reported to be due to consumption of contaminated canteloupes that left 146 people sick and caused 30 deaths in 28 states across the United States (http://www.foodsafetynews.com/2011/10/cantaloupe-listeria-outbreak-84-sick-15-dead).

Internalisation studies to date have focused mainly on either *E. coli* or *S. Typhimurium* (Deering et al. 2011a). The objective of this study was to investigate the potential of both Gram-negative (*E. coli* O157:H7, *S. Typhimurium*) and Gram-positive (*S. aureus*, *L. monocytogenes*) human pathogenic bacteria to internalise lettuce seedlings and mature plants in one study under similar controlled conditions. The internalisation of the specified pathogens was demonstrated using light and transmission electron microscopy (TEM), and viable plate counts with polymerase chain reaction (PCR) confirmation of pathogen identity. The background microflora was also isolated and identified using matrix-assisted laser ionisation–time of flight (MALDI–TOF).

**MATERIALS AND METHODS**

**Bacterial strain and inoculum preparation**

American Type Culture Collection (ATCC; Manassas, VA, USA) cultures *E. coli* O157:H7 (ATCC 35150), *L. monocytogenes* (ATCC 19115), *S. Typhimurium* (ATCC 14028) and *S. aureus* subsp. *aureus* (ATCC 12600) were used in this study. All cultures were maintained as lyophilised stocks and stored at −70°C with subcultures prepared 24 hours prior to use on Standard 1 medium. All media were obtained from Merck, Johannesburg, South Africa, unless stated otherwise. A single colony of each respective bacterium was subsequently inoculated into Tryptone Soy Broth (TSB) and shake incubated at 200 rpm at 37°C for 18 hours to obtain a final concentration of 10⁸ CFU/mL. The concentration was confirmed by dilution plating onto selective agar (MacConkey agar for *E. coli* O157:H7, Oxford
Listeria agar for *L. monocytogenes*, Xylose lysine deoxycholate agar for *S. Typhimurium* and Baird-Parker agar for *S. aureus*).

**Lettuce seedling germination, cultivation and inoculation**

Butterhead lettuce seeds (Kirchhoffs, Honeydew, South Africa) (132 in total: 24 seeds for each foodborne bacteria, 18 each set for an untreated control and for a crystal violet test to confirm uptake and translocation) were planted 0.5 cm deep into six separate seedling trays each containing sterilised autoclaved medium grade vermiculite (Hygrotech, Pretoria, South Africa). The seeds were germinated at 25°C in a dark growth chamber for 2 weeks and seedlings were watered daily with sterile distilled water and half strength nutrient solution (according to the manufacturer’s recommendations) (Ocean Agriculture (PTY) Ltd, Muldersdrift, South Africa).

Following germination, 12 hour light cycles were introduced and four seedling trays were watered daily with sterile distilled water containing the full strength nutrient solution inoculated with the respective pathogens (10⁵ CFU/mL). No pathogens were added to the fifth seedling tray, serving as the negative control. One gram of crystal violet (Merck) was added to 500 mL of sterile distilled water and used to water the sixth seedling tray. On days 3, 5, 7, 14, 21 and 28 after planting, three seedlings from each tray were harvested.

**Mature lettuce germination, cultivation and inoculation**

Butterhead lettuce seeds (Kirchhoffs) (106 in total: 19 seeds for each foodborne bacteria, 15 for control and 15 for crystal violet tests) were planted into seedling trays and germinated as described previously. Following germination, 12 hour light cycles were introduced and all seedling trays were watered daily with sterile distilled water containing full strength nutrient solution as before. No pathogens were added. Four weeks after planting, the seedlings were removed, rinsed with water to ensure removal of any excess and transplanted to the hydroponic system (University of Pretoria: Experimental Farm, Pretoria). Lettuce plants were grown in the hydroponic system at a temperature range of 25–30°C in sterile, water-tight containers with lids to prevent the leaves from touching the water. The control experiment was set up and crystal violet dye added as described previously. Sterile distilled water containing full strength nutrient solution and the respective bacterial inoculum (10⁵ CFU/mL) was used to water the experimental plants weekly. Before inoculation and 7, 14, 21 and 28 days after planting, three mature lettuce plants from each treatment were removed and the roots and leaves harvested.

**Weighing of seedlings and mature plants**

The harvested lettuce seedlings and mature plants were weighed prior to surface disinfection and microbiological analysis. Lettuce seedlings were weighed as whole plants which included the leaves, stems and roots. The mature lettuce plants were divided into roots and leaves by cutting the short stem at the base of the leaves. Roots and leaves were weighed separately.

