RESEARCH LETTER

Persistence and spread of *Salmonella enterica* serovar Weltevreden in soil and on spinach plants

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Abstract

Several outbreaks caused by pathogenic bacteria are related to the consumption of raw produce contaminated by animal manure. The majority of these outbreaks have been linked to *Salmonella* spp. We examined the ability of *Salmonella enterica* serovar Weltevreden to persist and survive in manure and soil as well as disseminate to, and persist on, spinach roots and leaves. Significantly higher numbers of *S.* Weltevreden inoculated into manure and applied to soil before planting spinach were found in soil than in pot cultures, where the pathogen had been inoculated directly into soil 14 days postplanting. Moreover, the pathogen seemed to disperse from manure to spinach roots, as we observed a continuous increase in the number of contaminated replicate pot cultures throughout the evaluation period. We also found that, in some cases, *S.* Weltevreden present in the phyllosphere had the ability to persist for the entire evaluation period (21 days), with only slight reductions in cell numbers. The results from the present study show that *S.* Weltevreden is capable of persisting in soil, roots and shoots for prolonged periods, indicating the importance of strict monitoring of untreated animal manure before considering its application to agricultural land.

Introduction

The use of animal manure as crop fertilizer contributes to the sustainable recycling of essential nutrients and organic matter required to maintain good soil quality. However, care must be taken to avoid soil and plant contamination with human pathogenic bacteria present in untreated animal manure as well as dissemination of the bacteria. A large part of the outbreaks caused by pathogenic bacteria is related to the consumption of raw produce contaminated with human pathogens such as *Salmonella* spp. (Semenov, 2008). *Salmonella* spp. are more persistent in soil compared with other bacterial pathogens (Guan & Holley, 2003), displaying long periods of survival (Zibilske & Weaver, 1978) and only slightly reduced cell numbers over time (Guo *et al.*, 2002a). *Salmonella* has been detected in fecal cultures from the majority of dairies (Kirk, 2003), posing a significant risk of further pathogen dissemination to soil and fresh plant produce through the application of untreated cattle manure to agricultural fields. In several cases, cows carried *Salmonella* asymptomatically, i.e. they did not have clear symptoms that humans infected with *Salmonella* show (Semenov, 2008).

Salmonella cells present in cattle manure have been shown to survive for at least 60 days at 4 and 20 °C (Himathongkham *et al.*, 1999), but were not detectable after 19 days at 37 °C. Upon application of contaminated manure to soil, *Salmonella* was shown to survive for up to 300 days, with higher initial bacterial inoculation doses normally resulting in extended survival periods of *Salmonella* in the soil (Jones, 1986; Baloda *et al.*, 2001; Islam *et al.*, 2004). Whether *Salmonella* can disseminate to plant roots depends on factors such as the site of colonization (Doyle & Erickson, 2008), i.e. whether bacteria colonize the root surface or exhibit endophytic colonization of roots and aboveground plant tissues. For example, *Salmonella enterica* has been shown to penetrate epidermal cell walls of barley roots (Kutter *et al.*, 2006) and has been detected in sterilized leaf samples from crops grown in soil contaminated with *Salmonella* (Franz *et al.*, 2007). The entry sites of the pathogens are believed to be around cracks (Wachtel *et al.*, 2002) and lateral root junctions (Cooley *et al.*, 2003; Dong

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et al., 2003; Warriner *et al.*, 2003), which display increased exudation of nutrients (Jablasone *et al.*, 2005). Internalized pathogens may move systemically through plants (Guo *et al.*, 2002b), but contamination of edible plant parts has been also reported to occur via movement along the plant surface (Cooley *et al.*, 2003). Bacteria that manage to reach leaf surfaces must contend with harsh conditions (i.e. lack of nutrients and sunlight), and the persistence of *S. enterica* is 30–40-fold lower in the phyllosphere compared with in the rhizosphere (Cooley *et al.*, 2003).

