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FS101056: The capacity and pathogenic potential of bacteria that internalise into plant tissue

Cell and Molecular Sciences

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1. Lay Summary

Fruit and vegetables are now recognised as a significant source of foodborne bacteria, in particular *E. coli* and *salmonella*. These bacteria are normally associated with farm animals and so can be found in meat or poultry, but are also capable of persisting in the wider environment and can use plants as alternative hosts. As a consequence, horticultural produce that is sold raw or minimally processed, e.g. salads or fruit sold as ready-to-eat, can become contaminated. Removal of the pathogens from the produce is one of the main challenges faced by producers, made more difficult as the bacteria have the potential to penetrate the internal tissues of the plant (termed internalisation) where they are protected from sanitation washes. As such, there are several key questions regarding the relevance of internalisation and its potential threat to consumers. We addressed these questions by determining the likelihood of internalisation and assessed whether bacteria that had become internalised were still able to cause foodborne illness (via a laboratory proxy). The work focused on the most relevant combinations of pathogen and produce: *Escherichia coli* O157:H7 with spinach and lettuce, and was carried out under laboratory conditions, taking commercial growth and production approaches into account.

Grower and Policy relevant findings:

- The current washing practices used in post-harvest production are ineffective at removing all external bacteria from plant surfaces and do not remove/inactivate any internalised bacteria. Therefore, any remaining bacteria (external and internal) present in greater numbers than the minimum infectious dose have the potential to cause disease.
- Multiple factors impact the likelihood of internalisation including the plant species, plant tissue type and plant growth media/substrate and as a consequence there is no single dominant factor that can be related to internalisation and as such used for control or mitigation.
- The growth of internalised bacteria was restricted or prevented completely in the three horticultural species tested (spinach, lettuce, tomato), suggesting that the normal plant

defence response is a key factor in preventing their growth. There is substantial evidence showing genotype-dependant differences in the plant defence responses of crops such as potato and barley (Foolan & Panthee, 2012). Although more research is needed in this area to provide sufficient genetic detail, the use of commercial varieties with robust plant defence responses could be a potential mitigation against bacteria.

- Internalised bacteria grew to high densities in the model plant tested (*Nictoiana benthamiana*), which indicates there may be other similarly susceptible species. Therefore, a screening approach to susceptibility would be beneficial.
- Internalised *E. coli* were not compromised in their ability to interact with and bind to human gut epithelial cells and hence are still likely to be able to cause disease.

2. Introduction

Verocytotoxigenic or Shiga toxin producing *Escherichia coli* (VTEC/STEC) is a foodborne pathogen that can cause serious disease ranging from haemorrhagic colitis to life threatening haemolytic uraemic syndrome (HUS) and central nervous system damage (Kaper et al., 2004). Although most cases were previously associated with contamination of meat and milk products, fresh fruit and vegetables are increasingly implicated as the sources of infection and on an international scale, ~20 % of foodborne STEC outbreaks are thought to have arisen from fresh produce (Greig and Ravel, 2009). It is now recognised that STEC are able to colonise plants as secondary hosts in a manner that has some key differences from their primary host, cattle (Holden et al., 2009). Indeed *E. coli* appears to have adapted to its hosts with strains belonging to phylogenetic group B1 being more able to colonise plants whereas phylogroups A and B2 are linked to an animal-associated lifestyle (Méric et al., 2013).

There is now a reasonable body of work to show that STEC and other foodborne pathogens, such as *Salmonella enterica* can exist both on external and internal tissues of plants (Deering et al., 2012; Hirneisen et al., 2012; Hou et al., 2013; Martinez et al., 2015; Wright et al., 2013). This ability presents a food safety threat in crop production, since internalised bacteria cannot be removed with standard sanitation practices, although other treatments such as ultrasound, irradiation and cold plasma can be effective (Bilek and Turantaş, 2013; Gomes et al., 2009; Ziuzina et al., 2015). In a comparative study, internalised bacteria were not affected by hypochlorite treatment, but were significantly reduced by ionising radiation (Niemira, 2007). However, widespread uptake of these approaches by the industry is limited, e.g. due to the levels of technology optimisation required for ultrasound (São José et al., 2014), or due to legislative regulations for irradiation, which for the UK is limited to dried herbs and spices, or requires labelling of imported produce (Food Irradiation (England) Regulations 2009, (2009 SI 1584)[1]).

[1] *This instrument applies to England only. Separate but parallel legislation applies in Scotland, Wales and Northern Ireland: The Food Irradiation (Scotland) Regulations 2009, 2009/1795 (W.162) and 2009/258 (NI).*

Internalisation into plants is defined as the ability of bacteria to penetrate into the internal tissues, where the bacteria normally reside in the extracellular spaces, in a tissue compartment termed the apoplast (Fig. 1) (Godfrey et al., 2010). The apoplast carries out essential functions relating to plant development and solute transport (Sattelmacher, 2001) and is an active site for interactions between plant pathogens (phytopathogens) and plant hosts (Xin et al., 2016). Some well-characterised phytopathogens can also reside close to the leaf surface in the stomatal pores (Yu et al., 2013) and other plant-associated bacteria are known to internalise within the cell (Turner et al., 2013). Internalisation of *E. coli* has been demonstrated by recovery of bacteria following surface sterilisation of the plant tissue (Deering et al., 2011; Erickson et al., 2014; Gomes et al., 2009; Hirneisen et al., 2012; Solomon et al., 2002; Warriner et al., 2003b; Wright et al., 2013).

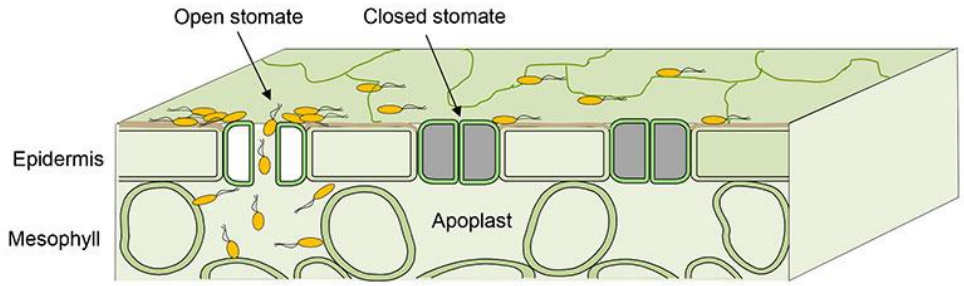


Figure 1: A diagram of a plant leaf cross-section showing open and closed stomata in the epidermis and bacterial invasion through open stomata into the leaf apoplast, adjacent to mesophyll cells. (Credit: Marlene Cameraon, with permission from Sheng Yang He, Howard Hughes Medical Institute).

We have previously demonstrated that a large proportion (81 - 91 %) of spinach plants maintained in synthetic liquid growth medium (termed hydroponic culture) support an internal population of bacteria, which accounts for approximately 0.5% of the total population of bacteria for either roots or leaves (Wright et al., 2013). Within the roots these bacteria were located inside the cell walls of epidermal and cortical cells and within the apoplast between the plant cells. Similarly, colonisation and invasion of 5-7 day old *Arabidopsis thaliana* roots by the *E. coli* O157:H7 strain Odwalla was observed along with proliferation of bacteria on the roots and shoots over a 3 day period (Cooley et al., 2003).

Although the migration of bacteria along the root to the crown was demonstrated, and bacteria were observed in the meristematic region (a formative tissue of undifferentiated cells) of the flowers, the actual transition from crown to shoot was not obvious (Cooley et al., 2003). Young lettuce seedlings grown in soil containing *E. coli* O157:H7 spiked manure were colonised on the surface, with some bacteria also located within the leaves (Solomon et al., 2002). As a second route of contamination, plants irrigated via the roots with contaminated water also yielded bacteria from the mature leaves (Solomon et al., 2002). However, the route by which bacteria migrate from roots to shoots remains unclear, particularly in relation to whether bacteria can move through the plant vasculature.