**Verification of uptake and distribution**

Seedlings and mature lettuce plants treated with crystal violet were cut vertically for visual verification of dye entrance, water uptake and distribution.

**Effectiveness of surface disinfection**

Butterhead lettuces (*Lactuca sativa* cv. Nadine) (28 days old) were separated into leaves and roots. Twenty-four leaves and roots were divided into two sets each (Set A surface disinfected and Set B non-surface disinfected). Leaves and roots within each set were subdivided into four subsets for each of the selected pathogens. Surfaces of leaves and roots in both Set A and Set B were inoculated with 50 μL respective bacterium inoculums using 10⁵ CFU/mL. Inoculation was followed by air-drying in the laminar flow hood for 5 min. Set A was subsequently surface disinfected by immersion into 80% (v/v) ethanol for 5 min, followed by immersion twice in sterile distilled water to remove residual ethanol and air-dried at room temperature in the laminar flow hood (*Solomon et al. 2002*). Set B was not surface
disinfected in order to determine the residual pathogen titre on the leaves and roots.

Ten grams of the individual plant parts were placed into sterile polyethylene bags with 90 mL quarter strength Ringer’s solution and homogenised for 5 min at 260 rpm in a Stomacher® 400 (Seward, Lasec SA (PTY) Ltd, Johannesburg, SA). The liquid phase was aseptically removed and filtered through a 0.45 μm cellulose nitrate filter (Sartorius, Johannesburg, SA). Each filter was added to 9 mL TSB, mixed by vortexing, serially diluted and plated onto the selective and Standard 1 agar in order to determine total viable bacterial counts. The plates were incubated at 37°C for 24 hours, counts recorded and transformed to log$_{10} (x + 1)$ CFU/g.

**Microbiological analysis of seedlings**

The lettuce seedlings and mature plants were surface disinfected as previously described. Seedlings inoculated with the respective bacterial pathogens and the control were chopped in a sterile Petri dish in 1 mL quarter strength Ringer’s solution using a sterile scalpel. Each seedling sample was subsequently serially diluted and plated onto selective agar and Standard 1 agar as described previously. The plates were incubated at 37°C for 24 hours, counts recorded and transformed to log$_{10} (x + 1)$ CFU/g.

**Microbiological analysis of mature plants**

Ten grams of both mature lettuce roots and leaves were weighed aseptically, separated and placed into sterile polyethylene bags together with 90 mL quarter strength Ringer’s solution and homogenised for 5 min at 260 rpm in a Stomacher® 400. The liquid phase was removed and filtered through a 0.45 μm cellulose nitrate filter. The filter was placed in 9 mL TSB, serially diluted, plated and incubated as described previously. Colony counts were recorded and transformed to log$_{10} (x + 1)$ CFU/g.

**DNA extraction and PCR**

In order to confirm the presence of *E. coli* O157:H7, *L. monocytogenes*, *S. Typhimurium* and *S. aureus* in both the lettuce seedlings and mature lettuce plants, PCR was performed using DNA extracted from the samples collected. The 0.45 μm cellulose nitrate filters were inoculated into TSB as described previously. Bacterial cells were cultured aerobically for 48 hours at 37°C with agitation at 200 rpm. The DNA was extracted using the Triton-X method described by Wang & Slavik (2005) with some modifications. One millilitre of the respective bacterial cultures was centrifuged at 6,000 x g for 5 min. The process was repeated if the pellet was too small. The pellets were washed three times using 1 mL sterile double distilled water and subsequently centrifuged at 16,000 x g for 5 min. The pellets were resuspended in 50 μL 1% (v/v) Triton X-100 (Sigma, Johannesburg, SA), boiled for 10 min at 100°C, cooled on ice for 10 min and centrifuged at 16,000 x g for 5 min. Three microlitres of Ribonuclease A (Roche, Johannesburg, SA) was added to the supernatant and incubated overnight at room temperature to remove RNA present. A 1.5 μL sample of the supernatant was used as a template for each PCR. Each 25 μL PCR reaction mixture contained 0.3 μL of BioTaq polymerase (5 U/μL), 1.5 μL MgCl₂ (50 mM), 0.75 μL dNTPs (10 mM of each), 2.5 μL NH₄ reaction buffer (10×) (all from Bioline, Celtic Molecular Diagnostics, Cape Town, SA), 1.75 μL Bovine Serum Albumin Acetylated (10 mg/mL) (Promega, Madison, WI, USA), 1.25 μL Dimethyl Sulphoxide (Saarchem, Merck) and 0.3 μL of each primer (Whitehead Scientific, Cape Town, SA). Primers used in this study are listed in Table 1. For control purposes, a PCR reaction mixture containing sterile double distilled water and all other reagents except template DNA was included. Thermocycling was performed using an Eppendorf Thermocycler (Merck) and the PCR conditions were as follows: 94°C for 2 min, followed by 37 cycles of 94°C for 30 s, 61°C for 45 s and 72°C for 1.5 min, with a final extension at 72°C for 7 min. PCR products were visualised following gel electrophoresis on 1% agarose gels.

