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Original paper

Investigation of Routes of *Salmonella* Contamination Via Soils and the Use of Mulch for Contamination Control during Lettuce Cultivation

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Foodborne illnesses associated with the consumption of fresh produce such as raw vegetables have become a major health concern worldwide in recent years. In the present study, we investigated the possible routes of *Salmonella* contamination in leafy lettuce via soil during cultivation. After 10-week cultivation of lettuce plants in soils inoculated with *S.* Enteritidis expressing green fluorescent protein (SE-EGFP), the bacterium was detected in soil inoculated with >10^4 cfu/g and from most lettuce leaves cultivated in soils inoculated with >4.4×10^7 cfu/g. As *Salmonella* was not detected in intact lettuce leaves or lettuce leaves with root injury cultivated in highly contaminated soils and after surface disinfection, the lettuce plants were not considered to internalize the bacterium. Overhead irrigation led to the contamination of one in 10 lettuce plants; however, all sets of three leaves of the plant were contaminated (>110 MPN/g). In an effort to prevent *Salmonella* contamination from soils, we investigated the effects of mulch on contamination levels during cultivation. Mulch effectively reduced *Salmonella* contamination levels of lettuce plants cultivated in highly contaminated soils.

Keywords: contamination, lettuce, *Salmonella*, soil, mulch

Introduction

The increasing consumption of raw and minimally processed fruits and vegetables, such as packaged and precut salads, etc., has been accompanied by an increase in the number of outbreaks of foodborne illnesses associated with fresh produce in developed countries (Sivapalasingam *et al.*, 2004; Doyle and Erickson, 2008). These outbreaks have raised interest in identifying pre- and/or post-harvest sources of contamination of raw and minimally processed fruits and vegetables with pathogens.

Fresh produce such as tomato, spinach, and lettuce are reported to be vectors of pathogens (Doyle and Erickson, 2008). Moreover, leafy vegetables are often contaminated with pathogenic bacteria. For instance, outbreaks of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in Europe and USA were linked to leafy vegetables such as lettuce and spinach (Horby *et al.*, 2003; Heaton and Jones, 2008; Parker *et al.*, 2012). Epidemic survey also showed a high possibility of leafy vegetables infected with *E. coli* O157:H7 at the farm (Parker *et al.*, 2012; Cooley *et al.*, 2007). In particular, Parker *et al.* (2012) reported that a large outbreak of *E. coli* O157:H7 in 2006 was linked to the consumption of ready-to-eat baby spinach, likely contaminated by soil and water. From those reports, it is considered that the cultivation period is a critical point in preventing contamination with pathogenic bacteria.

The use of partially decomposed compost, the mediation by insects or wild animals, and irrigation water are possible contamination sources of vegetables during cultivation (Beuchat, 1996). The

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use of partially decomposed compost can be easily restricted to avoid contamination. However, irrigation water, while essential for cultivation, is considered an important contamination source of leafy vegetables. In Japan, mulch is widely used for the cultivation of leafy vegetables to prevent damage caused by cold, frost, heat, or drought, and to prevent weeds. As mulch can prevent direct contact between vegetables and the soil, mulching is expected to be effective in preventing bacterial contamination in leafy vegetables. However, there are few reports regarding the effects of mulch on contamination levels of pathogenic bacteria in vegetables.

We previously showed that *Salmonella* survived in soil > 70 days during tomato cultivation (Mishima *et al.*, 2012). Once the soil is contaminated with bacterial pathogens, the pathogens can remain in the soil during cultivation and contaminate the leafy vegetables. In the present paper, we investigated several possible routes of *Salmonella* contamination in lettuce plants via soil during cultivation. Moreover, the use of mulch in preventing *Salmonella* contamination was also investigated.