Occurrence of internalisation under conditions that are relevant to horticulture and at ecologically appropriate inoculum levels has been demonstrated, although it appears to occur at a low frequency (Erickson et al., 2013; Erickson et al., 2010). This raises questions relating to the internalisation ability of foodborne pathogens and its relevance to food safety, i.e. do sufficiently high enough numbers of bacteria get into and stay within the plant material to pose a food safety threat to the consumer at the end of the food chain? There is little quantitative data comparing different horticultural species. The fate of internalised bacteria over time is unknown, whether they proliferate or succumb to the plant defence response. Foodborne outbreaks that have arisen from fresh produce have, by default, been caused by isolates capable of pathogenic disease, and there is some evidence that plant-associated isolates have increased levels of pathogenicity (CDC, 2006; Frank et al., 2011), but it is not possible to determine whether any of the plant-associated STEC outbreaks have arisen as a direct result of internalised bacteria. As such, we undertook this FSA/FSS-funded project FS101056, to investigate the relevance of internalisation to the fresh produce industry and public health agencies. Specific objectives were to:

- **A.** Determine how different factors affect the internalisation phenotype for STEC, by comparing a range of factors such as plant species and growth media
- **B.** Determine the internalisation capability of STEC into plants grown and processed under commercially relevant conditions
- **C.** Assess the viability of internalised bacteria

D. Determine the pathogenic potential of internalised bacteria in a tissue-culture-model of human infection

The work focused on STEC serotype O157:H7 isolate Sakai (*Stx* negative) (Michino et al., 1999; Watanabe et al., 1999) since it was derived from the largest outbreak associated with fresh produce (white radish sprouts), in combination with fresh produce species implicated in multiple STEC outbreaks - lettuce and spinach.

3. Material and Methods

The project made use of well-established molecular, microbiological and plant cell biology approaches. The techniques were largely spilt into the two main areas: basic microbiological counts from inoculated plant material for comparative analysis and internalisation; and molecular techniques to assess viability and pathogenicity potential.

The bacterial strains used were *E. coli* O157:H7 isolates Sakai (Yokoyama et al., 2000); TUV93-0 (Campellone et al., 2002) and H110320350 which was associated with the soil-contaminated outbreak in the UK, in 2012 (Perry et al., 2013). Where a fluorescently marked strain was required, bacteria were transformed with p*gyrA-gfp+* (chloramphenicol resistant) (Holden et al., 2007), which was found to be stably maintained in bacteria infiltrated into plant tissue for > 10 days. All strains were negative for expression of Shiga toxin (Stx), and as part of the project we removed the *stx* genes from strain H110320350, which was then termed ZAP1589. The original plan was to use strain ZAP1589 since it is a representative of a recent UK outbreak and has an association with plants, whereas the other two strains date from the 1990s. However, during our investigations with ZAP1589, we found that the strain is non-motile (although it produces H7 flagella), which we found detrimentally affected its ability to colonise plants in comparison to isolate Sakai. Attempts to restore motility were unsuccessful, therefore we proceeded with isolate Sakai, since it is derived from an extremely large-scale outbreak from white radish sprouts (Michino et al., 1999; Watanabe et al., 1999) and the research team have expertise in working with this

strain on plants. Where the work required comparisons between bovine-derived and plant-derived isolates, we used a *stx*- derivative of *E. coli* O157:H7 EDL933 (Perna et al., 2001) (isolate TUV93-0 (Campellone et al., 2002), since this isolate was obtained from a similar era to isolate Sakai. The project also included some work on the related pathogen *S. enterica* serovar Senftenberg, for which a clinical Scottish isolate was used from a large-scale outbreak in 2006 from basil (Elviss et al., 2009) and our previous work has shown that this isolate colonises tomato plants very well and can manipulate the host defence response.

The plants selected comprised salad vegetables (spinach, lettuce and tomato) and sprouted seeds, all of which have been associated with either STEC and/or *S. enterica* outbreaks. Growth conditions for the plants were designed to mimic commercial green-house conditions, i.e. in standard Mulitcote™ compost in 9 cm^3 disposable pots or on vermiculite supplemented with 0.5x Murashige and Skoog (MS) medium in 200 ml hydroponics pots, in glasshouses with 16/8 hour light/dark cycle, ~65 % humidity, maintained at 18-22 °C. Sprouted seeds were germinated in sterile distilled water, transferred to Petri dishes containing 0.5x MS medium and maintained at 25 °C.

The bacteria were routinely grown in rich, undefined medium lysogen broth (LB), and prior to plant-interaction assays were sub-cultured into defined medium of 3-(N-Morpholino)propanesulfonic acid sodium salt MOPS, amino acids and supplemented with glycerol, at 18 °C for ~ 18-20 hours. The density of the culture was measured and then adjusted either to an equivalent of 10^7 cfu/ml for the 'high dose' or 10^3 cfu/ml for the 'low dose'. Plants were inoculated in different ways depending on the tissue under investigation. To mimic contamination of root tissue, either plant pots were placed into a box containing 1 L of the bacteria suspended in sterile distilled water for compost-grown plants, or the plant growth medium was removed from hydroponics-grown plants and replaced with the same volume containing the bacteria suspended in 0.5 x MS. Leaves were inoculated by inverting the plant into a 1 L bacterial suspension for compost-grown plants, or drop-inoculated for hydroponics-grown plants.

For quantification of bacteria located within plant tissue, (i.e. internalised), the surface of the plant material was surface sterilised with 200 ppm calcium hypochlorite, which is within

the range of hypochlorite concentrations used commercially for a washing step during production, and washed at least three times in sterile distilled water. To determine whether surface sterilisation was complete, the plant material was placed on a petri dish containing solidified medium (LB) for ~15 seconds, before processing. The dishes were then incubated at 37 °C and any samples that contained bacterial colonies, i.e. incomplete surface sterilisation, were recorded for efficiency testing and the results of the count data discounted for internalisation analysis.

In planta growth rates: *E. coli* Sakai was transformed with the reporter plasmid to generate a strain termed GFP-Sakai. The bacteria were diluted to 10^3 cfu/ml in 0.5 x MS and 0.1 ml infiltrated into individual leaves, one per plant, by pressure injection and the plants maintained in an environmental cabinet. Leaves were harvested at random from 6 plants at the designated time points, ground in 1 ml PBS (phosphate buffered saline) and added to 5ml buffered peptone water (BPW) + chloramphenicol. This was serially diluted, 10-fold, to extinction with 3 x 1ml replicates for each dilution. Visible growth was assessed following overnight incubation at 37 °C and 10 µl spots were plated onto MacConkey (MAC) + chloramphenicol agar and incubated overnight as a means of selection and confirmation of GFP-Sakai. Colony growth was scored (+/-) and the MPN (minimum probable number) per ml of extract determined (Cochran, 1950), this was multiplied by 6 to determine the number in the whole leaf. Using the 6 replicate measurements for *N. benthamiana* (4 experiments), spinach and tomato (2 experiments each), the MPN estimates of GFP-Sakai bacteria, after $\log_{10}$ transformation, were analysed separately using a simple linear regression to estimate changes in population size during the first 20 days of the experiment .

Microscopy: mOrange-LTI6b plasmids, modified from EGFP-LTI6b markers (Kurup et al., 2005) were mobilised into *Agrobacterium* strain LBA4404 and used to transform *N. benthamiana* leaf segments (Horsch et al., 1985). Spectinomycin-resistant plants (termed mOrg-LTI-benth) were regenerated and screened for expression by fluorescence microscopy. Leaves were infiltrated with bacteria suspended in 0.5 x MS buffer at 10^7 cfu/ml by pressure injection using a 1ml syringe into the abaxial epidermis (cells of the lower side of the leaf) to introduce them into the apoplast and the plants maintained in an

environmental cabinet. Leaf segments were infiltrated with sterile distilled water, to displace air from the apoplastic spaces prior to mounting on microscope slides. The abaxial surface of the leaf was visualised using a Nikon A1R confocal laser scanning microscope mounted on a NiE upright microscope fitted with an NIR Apo 40x 0.8W water dipping lens and GaAsP detectors. Images represent false-coloured single sections, maximum intensity, 3D or orthogonal projections as indicated, produced using NIS-elements AR software. GFP (green) and chlorophyll (blue) were excited at 488 nm with the emissions being collected at 500-530 nm and 663-737 nm respectively. Excitation for either of the orange or red fluorescent markers (mOrange or mKate) was provided at 561 nm with emission at 570-620 nm (magenta).