**Identification of natural internal microflora using MALDI–TOF**

Purified bacterial cultures isolated from Standard 1 agar medium for both seedlings and mature lettuce plants were transferred in duplicate directly to the MALDI–TOF steel polished target plate (Bruker, Bremen, Germany) and
overlaid with the α-cyano-4-hydroxycinnamic acid matrix (Bruker). The target plate was subsequently analysed using Bruker MicroFlex LT MALDI–TOF in conjunction with Bruker Biotyper Automation Software and library. The MALDI–TOF was calibrated prior to use with the bacterial standard supplied by Bruker. Duplicate score values (SV) were recorded; SV were used to determine the accuracy of identification. A SV of between 1.999 and 1.700 was used to identify the genus name of the organism, and a value of above 2.0 was used to determine the genus and probable species of an organism.

Light and transmission electron microscopy – lettuce seedlings and mature plants

The primary root tip, branch point of newly developed lateral roots and the stem of the lettuce seedlings aged 1–4 weeks old were selected for sectioning. The primary root tip, branch point within the primary root at a mature lateral root junction and the stem node were selected for mature lettuce roots aged 4–8 weeks. For sectioning of the mature lettuce leaves 4–8 weeks old, the veins present at the base, middle and top of the lettuce leaves were selected. Following sectioning, the samples were fixed in 0.075 M phosphate buffer (pH 7.4) (Merck) containing 2.5% (v/v) glutaraldehyde (Merck). The samples were rinsed three times in 0.075 M phosphate buffer and fixed for 1 hour in 0.5% (v/v) aqueous osmium tetroxide (Merck). Subsequently, the samples were rinsed three times in 0.075 M phosphate buffer, followed by dehydration in a gradient of ethanol (50%, 70%, 90%, 100%, 100%, 100% (v/v)) (Merck) at 10 min intervals. The samples were infiltrated with 30% (v/v) Quetol (SPI-Supplies, West Chester, PA, USA) in ethanol for 1 hour, 60% (v/v) Quetol for 1 hour and a further 100% (v/v) Quetol overnight. Fresh pure Quetol was used to polymerise the samples at 60°C for 48 hours.

For light microscopy (LM), monitor sections (0.5 μm) were cut, stained in Toluidine blue (Merck) and mounted in immersion oil before being examined using the Nikon Optiphot Transmitted Light Microscope (Nikon, Japan). LM sections were selected randomly for viewing. Following positive identification of bacteria within these sections, samples were cut into ultra-thin sections with an ultramicrotome for TEM. The ultra-thin sections were contrasted in 4% (v/v) aqueous uranyl acetate (Merck) for 10 min, followed by Reynolds’ lead citrate (Merck) for 2 min and examined using the Jeol JEM-2100F Field Emission Electron Microscope (JEOL, Japan).

Pure bacterial cultures of the respective pathogens were prepared for morphological comparisons. A single pure colony of each respective bacterium was prepared and inoculated into TSB as described previously. The cells were cultured aerobically for 48 hours at 37°C with shaking at 200 rpm. One millilitre of each bacterial culture was centrifuged at 6,000 × g for 2 min and the process was repeated until a large enough pellet was obtained. The pellets were prepared for light and TEM as described previously. The following modifications were included for the TEM, samples were centrifuged at 6,000 × g for 2 min and resuspended between each step. Following the addition of fresh pure Quetol, the pellets were not resuspended but allowed to

### Table 1: Primers used for the detection of Escherichia coli O157:H7, Listeria monocytogenes, Salmonella enterica subsp. enterica and Staphylococcus aureus in lettuce seedlings and mature lettuce plants