Materials and Methods

Bacterial strains and culture conditions *Salmonella enterica* serovar Enteritidis IFO3313 was obtained from the National Institute of Technology and Evaluation-Biological Resource Center (Kisarazu, Japan) and electrophoretically transformed with a plasmid vector, pEGFP (BD Biosciences, San Jose, CA, USA), carrying the enhanced green fluorescent protein (EGFP) gene. The transformed strain was designated as SE-EGFP. One loopful of SE-EGFP was inoculated in 10 mL of Tryptic Soy Broth (Difco Laboratories Inc., Cockeysville, MD, USA) supplemented with 50 μg/mL of ampicillin (Nacalai Co. Ltd., Tokyo, Japan) and incubated with shaking at 37℃ overnight. Cells were harvested by centrifugation at 5,000 × *g* for 5 min, resuspended in sterile water, and then serially diluted with sterile water to concentrations of approximately 10^0, 10^1, 10^6, 10^7, 10^8, or 10^9 cfu/mL.

Preparation of SE-EGFP-contaminated soil Commercially available soil (peat-moss/perlite/vermiculite) was purchased from Oishi Bussan Co. Ltd. (Tokyo, Japan). The soil was sterilized by autoclaving at 121℃ for 20 min before inoculation. Approximately 100 g of soil was mixed with 50 mL of SE-EGFP at concentrations of 10^0, 10^1, 10^6, 10^7, 10^8, or 10^9 cfu/mL. The SE-EGFP concentrations in contaminated soil were approximately 10^{-1}, 10^0, 10^5, 10^6, 10^7, or 10^8 cfu/g after mixing. Plastic pots 7.5 cm in diameter were filled with 45 g of contaminated soil for lettuce cultivation.

Seed disinfection Seeds of *Lactuca sativa* var. *crispa* (Banshu RedFire) were obtained from Takii Seeds Co. Ltd. (Kyoto, Japan). The seeds were surface disinfected with 70% ethanol for 10 − 15 s, and then immersed in 0.2% sodium hypochlorite solution for 10 min. The seeds were then rinsed three times with sterile water.

Growth conditions of leafy lettuce Disinfected seeds were planted in pots with contaminated soil, and were grown at 20℃ with a 16-h light and 8-h dark photoperiod in a growth chamber (TOMY SEIKO Co. Ltd., Tokyo, Japan). Thirty mL of sterile tap water was added to the soil around the root daily. Furthermore, 30 mL of nutrient solution (0.2% Hyponex; Hyponex Japan Co. Ltd., Osaka, Japan) was applied to the lettuce plant once per week.

Mulching Two weeks after seed germination, the cultivation pot was covered with disinfected polypropylene film with a hole (3 − 4 cm diameter) during cultivation. Disinfection was carried out by spraying the film with 70% ethanol.

Generation of highly contaminated soil by addition of SE-EGFP suspension To examine the effect of high SE-EGFP contamination level in soil, the bacterial culture was suspended in sterile water at a concentration of approximately 10^9 cfu/mL and the suspension was used as irrigation water. To maintain an SE-EGFP concentration of $10^7 - 10^8$ cfu/g, 30 mL of contaminated water (10^9 cfu/mL) was added directly to the soil around the root, and without leaf contact, once every two weeks.

SE-EGFP contamination via artificial root injury To investigate whether root injury promotes SE-EGFP invasion via the plant vasculature, the main root of the lettuce plant, which had been cultivated in SE-EGFP contaminated soil (10^8 CFU/g), was severed. The root was severed 1 cm below the soil surface after 8 weeks of cultivation, and the lettuce plant was subsequently replanted in SE-EGFP-contaminated soil at approximately 10^7 CFU/g. Lettuce leaves were harvested 2 to 9 weeks after replantation. To investigate the internalization of SE-EGFP in lettuce leaves, samples were surface disinfected as described below.

Sampling methods for soils and lettuce plants During cultivation, 5 g soil samples were analyzed for SE-EGFP levels biweekly. Lettuce plants were harvested after the indicated cultivation periods. For microbial analysis, three sections of the outermost leaves from plants were used as a sample set.

Surface disinfection of lettuce leaves To determine whether SE-EGFP was internalized by lettuce or attached to the lettuce surface, harvested lettuce leaves were surface-disinfected with 100 mL of 70% ethanol for 1 min and 100 mL of 10% sodium hypochlorite for 1 min, at room temperature. Then, the leaves were rinsed three times in 100 mL of sterile water at room temperature for 1 min.