Salmonella enterica serovar Weltevreden causes gastroenteritis and is a very common causative agent of salmonellosis in south-east Asia (Bangtrakulnonth *et al.*, 2004; Vo *et al.*, 2006). In 2007, approximately 50 individuals living in Norway, Denmark and Finland became infected with *S.* Weltevreden due to the consumption of alfalfa sprouts (Emberland *et al.*, 2007). Seeds contaminated with *S.* Weltevreden bought from producers in infested countries were identified as the source of the outbreak, indicating that this bacterial strain is able to survive on plant seeds for prolonged periods. As *S.* Weltevreden 2007-60-3289-1 appears to have great potential as a food safety hazard, this strain was selected for evaluation of its capability to persist and survive in soil and spread onto spinach plant roots and leaves.

Materials and methods

Bacterial strains and growth conditions

The *S. enterica* ssp. *enterica* serovar Weltevreden strain 2007-60-3289-1, isolated from Danish alfalfa sprouts in 2007 (Emberland *et al.*, 2007), was provided by Dr Annette Nygaard Jensen (DTU-FOOD, Denmark) and used in the current experiments. *Salmonella enterica* serovar Weltevreden was grown in Luria–Bertani medium (1 L: 10 g tryptone, 5 g yeast extract, 5 g NaCl) and incubated at 37 °C overnight until an $OD_{600\,nm}$ of approximately 0.9 (early exponential phase) was reached. For inoculation of slurry and soil, bacteria were harvested, washed three times with 0.9% NaCl and resuspended in 0.9% NaCl.

Soil and cattle slurry

Cattle manure slurry (Table 1) was collected at an organic farm in Sandviken, Sweden, and stored at 4 °C for 4 weeks until use. Clay loam soil (Table 1) was collected at a biodynamic farm in Järna, Sweden, and stored at 4 °C for 4 weeks until use. Soil was collected from a 1 × 1 m square at a depth of approximately 0–20 cm, sieved (2 mm) and mixed before use. Chemical analyses were performed by Eurofins Lab (Kristianstad, Sweden).

Table 1. Chemical and physical properties of the manure and soil used in the pot culture experiments

	Cattle manure slurry	Clay loam soil
Dry matter	10.9%	88.9%
pH	7.0	6.6
Clay	–	36%
Sand	–	29%
Silt	–	35%
Organic content	–	4.2%
Ash content	2.3%	–
Tot-N	4.3 kg ton^{-1}	33 kg ha^{-1}
NH_4-N	2.2 kg ton^{-1}	0.159 mg 100 g^{-1} dry matter
NO_3-N	–	1.161 mg 100 g^{-1} dry matter
C/N ratio	20	–
P	0.59 kg ton^{-1}	1.6 mg 100 g^{-1} dry matter
K	4.2 kg ton^{-1}	–
Mg	0.79 kg ton^{-1}	–
K/Mg ratio	0.2	0.3
Ca	–	250 mg 100 g^{-1} dry matter

Bacterial inoculation of slurry and soil

Two separate experiments were performed (A and B). In Experiment A, *S.* Weltevreden was inoculated into cattle slurry at three different concentrations corresponding to 10^4, 10^5 and 10^6 cells g^{-1} soil before addition to soil that was subsequently planted with spinach seeds. A 220-mL aliquot of cattle slurry was mixed with a 22-mL bacterial suspension or 0.9% NaCl and added to 3 kg of soil. Each pot received 130 g of the mixture, and six organically produced spinach seeds (*Spinacia oleracea* variety Gamma) were sown at a depth of approximately 2 cm.

In Experiment B, *S.* Weltevreden was washed and resuspended in 0.9% NaCl and inoculated directly into the soil, 14 days after sowing at a bacterial density of 10^6 cells g^{-1} soil. Similar proportions of soil and slurry as in Experiment A were mixed, but all samples received 0.9% NaCl solution, and spinach seeds were sown in the soil/manure/saline mixture. Fourteen days after sowing, each pot in Experiment B received a 10-mL suspension of *S.* Weltevreden in 0.9% NaCl to obtain an approximate bacterial concentration of 10^6 cells g^{-1} soil. The suspensions were carefully added to soil around the plant and the lowest 2 cm of the stems.

Both experiments included a nonbacterial control with 0.9% NaCl, and spinach seeds were grown in plastic pots (6.5 × 6.5 × 5 cm). Each treatment was replicated five times and sampled four times, making a total of 20 pots per treatment.