Epithelial cell binding: Caco-2 human epithelial cells were maintained at 37 ^{o}C, 5 % CO_2. Prior to inoculation, the cells were harvested, counted and distributed in an 8-well multi-well plate, as described in (Fernandez-Brando et al., 2016). Bacteria were recovered from infiltrated *N. benthamiana* or spinach leaves, 24 hours post-infiltration (hpi) in 1 ml of PBS, subject to centrifugation at 1,000 rpm to remove plant debris, and concentrated 2-fold. 100 µl was used to inoculate each well, which was equivalent to 9 x 10^6 cfu/ml. As a positive control, bacteria were inoculated into minimum essential medium (MEM) tissue culture medium at 37 ^{o}C until the density reached 0.84, equivalent to 1 x 10^9 cfu/ml, then the cells were washed and 100 µl used to inoculate each well. At 2 or 4 hours, the wells were washed with PBS, and the cells recovered by scraping, then lysed with Triton X-100, and plated in 10-fold dilutions on MacConkey medium. A parallel set of wells contained a microscopy cover-slip that was removed and washed with PBS, fixed in 4 % PFA and stained with anti-O157 for the *E. coli* Sakai detection, and TRITC- Phalloidin for actin, as described in (Naylor et al., 2003).

Statistical Analyses: For assessment of internalised bacteria where the results are highly skewed by the number of samples in which no bacteria were detected, a decision was made to do a two phase analyses. First, the data were rescored as presence/absence of bacteria and analysed using logistic regression then the non-responding samples were removed and an unbalanced ANOVA was used to test for differences between treatments in samples

where bacteria were detected. Analyses were conducted separately for the two inoculum levels (10^3 and 10^7 cfu/ml). Genstat (VSN International) and Prism (GraphPad) were used for statistical analysis and data visualisation.

4. Results

1. Surface sterilisation efficiency of hypochlorite

The number of internalised bacteria is determined by removal of the external population, using a microbiocidal agent such as hypochlorite. In industrial production, chlorine is one of the most common disinfectants used, e.g. to control microbial populations in washing water. Chlorine in the form of hypochlorite of ~ 80 ppm (range 3 – 200, exposure time 30 seconds – 15 mins) is added to washing water in order to allow the water to be recycled and in use for several hours (FAO/WHO, 2008). In industry, the main process of washing produce to remove soil and surface-associated particles is via agitation. The purpose of adding hypochlorite is to keep the batch of water in use for a prolonged period, rather than as a plant sanitiser *per se*. Our initial attempts to remove surface-associated bacteria with 80 ppm of hypochlorite were ineffective. Therefore, we increased the dose by 2.5-fold to 200 ppm, which is the same as the upper limit used in industry, and although this increased efficiency, it varied substantially, dependent on the bacterial inoculum level and the plant tissue type, e.g. from a minimum of 37 % for spinach leaves inoculated with ~ 10^5 cfu/plant to 100 % for lettuce roots and leaves, both inoculated with ~ 10^2 cfu/plant (Table 1). The laboratory process included repeated, vigorous washing to remove trace hypochlorite, which is likely to exceed that used in industrial processes.

Therefore, a key finding of the project is that surface-associated bacteria will not be removed during the standard washing phase during industrial production, unless there are specific (and more effective) sanitation methods in place.

2. The likelihood of internalisation

A major goal of the project was to determine the likelihood of internalisation by STEC into plants and whether this would occur under conditions similar to those used in commercial production. Since contamination of STEC can occur either on the leaves or roots, e.g. from irrigation water, we assessed both. Leaves were inoculated by brief immersion into a bacterial suspension to mimic direct contact with irrigation water, and roots were inoculated indirectly by submersion of plant pots in a bacterial suspension to mimic irrigation water reaching plants via soil/compost. Our previous work has shown preferential colonisation of the roots by STEC compared to the leaves (Wright et al., 2013). We compared the ability of STEC to internalise into salad vegetables grown in standard compost with plants grown under hydroponics, i.e. on an inert substrate and in the presence of a liquid plant growth medium, since both growth conditions are used by the industry for horticultural produce. We also compared STEC in spinach and lettuce with *S. enterica* serovar Senftenberg in tomato to determine whether there were any clear distinctions between the plant-microbe systems.

The likelihood of internalisation was assessed from the proportion of plants that contained internal bacteria, and the level of internalisation was determined from the final counts determined 10 days post-inoculation (dpi). Both values varied substantially, dependent on the inoculation dose, the tissue inoculated, and the plant species (Table 1). A high variation between individual plant samples was evident, although the general trends showed higher total populations than internalised populations; the root harboured greater numbers than the leaves; the highest levels of bacteria were seen for *S.* Senftenberg on tomato roots for plant grown under hydroponics (Fig. 2). The greatest likelihood of internalisation after 10 days occurred for *N. benthamiana* leaves, although the greatest number of internalised bacteria occurred in spinach roots (Table 1). Sprouted seeds, (fenugreek and alfalfa), provided particularly favourable conditions for growth of *E. coli* Sakai over 2 days, yet virtually no internalised bacteria were detected (Fig. 3). It should be noted that removal of external bacteria from sprouted seeds was challenging and required 20,000 ppm hypochlorite (a saturated solution), which was still not 100 % efficient.

Table 1: Colonisation and internalisation data, and surface sterilisation efficiencies

Tissue[a]	Dose[b]	Time (days)	Positive plants[c] (n)	Total counts[d] (log_{10})	Internal counts[d] (log_{10})	Internal population[e] (%)	Sterilis. efficiency[f] (%)
Spinach var. Amazon							
Root	10^7	0	9 (10)	5.42 ± 0.20	2.31 ± 0.75	0.08	
		5	10 (11)	5.12 ± 0.37	2.62 ± 0.92	0.31	
		10	12 (14)	4.29 ± 0.67	2.37 ± 1.15	1.21	80
	10^3	0	5 (14)	1.45 ± 0.67	0.53 ± 0.79	31.43	
		5	1 (15)	0.82 ± 0.79	0.08 ± 0.29	3.60	
		10	4 (15)	1.17 ± 1.34	0.21 ± 0.44	0.17	97.7
Leaf	10^7	0	1 (1)	5.14 ± 0.38	3.66 ± N/A	2.28	
		5	4 (6)	3.51 ± 1.41	0.99 ± 0.85	0.08	
		10	9 (10)	3.81 ± 1.09	1.81 ± 1.06	1.28	37.7
	10^3	0	12 (12)	2.53 ± 2.71	1.73 ± 1.81	16.09	
		5	2 (10)	1.15 ± 1.45	0.27 ± 0.59	13.27	
		10	5 (13)	1.98 ± 2.44	1.50 ± 1.85	32.56	77.7
Lettuce var. All Year Round (AYR)							
Root	10^7	0	10 (10)	4.85 ± 0.43	2.19 ± 0.55	0.22	
		5	7 (10)	4.36 ± 0.54	2.17 ± 0.81	0.65	
		10	10 (13)	3.98 ± 0.35	1.87 ± 0.70	0.78	78.3
	10^3	0	11 (15)	1.73 ± 0.63	1.33 ± 0.89	39.53	
		5	11 (15)	1.69 ± 1.09	1.02 ± 0.67	21.42	
		10	5 (15)	2.11 ± 0.78	0.94 ± 1.40	6.82	100
Leaf	10^3	0	11 (15)	1.84 ± 0.40	0.81 ± 0.53	9.30	
		5	14 (15)	1.87 ± 1.02	1.77 ± 0.87	78.27	
		10	9 (15)	2.04 ± 1.95	0.92 ± 0.88	7.61	100
N. benthamiana							
Leaf	10^3	0	10 (15)	1.89 ± 0.65	0.86 ± 0.69	9.23	
		5	9 (14)	2.24 ± 1.20	1.66 ± 1.34	26.28	
		10	12 (15)	1.78 ± 1.58	1.34 ± 0.98	36.15	97.7