Homogenization of samples Soil or lettuce leaf samples were transferred to Stomacher bags and ten-fold diluted (one part lettuce to 9 parts buffered peptone water) with buffered peptone water (BPW) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). The soil and lettuce samples were then homogenized for 30 s using a Pulsifier (Microgen Bioproducts Ltd., Camberley, Surrey, UK) and a Masticator (IUL instruments, Barcelona, Spain), respectively.

Determination of viable bacterial counts and Salmonella counts One milliliter of each homogenized sample was serially diluted with sterile water and spread (100 μL) onto tryptic soy agar (TSA, Japan BD Company, Tokyo, Japan) containing 50 μg/mL ampicillin (TSA/Amp), deoxycholate-hydrogen sulfide-lactose agar (DHL, Nissui Pharmaceutical) in duplicate and incubated at 37℃

for 48 h. All colonies on TSA were counted as total viable bacterial counts; colonies expressing green fluorescence on TSA/Amp were counted as SE-EGFP counts; and red colonies were also counted as Enterobacteriaceae, black colonies on DHL were counted as *Salmonella* counts. Viable counts were expressed as colony forming units (cfu)/g.

To measure viable counts below the lower limit of detection of the plating method, the Most Probable Number (MPN) method was applied. In this method, 10, 1, and 0.1 mL of homogenates were inoculated into each 3 test tubes with 10 mL BPW and incubated at 37℃ for 20 ± 2 h. The culture (100 µL) was inoculated into 10 mL Rappaport-Vassiliadis broth (RV broth, Merck Ltd., Tokyo, Japan), and incubated at 42℃ for 22 ± 2 h. Finally, one loopful of the culture was withdrawn with a sterile plastic microloop, streaked onto DHL agar, and incubated at 37℃ for 24 ± 2 h. MPN values for *Salmonella* were determined using the MPN table and expressed as MPN/g.

Results

Survival of SE-EGFP in soil To investigate the survival of SE-EGFP in soil, SE-EGFP-inoculated soil was examined biweekly during the 70-day cultivation period. Figure 1 shows SE-EGFP survival in soil during lettuce cultivation. SE-EGFP soil levels were observed at an inoculum level-dependent manner during cultivation. The total viable counts of SE-EGFP-inoculated soil at 10^1, 10^4, and 10^6 cfu/g increased up to 10^8 cfu/g on day 14 and the counts remained more or less steady during the remainder of the cultivation period. Soil counts of SE-EGFP inoculated at 10^4 and 10^6 cfu/g increased to about 10^7 cfu/g on day 14 and decreased gradually to 10^5 cfu/g during the remainder of the cultivation period. At an initial inoculation of 10^1 cfu/g, SE-EGFP counts also increased to 10^5 cfu/g on day 28, then decreased gradually to 10^3 cfu/g on day 42, and were maintained until the end of the cultivation period. However, at an initial SE-EGFP inoculation of 10^8 cfu/g, counts decreased gradually to 10^6 cfu/g during the 70-day cultivation period.

Effects of soil SE-EGFP inoculum level on contamination of lettuce leaves To investigate the relationship between initial soil SE-EGFP level and lettuce leaf contamination, whole lettuce was examined for viable bacterial counts and *Salmonella* counts at harvest after 10-week cultivation. As shown in Table 1, soil viable counts ranged from 10^7 to 10^8 cfu/g independent of the initial inoculum size. *Salmonella* was detected in the soil at about 10^3 to 10^6 cfu/g, depending on the initial *Salmonella* inoculation level. Viable counts of whole lettuce ranged from 10^3 to 10^5 cfu/g regardless of the initial soil *Salmonella* level. However, *Salmonella* counts in the whole lettuce samples were less than the lower limit of detection in both the plating ($<10^2$ cfu/g) and MPN (<0.3 MPN/g) methods.