<table>
<thead>
<tr>
<th>Primer</th>
<th>Specificity</th>
<th>Sequence 5′–3′</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UidAa (30 pmol)</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>GCAGAACTGTGGAATTGGG</td>
<td>252</td>
<td>Cebula <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>UidAb (30 pmol)</td>
<td><em>Escherichia coli</em> O157:H7</td>
<td>CGCTTTTGACACCTTAAACC</td>
<td>252</td>
<td>Cebula <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>LMFP (20 pmol)</td>
<td><em>Listeria monocytogenes</em></td>
<td>AGCTCTTAGCTCCATGAGTT</td>
<td>450</td>
<td>Thomas <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>LMRP (20 pmol)</td>
<td><em>Listeria monocytogenes</em></td>
<td>TCGAGAATCGAGGTACTCAA</td>
<td>450</td>
<td>Thomas <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>SLDF (50 pmol)</td>
<td><em>Salmonella enterica</em> subsp. enterica</td>
<td>CCGTCTGAAAGCCTGTAGAT</td>
<td>787</td>
<td>S. Collignon, unpublished data</td>
</tr>
<tr>
<td>SLDR (50 pmol)</td>
<td><em>Salmonella enterica</em> subsp. enterica</td>
<td>GACACCTACGGGACTACTA</td>
<td>787</td>
<td>S. Collignon, unpublished data</td>
</tr>
<tr>
<td>SCN2F (30 pmol)</td>
<td><em>Staphylococcus aureus</em></td>
<td>TTGCATATGATGGCAATTTGT</td>
<td>655</td>
<td>S. Collignon, unpublished data</td>
</tr>
<tr>
<td>SCN2R (30 pmol)</td>
<td><em>Staphylococcus aureus</em></td>
<td>AACGTATACATACCGTAAA</td>
<td>655</td>
<td>S. Collignon, unpublished data</td>
</tr>
</tbody>
</table>
polymerise at 60°C for 48 hours. Samples were cut, contrasted, selected and viewed as described previously.

Data analysis

The data collected were analysed by analysis of variance procedures and means were separated using the Duncan’s multiple range test at the 5% level. Statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). To assess whether the same internalisation pattern observed in the lettuce seedlings could be seen with mature lettuce plants, plate counts for both roots and leaves were combined to give an overall whole plant analysis.

RESULTS

Effectiveness of surface disinfection

After surface disinfection of lettuce leaves and roots, a reduction in total viable bacteria numbers from 6.36 to 0.34 log CFU/g per lettuce leaf and from 6.92 to 0.30 log CFU/g per lettuce root was observed. Overall, the surface sterilisation effectiveness was 95% for both the lettuce leaves and roots.

Verification of dye uptake and distribution

Crystal violet dye could only be seen within the roots of seedlings grown in seedling trays (Figure 1). However, the dye was observed in both the roots and stems of mature lettuce plants grown in the hydroponic system (Figure 1). The root system was darkly stained, the stems were lightly stained and the leaf internal issue did not stain at all (Figures 1(e) and 1(f)).

Microbiological analysis of lettuce seedlings

Internalisation of E. coli O157:H7 in lettuce seedlings occurred after 3 days of irrigation with artificially contaminated water and could be detected for the entire 28 day duration of the experiment, ranging from 1.48 to 4.53 log CFU/g (Table 2). Statistical analysis showed that the number of internalised E. coli O157:H7 increased significantly from day 3 to day 5, with no significant differences in bacterial numbers noted from day 7 to day 28 (Table 2).

L. monocytogenes was internalised in lettuce seedlings following 5 days of irrigation as opposed to E. coli O157:H7 which was detected on day 3 (Table 2). The highest number of internalised L. monocytogenes was determined
for day 5 (3.46 log CFU/g), whereafter it gradually decreased from day 7 to day 14, while the pathogen could not be isolated from the internal seedling tissue for the remainder of the experiment (Table 2). Comparison of bacterial numbers determined from day 3 to day 14 using statistical analysis showed that they were similar, and not significantly different (Table 2).

*S. Typhimurium* was internalised in lettuce seedlings only after 7 days of irrigation as opposed to *E. coli* O157:H7 and *L. monocytogenes* which were detected on day 3 and day 5, respectively. Statistical analysis showed that the bacterial numbers determined from day 7 to day 28, ranging from 0.98 to 1.35 log CFU/g were similar and not significantly different (Table 2).

*S. aureus* was isolated from lettuce seedlings from day 3 to day 21 at similar levels, not significantly different, ranging from 1.24 to 2.83 log CFU/g. On day 28, *S. aureus* was not detected following plating onto selective agar and the absence of the pathogen was confirmed by PCR analysis (Table 2).

The identities of all presumptive colonies isolated from the respective selective media were confirmed by PCR analysis (results not shown). Similarly, the absence of the four pathogens in the control lettuce seedlings was confirmed using PCR analysis.