Table Footnotes:

a: plant roots were inoculated via the pot-soak method for 1h, or the leaves dipped into a bacterial suspension for 30s, for inoculation via roots or leaves, respectively.

b: the inoculum dose of *E. coli* Sakai expressed as cfu/ml

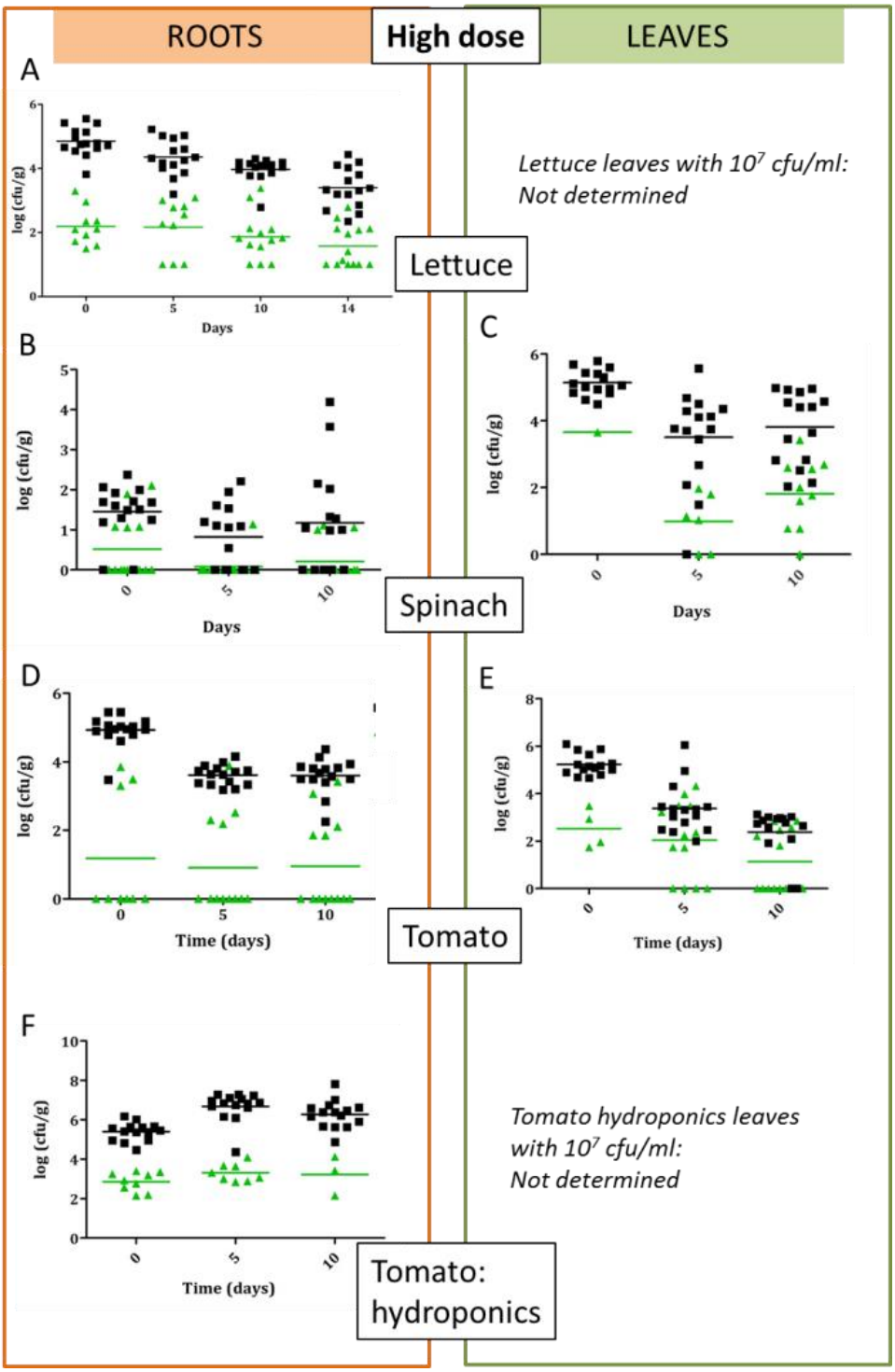
c: the number of plants containing internalised bacteria, along with the total number of plants assessed (experiments were repeated three times with a maximum of five replicate

plants, giving a possible maximum of 15, although due to poor sterilisation efficiency the usable number was sometimes lower).

d: the average counts for the total or just internalised population is given, +/- the SD

e: the population of *E. coli* Sakai recovered from surface sterilised leaves, expressed as a proportion of the total population

f: only one replication available, as it wasn't possible to remove all surface bacteria (out of 15 replications/attempts)


ROOTS
High dose
LEAVES
A
log (cfu/g)
Days
Lettuce leaves with 10^7 cfu/ml:
Not determined
Lettuce
B
log (cfu/g)
Days
C
log (cfu/g)
Days
Spinach
D
log (cfu/g)
Time (days)
E
log (cfu/g)
Time (days)
Tomato
F
log (cfu/g)
Time (days)
Tomato hydroponics leaves
with 10^7 cfu/ml:
Not determined
Tomato:
hydroponics