Sampling of soil and plant material

Pots were incubated in a phytotron at SLU, Uppsala, Sweden. The conditions in the climate chamber were set to mimick the weather conditions in June and July in Uppsala,

with a light/dark cycle of 18 h/6 h, temperatures of 20 °C/12 °C, relative humidity of 70% and light intensity of 400 μmol photons m^{-2} s^{-1}. Pots were watered every second day with nonsterile water to water-holding capacity. In Experiment A, pots were sampled at 0, 7, 14, 21 and 28 days postinoculation of *S.* Weltevreden and spinach seed planting. Pots in Experiment B were sampled at 0, 7, 14 and 21 days postinoculation.

DNA extraction

In both experiments, spinach plants were removed from the soil for DNA extraction. The soil in each pot was mixed, and an aliquot (10 g) was removed and stored at −20 °C before grinding with a mortar and DNA extraction. From each sample, 500 mg soil was used for extraction with the FAST DNA soil kit (MP Biomedicals). Plant roots and shoots were separated, and the roots carefully washed in sterile water to remove soil particles and bacterial cells that were not firmly attached to the surface. For the root and leaf samples, various concentrations of plant material (100–400 mg) were used for DNA extraction. These differences were considered when analyzing data. Before adding plant material to the FAST DNA soil kit, the plant parts were cut with a scalpel into pieces of approximately 5 mm and carefully mixed. On the early plant sampling occasions (days 0, 7 and 14) all plant material available was used. For the later sampling dates, the cut pieces were carefully mixed and subsamples were taken.

Real-time PCR

The real-time PCR assay was adopted from Nam *et al.* (2005). *Salmonella*-specific primers, StyinvA-JHO-2-left (5′-TCGTCATTCCATTACCTACC-3′) and StyinvA-JHO-2-right (5′-AAACGTTGAAAAACTGAGGA-3′), were selected for the amplification of a 119-base pair fragment of the *invA* gene (Hoorfar *et al.*, 2000). Real-time PCR was carried out on an IQ5 Multicolor Real-Time PCR Detection System (BioRad, Hercules, CA) in 20-μL triplicate reactions containing 1 × Flash SYBR® Green q-PCR Master mix (Finnzymes, Espoo, Finland), 1 × Rox reference dye (Finnzymes), 0.5 μM primers, 5 mM $MgCl_2$ and 20 ng of DNA from the soil/roots/leaves as template. The amplification program started with initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 15 s and elongation at 72 °C for 30 s and 5 min of final elongation at 72 °C. Melting curve analysis was performed over 55–95 °C, with increments set at 0.5 °C for 10 s (80 cycles). The DNA concentrations were determined spectrophotometrically (Nanovue, GE Healthcare). To generate DNA standards, the PCR *invA* gene fragment was inserted into PCR®4-TOPO® plasmids (Invitrogen, Carlsbad, CA) before linearization. The standards contained between 10^1 and 10^5 *Salmonella invA* gene copies calculated directly from the concentrations of linearized plasmids, resulting in a linear equation of $-3.315x+32.92$ with $R^2 = 0.999$. The efficiency was calculated as 92.8% on average, standard curves displayed similar slopes between runs (-3.406 to -3.671), and the melting curves revealed that amplified products were collected at similar temperatures (77.5–78.0 °C). To confirm the absence of potential PCR inhibitors, plasmid DNA, in combination with extracted soil/root/leaf DNA, was quantified and compared with the resulting gene copy numbers of plasmid DNA alone. In addition, soil DNA was diluted and the different concentrations quantified and analyzed.

Detection limits

To determine the detection limit of the real-time PCR assay, soil, root and leaf materials were inoculated with different quantities of bacterial suspensions containing *S.* Weltevreden corresponding to concentrations of 10^1–10^7 g^{-1} soil or plant material. For these analyses, DNA was extracted from 500 mg of soil, 100 mg of root samples and 200 mg of leaf material, in a similar way to that described above. DNA extracts were evaluated for their bacterial content using the real-time PCR assay targeting *S.* Weltevreden, as described previously. The limit of quantitation for the real-time PCR assay was calculated as 10^4 cells g^{-1} of soil, roots or leaves, respectively. Controls without templates resulted in negligible values.