To confirm the absence of *Salmonella* in lettuce leaves, sets of 3 outermost leaves from lettuce were subjected to determination of

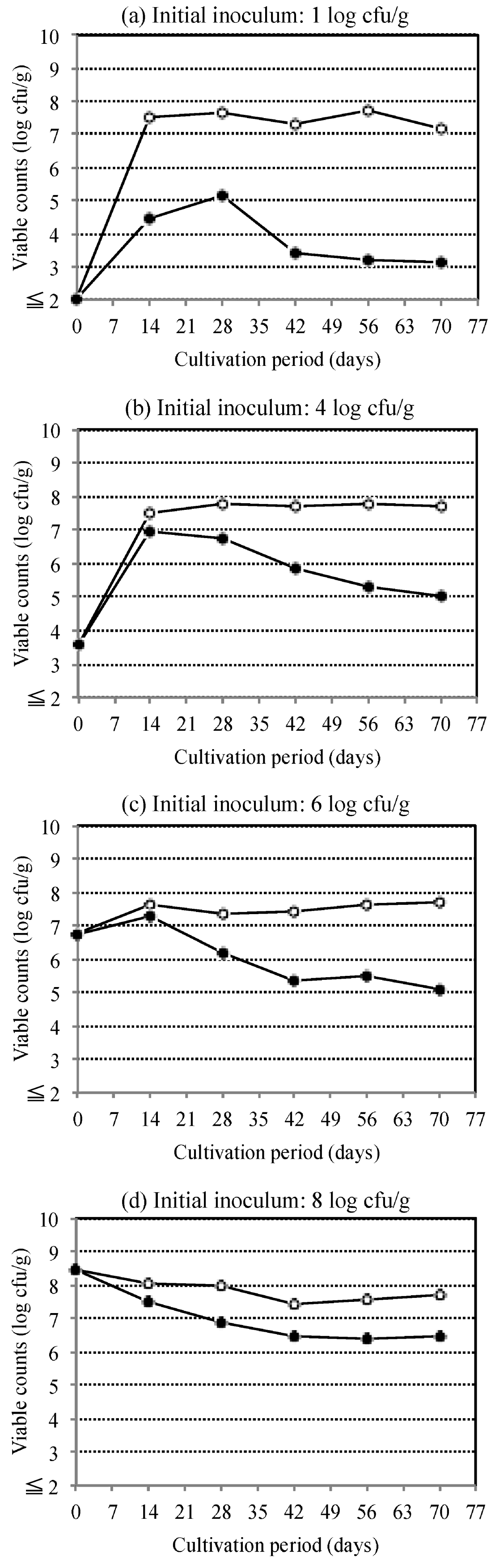

Fig. 1. Survival of SE-EGFP in soil during lettuce cultivation. Commercially available horticultural soil was inoculated with SE-EGFP at a final concentration of 10^1, 10^4, 10^6, 10^8 cfu/g. Leaf lettuces were cultivated as described in the text. Open circles and closed circles indicate total viable counts and SE-EGFP counts, respectively.

Table 1. Viable bacterial counts and *Salmonella* counts of whole lettuce cultivated in soil spiked at various SE-EGFP levels after 70-day cultivation.

Initial SE-EGFP inoculum (cfu/g)	Sample No.	Viable counts in soil (cfu/g)		Viable counts of whole lettuce (cfu/g or MPN/g)		
		Total	*Salmonella*	Total	*Salmonella*	
					Plating method	MPN
0	1	3.78×10^7	$< 10^2$	4.23×10^4	$< 10^2$	< 0.3
0.42	3	2.52×10^7	1.52×10^3	8.76×10^5	$< 10^2$	< 0.3
0.69	2	2.18×10^7	3.39×10^3	8.49×10^3	$< 10^2$	< 0.3
1.35	1	2.87×10^7	2.53×10^3	6.10×10^5	$< 10^2$	< 0.3
2.7×10^4	1	2.81×10^7	4.42×10^5	1.54×10^3	$< 10^2$	< 0.3
	2				$< 10^2$	< 0.3
3.1×10^5	1	2.99×10^7	7.18×10^5	4.46×10^3	$< 10^2$	< 0.3
	2				$< 10^2$	< 0.3
3.8×10^6	1	4.25×10^7	3.14×10^6	7.84×10^3	$< 10^2$	< 0.3
	2				$< 10^2$	< 0.3
4.4×10^7	1	4.23×10^7	7.03×10^6	2.35×10^4	$< 10^2$	< 0.3
	2				-	< 0.3
	3				$< 10^2$	< 0.3
2.1×10^8	1	1.08×10^8	7.20×10^6	2.38×10^4	-	< 0.3
	2				$< 10^2$	< 0.3