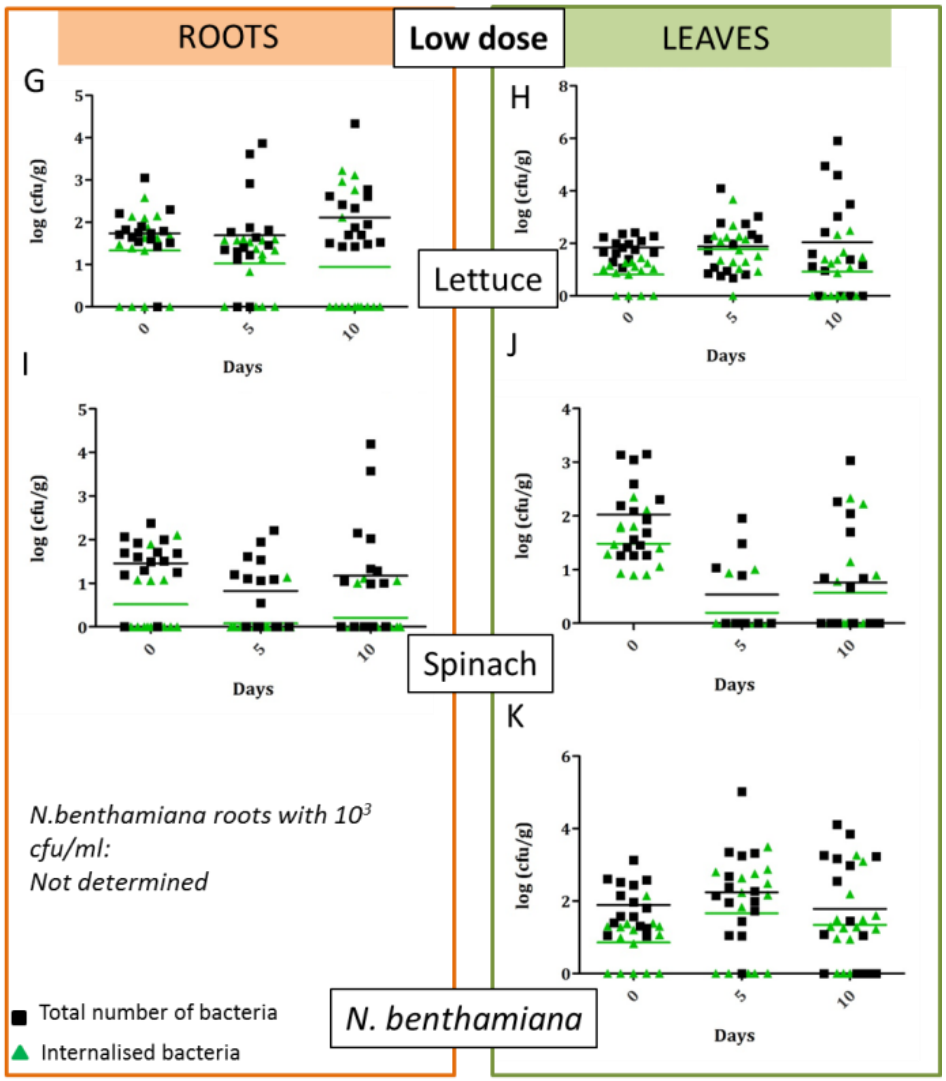


Figure 2: Colonisation and internalisation in leaves and roots of plants. The numbers of *E. coli* Sakai (A, B, C, G, H, I, J, K) or S. Senftenberg (D, E, F) inoculated at either high (A-F) or low (G-K) doses, recovered from the roots (A, B, D, F, G, I) or leaves (C, E, H, J, K) of lettuce (A, G, H), spinach (B, C, I, J), tomato (D, E, F) or N. benthamiana (K). Individual data points at shown for total number of bacteria (black squares) or just the internalised bacteria (green triangles), with the mean number shown by a black or green straight line. The numbers recovered are expressed as cfu/g (fresh weight), following inoculation with 107 or 103 cfu/ml.

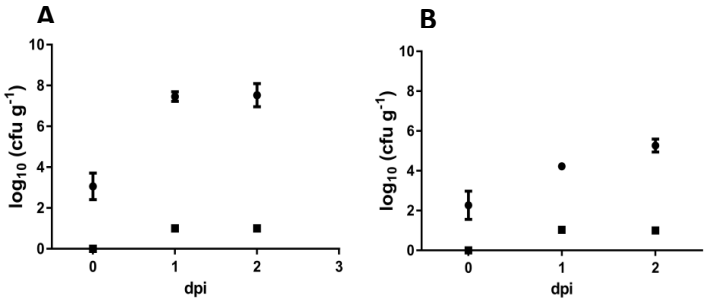


Figure 3: Colonisation and internalisation of *E. coli* Sakai into sprouted seeds. *E. coli* Sakai recovered from (A) alfalfa or (B) fenugreek sprouts, showing the total number (circles) or the internalised (squares) bacteria recovered. The average and the standard error (SE) are expressed as cfu/g (fresh weight).

Regression analysis was applied to determine any significant differences between the combinations of plant, dose and bacterial species. Firstly, to define any broad-scale differences, the data were analysed on a presence/absence basis rather than the absolute level (count) of bacteria that were detected. This approach revealed that although the variable 'plant species' had a significant effect ($p<0.01$) for the total bacterial population (i.e. external and internal), the difference was not evident for the internalised populations alone. However, taking into account only samples that contained detectable bacteria, i.e. just those plant with internalised bacteria, both the variables plant species and tissue type had an effect: the highest level of internalised bacteria being present in lettuce roots, which was significantly more than in lettuce leaves ($p<0.05$) (Fig. 4), and also for either tissue of spinach ($p<0.05$), where the mean levels were similar to that seen in lettuce leaves. These differences were only evident for the low inoculum dose of bacteria (10^3) and not for the higher dose (10^7). This is thought to be attributable to the 'carrying capacity' of plants that cause high levels of inoculum to drop and low levels to rise to the threshold level.

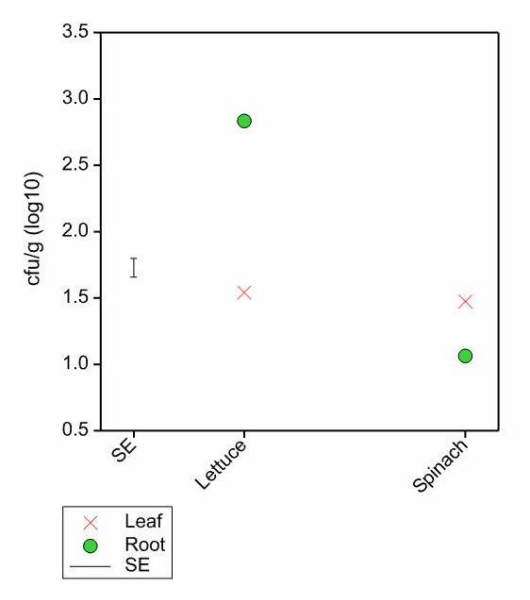


Figure 4: Internalised *E. coli* Sakai in lettuce or spinach tissues following inoculation with a low dose (103). The average of bacteria recovered is shown for two methods of inoculation, on roots (green filled circle) or leaves (red cross), together with the standard error of the mean (SE.).

Therefore, the main findings relating to the likelihood of internalisation of pathogens into plants are:

1. There is a degree of variability that occurs at the individual plant level for internalised bacteria. There are no obvious differences in the variation between plant species and the site of inoculation
2. There was some discrimination, however, for inoculation at a low dose (10^3) with lettuce roots harbouring higher levels of internalised bacteria than lettuce leaves, or either tissues of spinach.
3. Internalisation into sprouted seeds was not evident, although the extent of growth of the total population, i.e. on the surface of the tissue, was very rapid.

3. The viability of internalised STEC

The fate of internalised bacteria is not clear from evidence in the literature. Therefore, we artificially introduced *E. coli* Sakai into the leaves of spinach, lettuce and tomato. The model solanaceous species, *N. benthamiana*, was used as a comparator as it is genetically tractable and fluorescently marked genetic lines enable the localisation of internalised bacteria.

Bacterial viability was determined from expression of a plasmid-borne constitutive fluorescent reporter (validated at the start of the project) and from viable plate counts. We were able to quantify growth and persistence rates by using the MPN approach. There was a clear distinction in the ability of internalised *E. coli* Sakai to grow between the plant species, where there was exponential growth in *N. benthamiana* compared to no or minimal growth in spinach and tomato leaves (Fig. 5). Although bacterial growth was absent or minimal in these species, the cells were still viable and cultivatable, and appeared to be in a 'persistent' state of survival over the course of the three-week experiment. A similar pattern of persistence appeared to be present in lettuce (data not shown: variability in the numbers recovered, most likely due to difficulties introducing consistent numbers of bacteria into lettuce leaves, due to high degree of non-symmetric compartmental structures, reducing statistical confidence in the results).

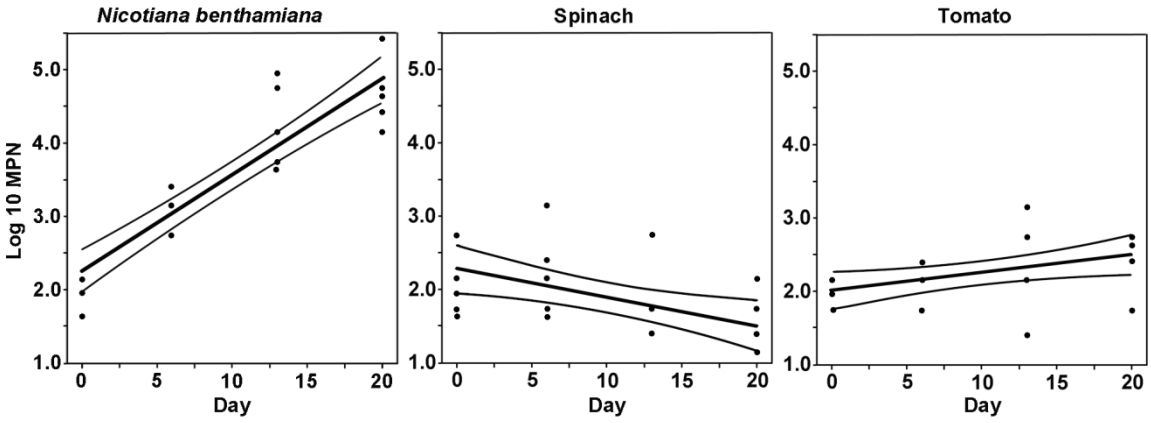


Figure 5: Estimates of infiltrated bacterial growth. *E. coli* Sakai recovered from infiltrated leaves of Nicotiana benthamiana, spinach or tomato and the numbers estimated by MPN. Individual data points are plotted for replicate plants and the slope of the curve fitted (straight lines, middle), bounded by 95% confidence limits (upper and lower lines). For each plant species, six replicate samples were assessed at each time point. The data represents one experimental replicate.