Statistical analyses

Differences in *invA* gene copy numbers between treatments and sites were tested for significance using one-way ANOVA and unpaired *t*-test (GRAPHPAD PRISM v. 5, GraphPad Software, San Diego, CA). For all analyses, $P < 0.05$ was considered the level of significance. Correlations between inoculation doses and bacterial cell numbers detected in soil and plant parts were evaluated using nonparametric Spearman correlation (GraphPad Software).

Results

Quantification of *S.* Weltevreden in soil

Salmonella enterica serovar Weltevreden was detected in soil samples at all sampling occasions and inoculation doses from both Experiments A and B (Fig. 1). The bacterial inoculation doses in Experiment A were positively correlated to the *invA* gene copy numbers detected in soil at all sampling occasions (day 0: $r = 0.94$, $P \leq 0.0001$; day 7: $r = 0.85$, $P \leq 0.0001$; day 14: $r = 0.93$, $P \leq 0.0001$; day 21: $r = 0.94$, $P \leq 0.0001$; day 28: $r = 0.89$, $P \leq 0.0001$). Data from Experiment A showed that *invA* gene copy numbers

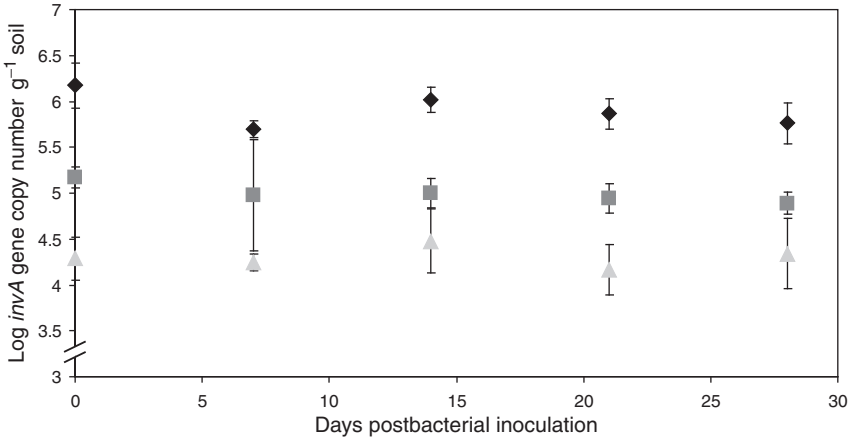

Fig. 1. Detection of the *Salmonella invA* gene by real-time PCR in soil after inoculation of *Salmonella enterica* serovar Weltevreden to cattle manure subsequently added to soil (Experiment A). Inoculation doses are 10^6 CFU g^{-1} soil (diamonds), 10^5 CFU g^{-1} soil (squares) and 10^4 CFU g^{-1} soil (triangles). Error bars represent the SD of five replicate samples.

did not drop significantly during the 4-week sampling period (Fig. 2). In Experiment B, the gene copy numbers decreased from 5.7 to 4.6 log between days 0 and 21 postinoculation ($P \leq 0.0001$) (Fig. 2).

The initial concentration (day 0 postinoculation) of *S.* Weltevreden differed significantly between Experiments A and B ($P < 0.0001$). In Experiment A, a mean value of 6.2 log gene copies g^{-1} soil was estimated from pots inoculated with 10^6 cells g^{-1} soil, whereas in Experiment B the corresponding value was 5.7 log gene copies g^{-1} soil. The significant differences ($P \leq 0.0001$) in *S.* Weltevreden cell densities between the two inoculation strategies (Experiments A and B; Fig. 2) persisted throughout the sampling period (21 days following bacterial inoculation), and even increased when the *invA* gene copy numbers decreased in Experiment B (4.6 cells g^{-1} soil on day 21). The increase in Experiment A was not as marked (5.9 cells g^{-1} soil on day 21).

Salmonella enterica serovar Weltevreden associated with spinach roots

In Experiment A, *invA* gene copies were only detected from plant roots grown in soil fertilized with manure slurry inoculated with the highest dose of *S.* Weltevreden, i.e. 10^6 cells g^{-1} soil. However, the bacteria were not consistently detected in all replicates on all sampling occasions (Table 2). At day 7 postinoculation, no replicate root samples contained detectable levels of *invA*. After 14 days, a positive PCR product was obtained from one of five replicates. This tendency increased throughout the sampling period, with four positive replicates at day 21 and positive products from all five replicate samples at day 28 (Table 2). In Experiment B, all replicates at all sampling occasions contained detectable amounts of *invA*, although the numbers significantly decreased from 6.0 log gene copies at day 1 to 5.0 log gene copies at day 21 postinoculation ($P \leq 0.0005$) (Fig. 3).