Lettuce seedlings grown in seedling trays with sterile growth medium and irrigated with sterile water (negative control) still retained natural microflora. Natural microflora titres in the lettuce seedlings were present at non-significantly different levels from day 3 to day 28 (Table 3). Identification of the natural internal microflora isolates using the MALDI-TOF revealed the presence of several well-known bacterial spp. in the lettuce seedlings. These included *Enterobacter cloacae* (SV: 1.941 & 2.091), *Enterococcus faecalis* (SV: 2.388 & 2.398), *Lysinibacillus fusiformis* (SV: 1.803 & 1.957), *Rhodococcus rhodochrous* (SV: 2.068 & 2.027), *Staphylococcus epidermidis* (SV: 2.208 & 1.830) and *Staphylococcus hominis* (SV: 2.055 & 2.080). *Enterococcus faecalis* and *S. hominis* were most abundant in the seedlings, followed by *R. rhodochrous*, *E. cloacae*, *L. fusiformis* and *S. epidermidis*.

Comparison of the weight of the lettuce seedlings infected with the four respective pathogens to that of the control seedlings showed no real significant difference (results not shown).

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**Table 2** | Average number of internalised human pathogenic bacteria in the lettuce seedlings over the 4-week detection period

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Log (CFU/g) of internalised foodborne bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>1.48ac</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1.24a</td>
</tr>
</tbody>
</table>

* ND = none detected. No foodborne bacteria were found to be internalised.
* The data collected were analysed by analysis of variance procedures, and means were separated using the Duncan’s multiple range test at the 5% level. Statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). *Means within rows followed by the same letters do not differ significantly (*P* = 0.05).

**Table 3** | Average number of natural microflora present in the control lettuce seedlings and mature lettuce roots, leaves and whole plants grown in seedling trays and a water culture hydroponic system, respectively, over a 4-week period

<table>
<thead>
<tr>
<th>Controls</th>
<th>Log (CFU/g) of natural microflora</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>Lettuce seedlings</td>
<td>4.01a</td>
</tr>
<tr>
<td>Mature lettuce roots</td>
<td>N/A</td>
</tr>
<tr>
<td>Mature lettuce leaves</td>
<td>N/A</td>
</tr>
<tr>
<td>Whole mature plants</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* The data collected were analysed by analysis of variance procedures, and means were separated using the Duncan’s multiple range test at the 5% level. Statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). *Means within rows followed by the same letters do not differ significantly (*P* = 0.05).
**Microbiological analysis of mature lettuce plants**

Similar to lettuce seedlings, natural internal microflora was present in the control roots and leaves of mature lettuce plants grown in sterile water (Table 3). Natural internal microflora was detected in the mature lettuce plants for the entire duration of the experiment (Table 3). Analysis of the control roots indicated that natural microflora titres were found to be not significantly different, ranging from 6.43 to 7.27 log CFU/g (Table 3). The bacterial titres in the control leaves were the highest on day 28 (6.92 log CFU/g) and significantly different from day 7 with the lowest bacterial count (4.83 log CFU/g) measured on day 21 (Table 3). Bacterial titres for the whole plants ranged from 6.60 to 7.78 log CFU/g (Table 3).

The natural internal microflora isolated from roots and leaves of mature lettuce plants were identified as *Arthrobacter creatinolyticus* (SV: 2.101 & 2.319), *Enterobacter cloacae* (SV: 2.320 & 2.238), *Lysinibacillus fusiformis* (SV: 1.944 & 2.083) and *Microbacterium arborescens* (SV: 1.967 & 2.297). In addition, *Enterococcus faecalis* (SV: 2.411 & 2.354) was found to be present in the mature lettuce roots. *Microbacterium arborescens* was found to be the most abundant in the mature lettuce roots followed by *A. creatinolyticus*, *L. fusiformis* and *E. cloacae*. In comparison, *E. faecalis* was found to be most abundant in the mature lettuce roots followed by *E. cloacae*, *L. fusiformis*, *M. arborescens* and *A. creatinolyticus*. None of the four pathogens was found to be present in mature lettuce plants prior to inoculation and transplanting.

*E. coli* O157:H7 was found internalised in both the roots and leaves of mature lettuce plants in the hydroponic system after 7, 14, 21 and 28 days (Table 4). No significant difference in internalisation levels for this pathogen in the roots were observed throughout the entire 28 day period, with viable plate counts ranging from 4.98 to 6.37 log CFU/g (Table 4). A significant increase of internalised *E. coli* O157:H7 from day 7 (3.70 log CFU/g) to day 14 (5.17 log CFU/g) was found in the lettuce leaves, followed by no significant difference for the remainder of the experiment. The *E. coli* O157:H7 internalisation levels in the whole plants ranged from 5.01 log CFU/g on day 7 to 6.38 log CFU/g on day 21 (Table 4).