Salmonella counts at harvest after 10-week cultivation. As shown in Table 2, *Salmonella* was not detected in every set of lettuce leaves cultivated in soil spiked at $<2.7 \times 10^4$ cfu/g. *Salmonella* was detected at 0.36 and 0.91 MPN/g from the sets of 1st – 3rd and 7th – 9th outermost leaves, respectively, in one out of 3 lettuce plants cultivated in soil initially inoculated with levels of SE-EGFP at 3.1×10^5 cfu/g. In addition, *Salmonella* was detected in almost all of the sample sets, from 1st to 15th outermost leaves of one of two lettuces cultivated in the soil spiked at 4.4×10^7 cfu/g. At an initial soil contamination level of 2.1×10^8 cfu/g, *Salmonella* was detected from the 1st – 15th outermost leaves from one lettuce.

Effect of mulching on Salmonella contamination of lettuce plants cultivated in contaminated soils To investigate the effects of mulching on contamination control, soils spiked at 10^5, 10^6, 10^7, or 10^8 cfu/g were used for lettuce cultivation with mulching. After 10-week cultivation, *Salmonella* levels in sets of 3 outermost leaves from lettuce were investigated (Table 3). *Salmonella* levels from all samples were below the detection limit (0.3 MPN/g), suggesting that mulching reduced contact between the lettuce and soil, and consequently reduced the contamination of lettuce with *Salmonella*.

Effect of mulching on Salmonella contamination of lettuce plants cultivated in highly contaminated soil The effect of mulching on *Salmonella* contamination in lettuce plants cultivated in highly contaminated soil was investigated. The soil was spiked with SE-EGFP at 10^8 cfu/g and then, to maintain SE-EGFP soil contamination levels in the range of $10^6 – 10^8$ cfu/g, 30 mL of a SE-EGFP suspension (10^9 cells/mL) was added to the soil biweekly during cultivation. After 10 – 17 weeks of cultivation, lettuce plants were harvested and divided to several sets of 3 outermost leaves for determination of *Salmonella* counts. Viable *Salmonella* counts of the leaves were measured by the MPN method before and after surface disinfection (Table 4). In non-disinfected lettuce plants cultivated without mulch, *Salmonella* was detected in 7, 5, 3, and 2 out of 8 plants for the set of 1st – 3rd, 4th – 6th, 7th – 9th, and 10th – 12th outermost leaves, respectively. The contamination levels of outer leaves were higher than those of inner leaves. The detected levels were in the range of 0.73 to >110 MPN/g in all sets from the 1st – 12th outermost leaves. On the other hand, in the case of mulched non-disinfected lettuce plants, *Salmonella* was detected in 3 and 2 out of 7 plants for the sets of 1st – 3rd and 4th – 6th outermost leaves, respectively. These results show that mulching can effectively reduce contamination of lettuce plants. In regard to surface disinfected samples, *Salmonella* was not detected in 35 samples regardless of mulching, except for one plant. Even under cultivation in highly contaminated soils, the possibility of in-

Table 2. *Salmonella* counts of sets of 3 outermost leaves from lettuce cultivated in soil spiked at various SE-EGFP levels. Counts were determined by the MPN method after 70-day cultivation.

Initial SE-EGFP inoculum (cfu/g)	Sample No.	*Salmonella* counts of leaves (MPN/g)						
		Outermost leaves						
		1st–3rd	4th–6th	7th–9th	10th–12th	13th–15th	16th–18th	19th–21st
0	2	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
0.42	6	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	–	–
0.69	5	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	–	–
1.35	4	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	–	–
2.7×10^4	3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	–
3.1×10^5	3	0.36	< 0.3	0.91	< 0.3	< 0.3	< 0.3	–
	4	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	–
	5	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	–	–
3.8×10^6	3	2	< 0.3	46	9.3	< 0.3	< 0.3	–
4.4×10^7	4	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	2.1
	5	0.93	< 0.3	0.3	0.36	24	–	–
2.1×10^8	4	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	–
	5	2.3	0.3	45	1.5	24	< 0.3	< 0.3

ternalization of *Salmonella* in lettuce plants was thought to be low.