Salmonella enterica serovar Weltevreden on spinach leaves

We detected no *invA* gene copies on spinach leaves at any of the sampling dates in Experiment A. In contrast, in Experiment B, *S.* Weltevreden was detected on spinach leaves from all five replicates at days 0 and 7 postinoculation (Table 3). At day 14, *invA* gene copies were identified in two of five replicates, and at the last sampling date (day 21; Table 3) the corresponding number was one of five replicates. Moreover, there was a significant decrease in the number of *invA* gene copies estimated in leaves between days 0 and 7 ($P < 0.05$).

Discussion

Our results revealed that *Salmonella* is able to persist in soil, showing only a slight reduction in bacterial numbers over a 4-week evaluation period (Fig. 1). As no significant decline in bacterial cell numbers was found over the time-span of the present study, which might have indicated the presence of dead cells, we concluded that the cells detected are most likely living. The minor reduction in *S.* Weltevreden cell numbers during the evaluation period in the current study emphasizes the importance of keeping the intervals between manure application and plant harvest as long as possible, as the survival of *Salmonella* in soil is time dependent (Doyle & Erickson, 2008). Moreover, the density and survival length of *S.* Weltevreden in soil correlated well with bacterial inoculation levels, with larger numbers of initial bacteria resulting in higher bacterial densities in soil throughout the sampling period (Fig. 1). This finding is in agreement with several reports showing that the number of *Salmonella* cells initially present influences the length of survival in soil, sometimes as much as up to several months after manure spreading (Jones, 1986; Baloda *et al.*, 2001).

In the current study, we evaluated the persistence of *S.* Weltevreden in soil and on spinach roots and leaves using two different bacterial inoculation strategies. The

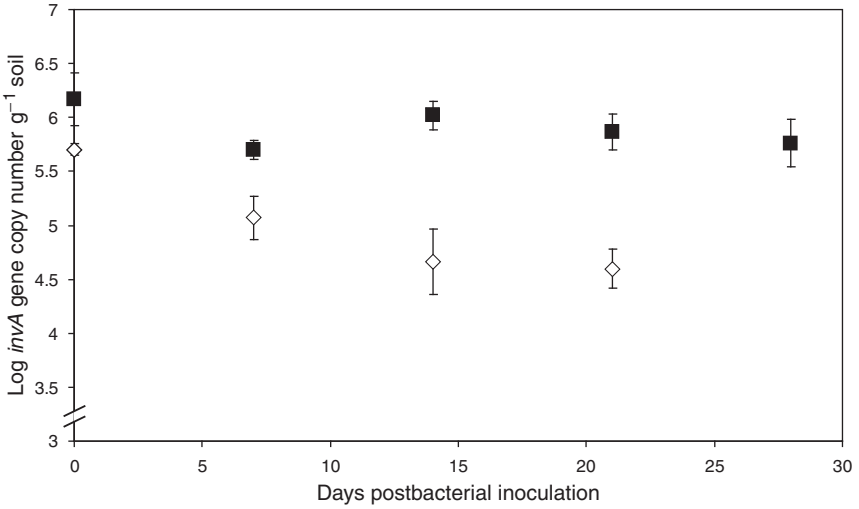

Fig. 2. Comparison of *invA* gene copy numbers in soil inoculated with *Salmonella enterica* serovar Weltevreden just before sowing spinach seeds (squares; Experiment A) and 2 weeks postsowing (diamonds; Experiment B). Bacterial inoculation dose: 10^6 CFU g^{-1} soil. Error bars represent the SD of five replicate samples.

Table 2. Number of replicate samples containing detectable *Salmonella enterica* serovar Weltevreden populations in roots from Experiment A

Days	10^6 g^{-1} soil	10^5 g^{-1} soil	10^4 g^{-1} soil
0	NA	NA	NA
7	0 (5)	0 (5)	0 (5)
14	1 (5)	0 (5)	0 (5)
21	4 (5)	0 (5)	0 (5)
28	5 (5)	0 (5)	0 (5)

Values in parentheses represent total number of samples.
NA, not analyzed.