Visualisation of the internalised *E. coli* Sakai in *N. benthamiana* showed the formation of large colonies, which contained components characteristic of a biofilm matrix, i.e. curli fibres (*E. coli* Sakai does not express the genes for curli under *in vitro* conditions), cellulose and extracellular DNA (Fig. 6). In contrast, such colonies or associated biofilm formation

was not evident in spinach, lettuce or tomato leaves, demonstrating a completely different interaction of the internalised bacteria.

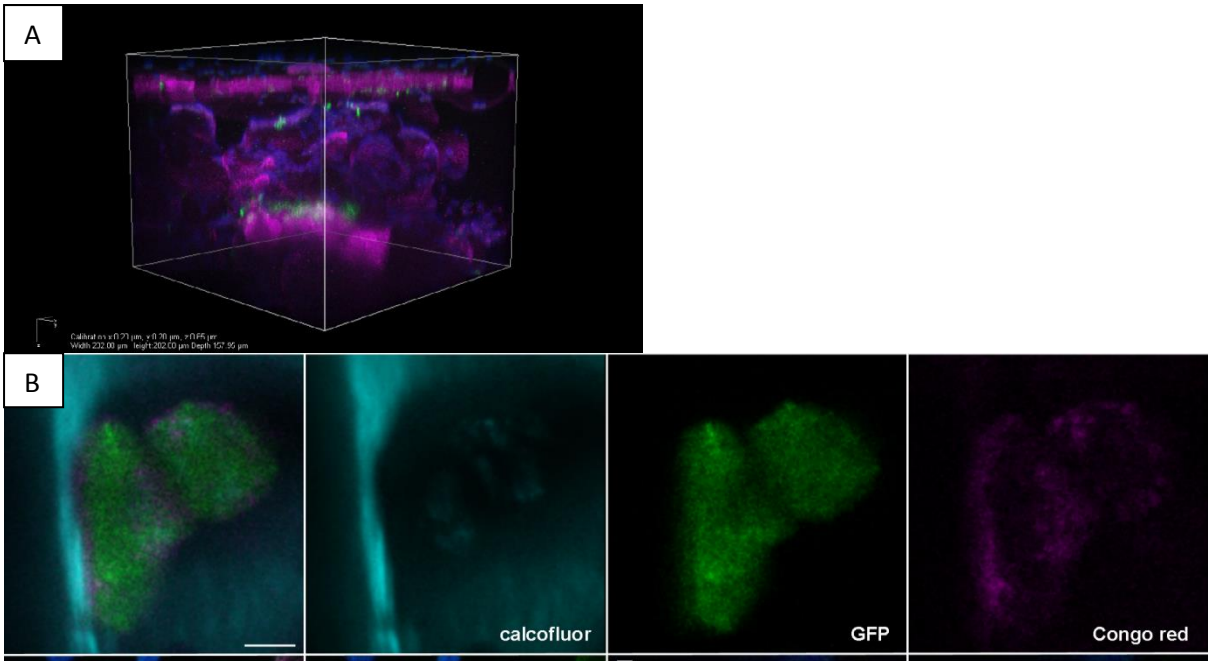


Figure 6: Internalised *E. coli* (Sakai) colonies in the apoplastic spaces in N. benthamiana. (A) A 3-D rendered microscopy image where the bacteria are labelled with GFP (green), the plant cell membranes are expressing a red-fluorescent marker (magenta), and the chloroplasts emitting auto fluorescence (blue). (B) *E. coli* (Sakai) colonies (green) in unlabelled (WT) N. benthamiana leaves, stained with calcoflour for cellulose (pale blue) or Congo red for curli (magenta), and GFP-Sakai detection (green). The left-most image is a merge of the three single-channel images.

The main findings from the viability experiments are:

4. Internalised bacteria remain viable and cultivable over at least 3-weeks, in three salad vegetables species
5. Internalised bacteria persist and either do not grow at all, or can only do marginally in spinach, lettuce and tomato leaves
6. Internalised bacteria can proliferate in *N. benthamiana* and form colonies encased in a biofilm matrix.

4. The pathogenicity potential of internalised STEC

One of the questions surrounding internalised bacteria in fresh produce is whether their ability to cause disease has been affected. To test this aspect, we looked at the expression of key virulence genes, both in plant extracts and *in planta*, and then at their ability to attach to human intestinal epithelial cells. Virulence genes were selected that relate to the type 3 secretion system (T3SS) (*ler*, *sepZ*, *tir*), iron uptake (*chuA*) and one of the Shiga toxin genes (*stx2*), since these have been shown to be involved in pathogenicity in animal hosts (Quinones et al., 2012). The T3SS is encoded by the locus for enterocyte effacement (LEE), under the control of the master regulator, Ler, located in the first transcript of the operon, *lee1*. The expression of these genes was measured directly and found to be either not induced or significantly repressed when the *E. coli* Sakai were incubated in apoplastic fluid, derived from spinach or *N. benthamiana*, compared to tissue culture medium that is known to prime expression of these genes (Roe et al., 2004). We also used reporter fusions of the genes, linked to GFP expression, which allowed us to examine their regulation in living plants. In contrast to that observed for the *in vitro* extract, expression of *ler* was shown to be induced when the bacteria were infiltrated into *N. benthamiana*. There was also some evidence of expression of the promoter for *tir* and *eae*, but only *in planta* and not *in vitro* in the apoplastic fluid (Fig. 7).