Effect of root injury on Salmonella internalization of lettuce in highly contaminated soil Table 5 shows the effect of root injury on *Salmonella* internalization in lettuce tissues cultivated in soil spiked with SE-EGFP at $10^6 - 10^8$ cfu/g. *Salmonella* was detected in the range from 0.3 to >110 MPN/g on sets of 3 outermost leaves of 6 out of 8 plants. The *Salmonella* contamination level of lettuce was lower in the inner leaves compared with the outer leaves. However, *Salmonella* was not detected in any sets of 3 outermost leaves of 8 plants after surface disinfection. These results indicate that root injury does not affect the internalization of *Salmonella* and soil contamination of lettuce occurs only on the plant surfaces, not internally via the plant vasculature.

Effect of overhead irrigation on Salmonella contamination of lettuce cultivated in contaminated soil To study the effect of water splash on SE-EGFP contamination of lettuce cultivated in highly contaminated soil, to which contaminated water ($10^7 - 10^8$ cfu/mL) was added biweekly in addition to initial inoculation, overhead irrigation with sterilized water was conducted daily during cultivation. Concurrently, the effect of mulching was also investigated. After 10 − 15 weeks of cultivation, lettuce plants were harvested. As shown in Table 6, without mulching, *Salmonella* was detected in all sets of at least one plant at >100 MPN/g. Even the innermost leaves (the 22nd − 24th) were contaminated with *Salmonella*. On the other hand, in the case of mulching cultivation, *Salmonella* was detected from only one set (4th − 6th) of leaves at 4.3 MPN/g. These results indicate that overhead irrigation causes *Salmonella* contamination in cultivated lettuce and that mulching can effectively reduce contamination levels in lettuce.

Discussion

The present study was carried out to investigate possible routes of *Salmonella* contamination in leaf lettuce during cultivation. Once the soil is contaminated with pathogens such as *Salmonella*, there is the possibility that the bacteria persist in the soil throughout the cultivation period and contaminate the edible portions of vegetables by contact with contaminated soil. We first investigated *Salmonella* survival in soil during the 70-day cultivation period. As shown in Fig. 1, SE-EGFP counts increased once and were gradually reduced during the 70-day cultivation. Our previous study also showed that *Salmonella* survived in soil for >70 days during tomato cultivation (Mishima *et al.*, 2012). The soil did not completely desiccate during cultivation, since irrigation was carried out daily. This might be one of the reasons for the survival of SE-EGFP in soil for 70 days. Islam *et al.* (2004) investigated the fate of *Salmonella* on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Salmonella* survived for 200 days even in soil, which had been inoculated

Table 3. Effect of mulching on *Salmonella* counts in lettuce leaves cultivated in soil spiked with various levels of SE-EGFP. Counts were determined by the MPN method after 70-day cultivation.

Initial SE-EGFP count (cfu/g)	Outermost leaves	*Salmonella* positive plants/Total plants	*Salmonella* counts in leaves (MPN/g)
10^5	1st-3rd	0/3	< 0.3
	4th-6th	0/3	< 0.3
	7th-9th	0/3	< 0.3
	10th-12th	0/3	< 0.3
	13th-15th	0/3	< 0.3
10^6	1st-3rd	0/3	< 0.3
	4th-6th	0/3	< 0.3
	7th-9th	0/3	< 0.3
	10th-12th	0/3	< 0.3
	13th-15th	0/3	< 0.3
10^7	1st-3rd	0/3	< 0.3
	4th-6th	0/3	< 0.3
	7th-9th	0/3	< 0.3
	10th-12th	0/3	< 0.3
	13rd-15th	0/2	< 0.3
10^8	1st-3rd	0/3	< 0.3
	4th-6th	0/3	< 0.3
	7th-9th	0/3	< 0.3
	10th-12th	0/3	< 0.3
	13th-15th	0/3	< 0.3

Table 4. Effects of mulching and surface disinfection on *Salmonella* counts of lettuce cultivated in highly contaminated soil. The SE-EGFP concentration was maintained at $10^6 - 10^8$ cfu/g by treating with the bacterial suspension biweekly. Counts were determined by the MPN method after 70-day cultivation.