Table 3. Number of replicate samples containing detectable *Salmonella enterica* serovar Weltevreden populations on leaves from Experiment B

Days	10^6 g^{-1} soil
0	5 (5)
7	5 (5)
14	2 (5)
21	1 (5)
28	NA

Values in parentheses represent total number of samples.
NA, not analyzed.

experimental conditions for the two experiments were similar except for the bacterial inoculation procedures, which occurred at different time periods. Our objectives were to compare general tendencies between the experiments that might possibly be explained by the different application times of *S*. Weltevreden. In Experiment A, in which different concentrations of bacteria were inoculated into cattle manure slurry before application to soil, the numbers of *S*. Weltevreden detected in soil at all sampling occasions were significantly higher than the corresponding values in Experiment B, where the bacteria were added in saline solution directly to the soil at 14 days postplanting and fertilizing (Fig. 2). The early differences in cell densities in the soil observed between the two inoculation strategies may be attributed to a better developed spinach root system in Experiment B, leading to more pronounced effects of the rhizosphere on *S*. Weltevreden stimulation. Improved soil nutrient status through exudation may yield general bacterial stimulation (Lugtenberg *et al*., 2001), resulting in increased competition for preferred colonization niches between other microorganisms and therefore potentially harsher conditions for *S*. Weltevreden. On the other hand, increased secretion of root exudates has previously been shown to promote the survival of *Salmonella* more specifically (Reijs *et al*., 2004). As manure slurry from the same sampling site was added to the pots in both experiments, no large variations in nutrient and/or organic material content should have affected the persistence of *Salmonella* in the experiments. However, as the manure in Experiment B was added to the pots 2 weeks before bacterial inoculation, some nutrients may have been degraded during this time, which could be one explanation for the differences in bacterial persistence observed between the experiments. Nevertheless, high numbers of the pathogen in the rhizosphere may represent an increased risk of internal plant contamination via roots (Klerks *et al*., 2007).

Spinach roots evaluated for the presence of *S*. Weltevreden in the current study were thoroughly rinsed with sterile water several times to remove bacteria loosely bound to the root surface. Consequently, *S*. Weltevreden detected in root samples were either firmly attached to the root surface or were living endophytically inside the root tissues. In Experiment A, where manure slurry was inoculated with *S*. Weltevreden, only the highest inoculation dose (10^6 cells g^{-1}

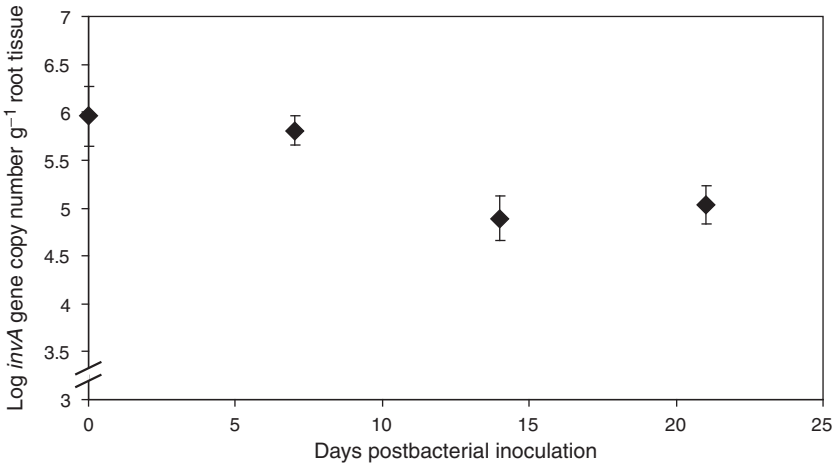

Fig. 3. Copy numbers of *invA* genes on spinach roots after inoculation with *Salmonella enterica* serovar Weltevreden at a level of 10^6 CFU g^{-1} soil 2 weeks postsowing (Experiment B). Error bars represent the SD of five replicate samples.