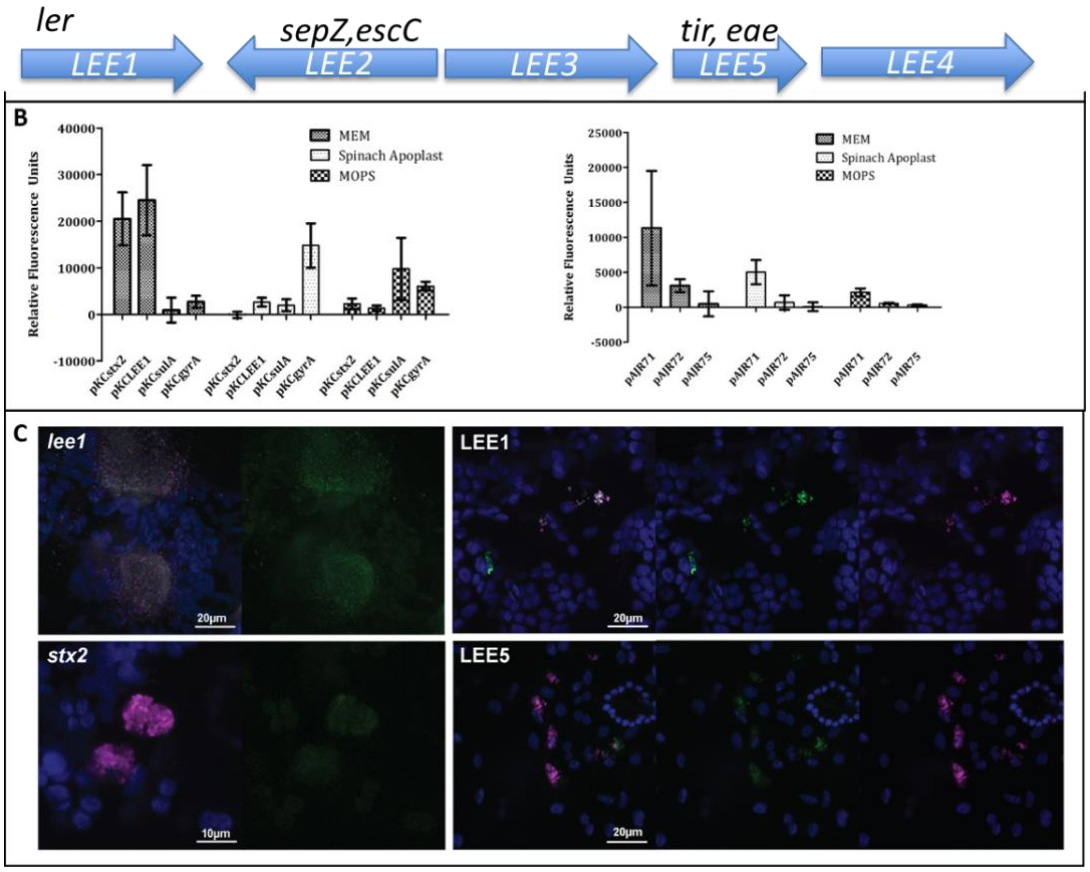


Figure 7: Expression profiles of *E. coli* Sakai virulence genes. A genetic map (A) of the T3SS locus, indicating the location of the first coded transcript in the LEE1 operon, ler (measured from pKCLEE1 and pAJR71, transcriptional and translation fusions, respectively), sepZ/escC and tir/eae (measured from pAJR72 and pAJR75 translational fusions, respectively). The relative expression levels in vitro (B) in tissue culture media @ 37 oC (MEM), MOPS growth media @ 18 oC (MOPS) or apoplastic fluid @ 18 oC (spinach apoplast). Expression levels from infiltrated bacteria expressing reporter fusions (C) in N. benthamiana for ler, stx2 and tir/eae.

To determine whether or not internalised STEC retain pathogenicity, we tested the ability of *E. coli* Sakai recovered from infiltrated spinach or *N. benthamiana* leaves to bind to human intestinal epithelial cells (Caco-2), since this is dependent on adhesion via T3SS. This population was compared to bacteria grown in tissue culture medium to prime expression of virulence genes. Elicitation of the T3SS varies widely between *E. coli* O157:H7 isolates (Roe et al., 2004) and since isolate Sakai is classed as a 'low secretor', we included another isolate TUV93-0, which is known to be a 'high' secretor of the T3SS. This choice also had the benefit of being about to compare isolate Sakai, derived from a plant-associated outbreak, with an isolate derived from a beef-burger associated outbreak. The viability of isolate

TUV93-0 was found to be the same as isolate Sakai in *N. benthamiana* and followed a similar pattern of *in planta* growth (not shown, but as per Fig. 4).

The numbers of bacteria used to inoculate the human cell line was ~ 2 orders of magnitude lower for those recovered from the plant samples compared to bacteria grown under *in vitro* conditions, although this was the maximal number that could feasibly be recovered from the plant tissue. Subsequently, the number of bacteria recovered from the human cell line was low; with some replicate samples generating a '0' return. Despite this, it was possible to assess the effect of plant infiltration by comparing the proportions to *E. coli* TUV93-0 pre-incubated in tissue culture media. The level of adherence of *E. coli* Sakai was ~ 38 % of isolate TUV93-0 at 2 hours, as expected. However, for *E. coli* Sakai pre-infiltrated into *N. benthamiana*, the level of adherence compared to *E. coli* TUV93-0 was also ~ 45 %, and after 4 hours this value was greater than for *E. coli* Sakai in tissue culture medium (MEM) (Fig. 7). The available data for *E. coli* Sakai or TUV93-0 pre-infiltrated into spinach resulted in comparable levels of adherence, of 25% and 34 %, respectively, although derived from a low number of bacteria recovered from the cell line.

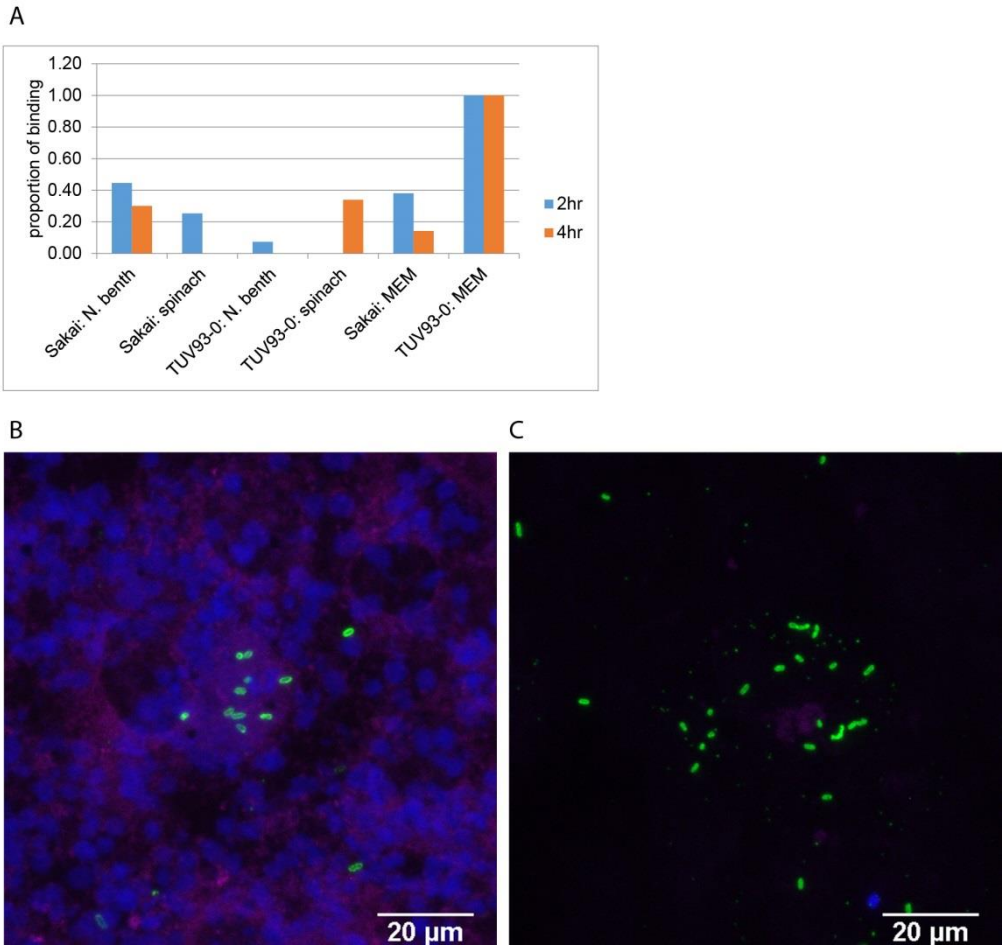


Figure 7: Adherence of *E. coli* O157:H7 isolates Sakai and TUV93-0 to human intestinal epithelial cells. Adherence of bacteria to the cell line two and four hours after inoculation, either from bacteria pre-infiltrated into plants (N. benthamiana, spinach) at 18 °C for 24 hours, or pre-cultured in tissue culture medium (MEM @ 37°C for ~ 4hours). The inoculum levels of ~ 3.8 x 105 cfu/g for the plant-infiltrated samples and ~1.1 x 108 for the MEM samples. (A) The numbers of bacteria expressed as a fraction of the initial inoculum (i.e. 2hr/0hr), relative to the positive control of isolate TUV93-0 in MEM to yield the 'proportion of binding'. Light microscopy imaging of bacteria labelled in green on cell line (Caco-2) either derived from infiltrated plants (B) with chloroplast auto fluorescence in blue, or from tissue-culture broth (C). F-actin was stained with phalloidin (magenta): Attaching and effacing lesions were not visible or masked by chloroplasts.