Mulching	Outermost leaves	Before disinfection		After disinfection	
		Salmonella positive plants/Total plants	*Salmonella* counts of leaves (MPN/g)	*Salmonella* positive plants /Total plants	*Salmonella* counts of leaves (MPN/g)
No	1st-3rd	7/8	0.91 - >110	0/8	< 0.3
	4th-6th	5/8	0.73 - >110	0/8	< 0.3
	7th-9th	3/8	2.3 - >110	0/8	< 0.3
	10th-12th	2/8	24 - >110	0/8	< 0.3
	13th-15th	0/2	< 0.3	0/2	< 0.3
Yes	1st-3rd	3/7	0.36 - >110	1/27	0.3
	4th-6th	2/7	2.3	0/27	< 0.3
	7th-9th	0/7	< 0.3	0/27	< 0.3
	10th-12th	0/6	< 0.3	0/27	< 0.3
	13th-15th	0/1	< 0.3	0/3	< 0.3

once with *Salmonella* (10^5 cfu/g) suspended in irrigation water. Furthermore, Zhang *et al.* (2009) investigated the presence of *E. coli* O157:H7 on and in leaves of lettuce grown in inoculated soil. They showed that all samples from soil inoculated at 6 log cfu/g were positive for *E. coli* O157:H7 at 17 days after inoculation. Of 60 samples, 54 and 45 were positive for *E. coli* O157:H7 at 45 and 60 days after inoculation, respectively. With soil inoculation at 3 log cfu/g, all soil samples were positive for *E. coli* O157:H7 in the 26-day post inoculation samples, but only 16 of 60 samples were positive at 60 days. Our results and those of Zhang *et al.* (2009) indicated that all the soils were positive for Gram-negative foodborne pathogens such as *Salmonella* and *E. coli* O157:H7 for 60 to 70 days after soil contamination, even at low concentrations.

We investigated the relationship between spiking with *Salmonella* and contamination of lettuce (Tables 1 and 2). Contamination was not confirmed by investigating whole lettuce (Table 1). However, as shown in Table 2, *Salmonella* was detected by investigating sets of 3 outermost leaves. The *Salmonella* contamination level of the lettuce leaves at harvest increased with increasing initial inoculum levels of *Salmonella* in the soil. Not only the outer leaves but also the inner leaves of lettuce were contaminated with *Salmonella* when cultivated in soil highly contaminated with *Salmonella.* These results indicate that lettuce grown in soil containing *Salmonella* or *E. coli* O157:H7 may become contaminated throughout the growing season. Gagliardi and Karns (2002) revealed that *E. coli* O157:H7 survived for 25 to 41 days in fallow

Table 5. Effect of root injury on SE-EGFP counts of lettuce cultivated in soil spiked with about $10^6 - 10^8$ cfu/g. Counts were determined by the MPN method after 70-day cultivation.

Outermost leaves	Before disinfection		After disinfection	
	Salmonella positive plants / Total plants	*Salmonella* counts of leaves (MPN/g)	*Salmonella* positive plants / Total plants	*Salmonella* counts of leaves (MPN/g)
1st–3rd	6/8	0.3 - > 110	0/8	< 0.3
4th–6th	5/8	0.91 - > 110	0/8	< 0.3
7th–9th	5/8	0.91 - > 110	0/8	< 0.3
10th–12th	4/8	0.36 - 110	0/8	< 0.3
13th–15th	-	-	0/1	< 0.3

Table 6. Effect of mulching on overhead-irrigation-induced SE-EGFP contamination of lettuce. Counts were determined by the MPN method after 70-day cultivation.

Mulching	Outermost leaves	*Salmonella* positive plants/ Total plants	*Salmonella* counts of leaves (MPN/g)
No	1st-3rd	2/10	0.3- > 110
	4th-6th	1/10	> 110
	7th-9th	1/10	> 110
	10th-12th	1/10	> 110
	13th-15th	1/5	> 110
	16th-18th	1/4	> 110
	19th-21st	1/3	> 110
	22th-24th	1/2	> 110
Yes	1st-3rd	0/6	< 0.3
	4th-6th	1/6	4.3
	7th-9th	0/6	< 0.3
	10th-12th	0/6	< 0.3
	13th-15th	0/3	< 0.3
	16th-18th	0/1	< 0.3