Internalisation levels of *L. monocytogenes* in the roots ranged from 2.97 to 5.75 log CFU/g over the 28 day period. The pathogen levels determined for days 7, 14 and 28 were similar and not significantly different (Table 4). *L. monocytogenes* titres fluctuated in the lettuce leaves from 2.26 log CFU/g (lowest) to 6.01 log CFU/g (Table 4). *L. monocytogenes* titres fluctuated in the whole plants, ranging from 4.33 log CFU/g on day 21 to 6.11 log CFU/g on day 7 (Table 4).

Internalisation levels of *S. Typhimurium* in the roots of mature lettuce plants were not significantly different from day 7 (4.16 log CFU/g) to day 21 (4.73 log CFU/g), however a significant increase was observed on day 28 (5.87 log CFU/g) (Table 4). Similar to *L. monocytogenes*, the detection levels of *S. Typhimurium* in the lettuce leaves were found to fluctuate over the 28 day period ranging from 0.84 to 3.79 log CFU/g and were not significantly different.

**Table 4** Average number of internalised human pathogenic bacteria in mature lettuce roots, leaves and whole plants grown in a water culture hydroponic system over a 4-week period

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Log (CFU/g) of internalised bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>4.98a</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>5.21ab</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>4.16b</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>4.05b</td>
</tr>
</tbody>
</table>

*Means within rows followed by the same letters do not differ significantly (P = 0.05).*
(Table 4). Whole plant analysis revealed a continuous gradual increase in internalised levels of S. Typhimurium from day 7 (4.34 log CFU/g) to day 28 (5.89 log CFU/g). Statistical analysis showed that the internalisation levels were similar and not significantly different (Table 4).

The internalisation levels of S. aureus in lettuce roots were not significantly different from day 7 (4.06 log CFU/g) to day 21 (4.48 log CFU/g), however a significant increase was observed on day 28 (7.1 log CFU/g), which is a similar pattern to that observed for S. Typhimurium in the mature lettuce roots (Table 4). The levels of internalised pathogen in the lettuce leaves increased significantly from day 7 (4.63 log CFU/g) to day 14 (5.87 log CFU/g), whereafter a significant decrease was observed on day 21 (3.82 log CFU/g) (Table 4). Bacterial titres found for the whole plants ranged from 4.64 to 5.96 log CFU/g from day 7 to day 21, with a significant increase to 7.1 log CFU/g found on day 28 (Table 4).

Comparison of the weight of both the roots and leaves of the pathogen inoculated mature lettuce plants to that of the control mature lettuce plants showed no real significant differences.

All presumptive pathogen isolates detected in mature plants by plate counts on the respective selective media were confirmed by PCR analysis using pathogen-specific primers. The absence of the four human pathogenic bacteria in the mature control plants was confirmed by PCR analysis of the microflora isolated from the plants.

Light and transmission electron microscopy

Pure cultures of the respective pathogens were used as a reference point when viewed under the LM in order to confirm the presence of the pathogens in the lettuce root and leaf cell (Figure 2). Long rod-shaped bacteria were present in both mature lettuce leaves and roots inoculated with E. coli O157:H7 (Figure 2(a)); short rods for L. monocytogenes (Figure 2(b)) and S. Typhimurium (Figure 2(c)); and cocci-shaped bacteria for S. aureus (Figure 2(d)).

TEM revealed long rod-shaped bacteria (5.20 μm in length) in mature lettuce leaves and roots contaminated with E. coli O157:H7 (Figure 3(a)). Short rod-shaped bacteria (1.25 μm in length) could be observed in mature lettuce leaf and root cells contaminated with L. monocytogenes (Figure 3(b)). Furthermore, short rods averaging 1.65 μm were observed in the cells of mature lettuce leaves and roots contaminated with S. Typhimurium (Figure 3(c)). Lastly, cocci-shaped bacteria averaging 1 μm were observed in the cells of the mature lettuce leaves and roots contaminated with S. aureus (Figure 3(d)).

To confirm that the rod- and cocci-shaped structures observed in the mature lettuce leaves and roots were indeed bacteria, the images were magnified to confirm the presence of a double cellular bacterial cell membrane (images not shown). Cell shape and size of the waterborne pathogens were confirmed using the control pathogens prepared. The structures observed in the contaminated lettuce leaves and roots were similar and characteristic to those observed in the control micrographs (Figure 3). In addition to the presence of bacterial cells, the cells could be seen to be dividing and thus multiplying inside the lettuce plant, as seen in Figure 3.