Therefore, the main findings from the pathogenicity potential experiments are:

7. The plant status i.e. living or cut, influences bacterial virulence gene expression, so that genes are repressed *in vitro* in extracts, but conversely, some are expressed *in planta*
8. There appears to be little or no detrimental effect in the ability of internalised *E. coli* isolate Sakai to adhere to human intestinal epithelial cells.

5. Discussion & implications

The main aim of this project was to determine the relevance of internalisation of foodborne bacteria into plant tissue, in the context of food safety. We were able to show that internalised *E. coli* O157:H7 remain viable in fresh produce plants and in one of the species tested, can grow. This has been implicated by others (Deering et al., 2012; Eblen et al., 2004; Gomes et al., 2009; Warriner et al., 2003a), but a growth or persistence rate has not been reported previously. Viability and persistence of internalised bacteria was demonstrated after a period of three weeks, which is relevant to the harvestable age of leafy vegetables such as lettuce and spinach.

There was a wide degree of variation in the likelihood of *E. coli* Sakai internalising into plants, which was influenced, in part, by the route of contamination, (i.e. mimicking irrigation with contaminated water to the plant roots or direct contact of irrigation water on the leaves). The plant species also influenced internalisation (i.e. lettuce > spinach), and the tissue type for lettuce (roots > leaves). However, it was notable that there was up to two-orders of magnitude difference in the numbers of internalised bacteria between individual plants, which implies that just a few plants may have the potential to cause higher levels of cross-contamination within a batch during the post-harvest processing stage. This is likely to occur due to natural variation between individual plants that impacts plant-microbe interactions. Some differences were also seen between the bacterial species *E. coli* and *S. enterica*, and although the work here focused on a single *E. coli* O157:H7 isolate, a larger number would need to be screened to define any isolate-specific differences. It was also

notable that there were marked differences in the internalised bacteria when the population were introduced artificially by infiltration (Fig. 4), compared for the more 'natural' route mimicking spiked irrigation water (Table 1 / Fig. 1): whereas the population appeared to decline in the former, some individual plants harboured higher numbers in the latter. This suggests that the external population is a source of internalised bacteria but this is not a static situation and can vary over time. Related to this, the increased number of the total population after 10 days, e.g. on lettuce roots, also indicates growth of the external population. Although roots of these species are not consumed, mechanised harvesters can introduce root tissue, which subsequently requires removal during trimming and packaging, thus running the risk of microbial cross-contamination to edible tissue (Reed, 2011).

Sprouted seeds are produced in quite a different manner to leafy vegetables, under conditions that are conducive for bacterial proliferation. Indeed, high levels of growth of *E. coli* Sakai were observed, but it was notable that internalisation did not appear to occur. However, it was not easy to assess confidently given the extremely high levels of hypochlorite that were required to remove the external population of bacteria (> 20,000 ppm), which inevitably damaged the plant tissue.

The ability of foodborne pathogens to cause disease in susceptible human hosts is dependent on expression of virulence genes, many of which are known to be tightly regulated in response to mammalian-host environments (Umanski et al., 2002). A key virulence determinant of STEC is the type three secretion system (T3SS), which is used to manipulate the mammalian host defence and hence facilitate colonisation. Other virulence determinants include the bacteriophage-encoded Shiga toxin and iron-acquisition genes, such as siderophores that are induced with iron level restrict growth. We found that expression of all three categories was repressed or not induced when the bacteria were incubated with *in vitro* extracts of plant apoplastic fluid, at 18 °C, presumably as a result of the physico-chemical conditions. Surprisingly, the master regulator of the T3SS (*ler*, present on the *LEE1* operon), and potentially the genes for intimate attachment were shown to be expressed for internalised bacteria in the model plant species *N. benthamiana*. This means that the T3SS could still be 'primed' for expression and *in vivo* function in internalised

bacteria. Indeed, there appeared to be little difference in the ability of *E. coli* isolate Sakai to bind to human intestinal epithelial cells whether it was derived from an internalised population (at 18 °C), or whether it was derived from a tissue culture population (at 37 °C). Although it was only possible to measure T3SS-dependent attachment as a proxy for pathogenicity here, the data indicates that internalised STEC retain their pathogenic potential are not detrimentally affected following interaction with plant tissue. This finding is in-line with data for spinach-outbreak associated STEC strains from the USA, which resulted in levels of severe disease (HUS) that were significantly higher than in previous outbreaks (CDC, 2006).

In conclusion, the main findings from the project are:

1. Surface-associated bacteria will not be removed during the standard washing phase that is used in production of leafy greens (vigorous washing in the presence of ~ 80 ppm hypochlorite)
2. There is a large degree of variability in level of internalised bacteria on an individual plant basis
3. In this study, lettuce roots harboured significantly higher levels of internalised bacteria than lettuce leaves, or either tissues of spinach (at the low inoculation dose of 10^3 cfu/ml)
4. Internalisation did not appear to occur in sprouted fenugreek or alfalfa, although rapid and high levels of growth of the total population were supported
5. Internalised bacteria remain viable and cultivable over at least 3-weeks in the leaves of lettuce, spinach and tomato
6. Internalised bacteria persist and either grow only marginally, or not at all in spinach, lettuce and tomato leaves
7. Internalised bacteria grow in *N. benthamiana* and form colonies encased in a biofilm, comprising curli, cellulose and extracellular-DNA
8. The status of the plant (living or plant extracts) influences bacterial virulence gene expression, so that although they are repressed or not induced in extracts, some are expressed *in planta*.

9. There appears to be little or no detrimental effect of the ability of internalised bacteria to adhere to human intestinal epithelial cells compared to bacteria that have not been internalised.

Thus, the implication of the project to the fresh production industry and public health authorities is that internalisation of STEC into fresh produce crop plants is a relevant issue that needs to be considered, e.g. in risk assessment. Since the plant-microbe interactions are dynamic and specific, it is not possible to provide a common value that describes the rate or likelihood of internalisation; instead it is governed by the complete system of bacteria, plant species, and tissue type as well as growth conditions. Therefore, our data supports the current FSS foodborne illness strategy proposal of targeting interventions at the likely source of contamination, which are likely to have more impact rather than trying to control bacteria that have already established an interaction and colonised the plant host.

6. Future work and recommendations

- The industry should introduce sanitation measures at the end of the processing line, e.g. during bagging, that have a proven track record (Karaca and Velioglu, 2007; Scholtz et al., 2015) in reduction of produce-associated microorganisms. Although this will require optimisation largely dependent on the type of produce being handled, it will overcome the limitations of washing alone.
- Any sanitation measures introduced need to be effective on internalised bacteria, or at the very least, on bacteria that are still classed as 'external' but have not been washed off and are protected from chemical sanitisers.
- Risk assessments are required that take into account the complete route of transmission, from contamination at source, i.e. during plant growth and development.

- Since passage through the plant system does not appear to impact pathogenicity, straight-forward detection of the bacteria can be taken as a proxy for the potential to cause disease, notwithstanding reported virulence differences between STEC isolates
- Good agricultural practices should be adhered to for reducing the risk of contamination in the field, especially from irrigation water. These are "Protecting our water, soil and air" (DEFRA) for England & Wales; Agricultural regulation and guidance (SEPA) for Scotland.
- It is worth screening a larger number of edible plant species to determine whether *in planta* growth of pathogenic microorganisms occurs in other plant species

There is a need to consider the total population that remains following any intervention as the 'potential pathogenic load' of a crop or processed batch, since the external population, which can be resistant to or avoid sanitation agents, could be a potential source for internalisation.

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