soils; >500 days in frozen soil; and 47 to 96 days on rye (*Secale cereale* L.), alfalfa (*Medicago sativa*), hairy vetch (*Vicia sativa* L.), and crimson clover (*Trifolium incarnatum* L.). In an *E. coli* O157:H7 survival study in Dutch soils, the calculated detection time by the Weibull model was 54 to 105 days. Franz *et al.* (2008) revealed that *E. coli* O157:H7 populations declined more rapidly under more oligotrophic soil conditions, which can be achieved by using manure with a relatively high carbon-to-nitrogen ratio and consequently, a relatively low rate of nutrient release. The pH and fiber content of manure used in soils also affected the survival of *E. coli* O157:H7 and *Salmonella* in soil (Franz *et al.*, 2005). It seems that soil composition, temperature, and moisture content can influence pathogen survival, with nutritionally-rich soil in combination with moisture significantly increasing the soil persistence of *E. coli* O157:H7 (Vidovic *et al.*, 2007). Considering that these factors can contribute to the contamination and persistence of foodborne pathogens, measures should be taken to prevent soil contamination with Gram-negative foodborne pathogens, such as *Salmonella* and *E. coli* O157:H7, from the time of seeding to harvesting to reduce the risk of contamination of lettuce at harvest.

In Tables 4 and 5, we investigated the possibility of internalization of *Salmonella* in lettuce leaves from contaminated soils by comparing *Salmonella* counts before and after surface disinfection. *Salmonella* counts of intact (non-injured) lettuces and lettuces with artificially-injured roots are shown in Tables 4 and 5, respectively. In both intact and root-injured lettuces, *Salmonella* was detected before disinfection; however, no *Salmonella* was detected after disinfection. Sodium hypochlorite solution is a sanitizer that is widely used for disinfecting vegetables and fruits. Furthermore, the solution is not thought to penetrate the interior of lettuce leaves because of its rigid structure, e.g., the cuticular layers. Thus, it has been used to investigate the internalization of bacteria into plants (Jablasone *et al.*, 2005; Mitra *et al.*, 2009). In the present study, by using ethanol and sodium hypochlorite solution, the surfaces of leaf lettuce were disinfected. After surface disinfection, *Salmonella* was not detected in most lettuce plants except for one (Table 4). *Salmonella* was detected from a set of the 1st - 3rd outermost leaves in one out of 27 disinfected plants, one of the corresponding leaves was etiolated and the injured part of the leaf was likely contaminated with *Salmonella*. Taken together, although *Salmonella* in soils contaminates the surfaces of lettuce leaves during cultivation, it appears that *Salmonella* is not internalized by intact lettuce plants. Zhang *et al.* (2009) reported that all 512 surface-disinfected leaf lettuce samples, regardless of plant age when inoculated or analyzed, type of lettuce, strain of *E. coli* O157:H7 in the inoculum, or water or cow manure extract used as an inoculum carrier, were negative for *E. coli* O157:H7. Taken together with our results, this indicates that the internalization of Gram-negative foodborne pathogens in lettuce tissues from the contaminated soil in which plants are grown does not occur.

During cultivation, water and soils, which are splashed by overhead irrigation and rainfall, might transfer contamination from the soil to the edible parts of vegetables. To analyze such contamination routes, the effect of mulching on contamination from spiked soils was studied. As shown in Tables 3 and 4, mulching reduced *Salmonella* contamination from soils to the edible parts of leaf lettuce as expected. Mulching likely reduced the opportunity for contact between the soil and the edible parts of lettuce plants. In the USA, overhead irrigation by sprinkler systems accounts for 58% of lettuce cultivation (Solomon *et al.*, 2002). Overhead irrigation using such systems has the possibility of increasing the contamination levels of leafy vegetables with pathogens. Firstly, the weight of the irrigation water might allow contact between the lettuce leaves and the soil, leading to bacterial contact with the leaves. Secondly, the water splash containing soils and pathogenic bacteria might contact the leaves. In the present study, we employed mulch to prevent contact between contaminated soils and lettuce leaves. As shown in Table 6, overhead irrigation resulted in *Salmonella* contamination in only one lettuce plant. However, mulching certainly reduced contamination rates. The present study clearly showed that cultivation with mulch effectively prevented bacterial contamination of leafy vegetables.

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