DISCUSSION

This study clearly demonstrated the internalisation potential of E. coli O157:H7, S. Typhimurium, L. monocytogenes and S. aureus in lettuce seedlings and mature hydroponically grown lettuce plants. Although the Staphylococcus genera are commonly encountered in the endophytic population of plants, the ability of the S. aureus human pathogen to internalise in plants has, to our knowledge, not been reported to date. Sixty and seventy per cent, respectively, of studies summarised in a review paper by Deering et al. (2011a) reported the internalisation of E. coli O157:H7 and Salmonella spp. in fresh produce. In contrast, 40 and 50% of studies respectively reported that they found no evidence of internalisation of these pathogens. In our internalisation study, sterile distilled water was spiked with either one of the four human pathogenic bacteria (10⁵ CFU/mL) and used to irrigate the lettuce seedlings grown in vermiculite and mature lettuce plants grown in a hydroponic system. Similar to our study, hydroponic systems were also used in other studies to investigate the internalisation potential of human pathogens in tomatoes (Guo et al. 2004), spinach (Warriner et al. 2005), maize (Bernstein et al. 2007) and lettuce (Franz et al. 2007). Other internalisation studies used
soil (Warriner et al. 2003; Bernstein et al. 2007; Mootian et al. 2009; Sharma et al. 2009; Erickson et al. 2010a) or compost (Erickson et al. 2010c) as growth medium, or directly inoculated leaf surfaces (Zhang et al. 2009) or seed (Jablasone et al. 2005; Miles et al. 2009).

Methods used to date to show internalisation of *E. coli* O157:H7 and *Salmonella* spp. within plants included the following: plating onto appropriate growth media, LM, scanning electron microscopy (SEM), TEM, immunolocalisation, fluorescence in situ hybridisation (FISH) and immunoblot tissue printing (Deering et al. 2011a). In this study, methods selected to confirm internalisation included enumeration of viable bacteria by plating onto selective growth media, PCR confirmation of pathogen identity, LM as well as TEM. To verify the presence of the pathogens, a representative colony for each of the test organisms was included in the LM and TEM study to compare bacterial cell wall structure and cell size. In order to verify the absence of the four human pathogens in uninoculated lettuce leaves the natural viable background microflora was determined and the isolates identified using MALDI-TOF analysis. It is clear from published data that each method used to investigate potential internalisation of microbes has its limitations, however using a combination of methods supports the findings in this study.

Concentrations ranging from as high as $10^9$ CFU/mL of *E. coli* O157:H7 and *S. Typhimurium* (Franz et al. 2007) to as low as $10^2$ CFU/mL of *E. coli* O157:H7 and *S. Typhimurium* (Jablasone et al. 2005) have been used to inoculate lettuce seeds in previous studies. Erickson et al. (2010b) reported no internalisation when lettuce leaves were inoculated with $10^6$ log CFU/mL (4.4 log CFU per leaf) *E. coli*
O157:H7, but it did occur when inoculated with $10^8$ CFU/mL (6.4 log per leaf). The inoculation concentration used in this study was $10^5$ CFU/mL. An interesting observation was that even though initial inoculation concentrations differ between studies, the final concentration of internalised organisms was similar. In the present study, internalisation levels of \textit{E. coli} O157:H7 remained relatively constant, reaching a high of 4.53 log CFU/g, suggesting the seedlings had reached a maximum carrying capacity (Table 2). This is consistent with the study by Franz \textit{et al.} (2007) where a similar carrying capacity of 3.95 ($\pm$1.02) log CFU/g of \textit{E. coli} O157:H7 in lettuce seedlings was recorded. In a recent study by Erickson \textit{et al.} (2010), 2.9 ($\pm$1.1) log CFU/g internalised \textit{E. coli} O157:H7 was recorded for lettuce leaves, which is similar to previously reported internalisation levels.

Two major routes of human pathogenic bacteria colonisation and internalisation have been reported to date: (1) entrance through natural plant openings such as stomata, lenticels, sites of lateral root emergence and/or biological or physically damaged sites; (2) following irrigation of plants, soaking of seeds or washing of fresh produce during processing after harvest (Solomon \textit{et al.} 2002; Jablonske \textit{et al.} 2005; Cooley \textit{et al.} 2006; Deering \textit{et al.} 2011). In the case of the latter, bacteria are pulled along with the movement of water as the water enters internal plant tissues. The four human pathogens tested in this study entered the plants through water uptake, as was evident due to the tracking of crystal violet dye uptake in the roots and stems.