soil) resulted in detectable pathogen levels associated with roots (Tables 1 and 2). As the number of replicate pots (between 0 and 5) containing roots positive for *S.* Weltevreden consistently increased during the evaluation period (Tables 1 and 2), we conclude that, with time, more *Salmonella* cells colonized spinach roots. Entry sites consisting of cracks in the seed coats (Wachtel *et al.*, 2002) or lateral root junctions in seedlings (Cooley *et al.*, 2003; Dong *et al.*, 2003; Warriner *et al.*, 2003) may present increased nutrient exudation, and hence may function as triggers for mobilization of *S.* Weltevreden to these sites (Cooley *et al.*, 2003; Dong *et al.*, 2003; Jablasone *et al.*, 2005). Alternatively, *S.* Weltevreden may associate with the root surface through adhesive properties (Wachtel *et al.*, 2002), specific selective advantages (Jacobsen, 1997) and well-developed colonization ability (Dong *et al.*, 2003), which may represent a potential strategy for contamination of plants and further dissemination into edible parts along the root and shoot surfaces.

As the number of replicate spinach plant roots infected with *S.* Weltevreden increased in Experiment A during the evaluation period and bacterial numbers in individual root systems tended to increase rather than decrease over time, it is likely that the bacteria observed were viable, rather than resting or dead. In Experiment B, where *S.* Weltevreden was added in saline solution directly to soil after plant emergence, the pathogen was detected in all replicates at all sampling occasions (Fig. 3). However, bacterial numbers present in the roots decreased significantly from day 0 to day 21 postinoculation, in contrast to the trend of increased cell numbers with time in Experiment A. When roots started to develop in the pots in Experiment A, *S.* Weltevreden may have benefited from increased nutrient levels available in roots accessible via lateral root junctions or breaks, leading to proliferation. The more pronounced decline in bacterial numbers in roots in Experiment B, compared with Experiment A, was similar to the trends seen in soil between the two experiments. This indicates that *S.* Weltevreden inoculated into soil after plant emergence faced more competition from the indigenous microbial communities for nutrients and colonization sites compared with *S.* Weltevreden applied to the soil before planting.

Salmonella can contaminate crops in fields through leaves or other aerial surfaces (Doyle & Erickson, 2008). In the current study, no *S.* Weltevreden cells were recovered above the threshold level on leaves when added to the soil in a manure mixture (Experiment A). However, when bacteria were added in saline solution and added directly to soil 14 days after sowing and fertilization, cells were detected in all replicate pots on days 0 and 7 postinoculation (Experiment B). However, as *S.* Weltevreden was detected on leaves on the day of bacterial addition, this finding may have been an artifact resulting from negligent inoculation, i.e. unintentional application of bacteria to the leaves. Alternatively, *S.* Weltevreden may have potentially mobilized to spinach leaves through direct contact between leaves and soil/manure slurry as well as aerosol dispersion (Doyle & Erickson, 2008). Nevertheless, it is interesting to note that *S.* Weltevreden persisted on leaves in some of the replicates up to 21 days postinoculation, which may present a significant food safety hazard. Moreover, the lower cell densities of *S.* Weltevreden detected on leaves than in/on roots were consistent with previous reports showing 30–40-fold lower levels of *S. enterica* on leaves in relation to roots (Cooley *et al.*, 2003). It would be interesting to see whether *S.* Weltevreden actively proliferates on the plants or whether it simply survives there without further growth. The metabolic activity of *S.* Weltevreden under varying conditions could be assayed using molecular tools (Artursson *et al.*, 2005). The potential of pathogenic bacteria to exist and survive on plant surfaces is affected by adjacent physicochemical conditions and fluctuations in these environments (Brandl *et al.*, 2004), indicating that the quantity and composition of root and leaf exudates, along with other

parameters, play a major role in influencing the persistence or decline of *S*. Weltevreden.

In conclusion, our results showed a great persistence of *S*. Weltevreden in soil, roots and on leaves, which further emphasizes the importance of strict monitoring of untreated animal manure before considering application to agricultural land. Moreover, the pathogen appeared to be mobilized from manure to spinach roots, as the number of contaminated pot cultures steadily increased throughout the evaluation period. Consequently, introduction of enteropathogenic bacteria via manure into the food chain should be avoided and more precise safety guidelines prepared for defining actions to minimize contamination of plant produce.

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