Several mechanisms of human pathogen internalisation in plants have been postulated and are extensively studied at the moment (Kroupitski \textit{et al.} 2009). The first major obstacle
for entry of human pathogenic bacteria into plant tissues is the rigid and chemically complex host cell wall (Aparna et al. 2009; Deering et al. 2011b). The ability of plant pathogens to produce enzymes such as cellulases, xylanases, pectinases and proteases that break down cell walls, has been reported to provide a point of entry for the pathogen (Aparna et al. 2009; Deering et al. 2011a). Salmonella is a member of the Enterobacteriaceae family that includes the soft rot Erwinia, which produce cell wall degrading enzymes. A cellulase able to degrade cellulosic substrates has been isolated from S. Typhimurium (Yoo et al. 2004). Additionally, Salmonella isolated from infected Arabidopsis plants were equally virulent for human cells and mice, which indicates the Salmonella infection mechanisms for plants and animals are similar (Schikora et al. 2011). Salmonella spp. induce phagocytosis in humans and once internalised, destroy the cell.

In contrast to the Salmonella infection mechanism, human pathogenesis studies showed that E. coli O157:H7 did not destroy the host cell by phagocytosis. E. coli O157: H7 adhere to the extracellular regions of the host cell, ultimately causing damage to the cell structure through complex processes, resulting in inflammation and the inability of the cell to absorb nutrients, thereby enabling the pathogen to enter the host cell (Deering et al. 2011b).

There are no reports in the literature of S. aureus cellulosic degrading enzyme isolation and characterisation. However, a putative endo-1,4 beta-glucanase has recently been identified for S. aureus RF122 (EMBL Bank AJ958182). Interestingly, mutants of S. aureus strain with human pathogenesis genes inactivated, were attenuated in their ability to infect A. thaliana. These results indicate that as was observed for Salmonella, S. aureus infection mechanisms for plants and animals might be similar. Although S. aureus has not been implicated in foodborne disease outbreaks, comparison to infection mechanisms in humans could facilitate the elucidation of internalisation mechanisms of Gram-positive bacteria in plants.

Using L. monocytogenes expressing green fluorescent protein (GFP), the pathogen was visualised in the intercellular spaces of A. thaliana leaves, suggesting internalisation through stomata (Milillo et al. 2008). The internalisation of L. monocytogenes in the lettuce plants observed during this study is of concern, since it may pose a health risk.

The recent Listeriosis outbreak due to contaminated cantaloupe consumption in the USA emphasises the importance of the findings in this study (http://www.foodsafetynews.com/2011/10/cantaloupe-listeria-outbreak-84-sick-15-dead).

Future work should focus on the possibility of contamination under field conditions. Enhanced knowledge of the comparative behaviour of human bacterial pathogens and both epiphytic and endophytic bacteria through co-inoculation, as well as plant-microbe interactions will provide valuable information on both plant and bacterial factors involved in internalisation. Further work is also needed to better understand the mechanisms involved in the ability of S. aureus and L. monocytogenes to survive as endophytes in fresh produce and migrate through plant cell tissues. This should be greatly facilitated by the large amount of genomic and proteomic data now available to shed light on the role of numerous genes that still have unknown functions. Some of these genes may be important in crop plants grown in nature and therefore may provide clues to developing new methods to detect contamination of products with human bacterial pathogens.

CONCLUSIONS

In this study, the potential of human pathogenic bacteria (Gram-positive and Gram-negative) to internalise lettuce seedlings grown in vermiculite in seedling trays and hydroponically grown mature plants in a pre-harvest environment, was confirmed. Regardless of the modes of infiltration, internal tissues of the edible portions of fresh produce can provide a microenvironment for bacteria where they are protected from removal, allowing colonisation and potential survival. Improved monitoring and water quality control is therefore needed to avoid fresh produce contamination. This study highlights the potential impact of using contaminated agricultural water in fresh produce production and processing systems on food safety. Although the controlled environment used to study potential internalisation does not mimic the complexity of the natural environment, it does show the potential of endophytic presence of human pathogens in plant tissue and provides additional support for similar internalisation studies.
ACKNOWLEDGEMENTS

This study was undertaken as part of a Water Research Commission (WRC) solicited project co-funded by DAFF: ‘An investigation into the link between water quality and microbiological safety of fruit and vegetables from the farming to the processing stages of production and marketing’ (WRC Project No. K5/1875/4, Water Research Commission 2009). In addition, student support is gratefully acknowledged from the National Research Foundation and the Consumer Goods Council of South Africa, the personnel of the Laboratory of Microscopy and Microanalysis, in particular Mr A. Hall. Editorial comment from Dr W. Janisiewicz is gratefully acknowledged.

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First received 7 September 2012; accepted in revised form 27 February 2013. Available online 11 April 2013