Seamons, Colleen

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In response to the call for comments for approaches to enhance the microbiological safety of sprouted seed I have enclosed several publications of our research in this area over the years. The research has primarily focused on seed decontamination and sampling of spent irrigation water.

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Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on Mung Beans, Alfalfa, and Other Seed Types Destined for Sprout Production by Using an Oxychloro-Based Sanitizer

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ABSTRACT

The efficacy of a stabilized oxychloro-based food grade sanitizer to decontaminate seeds destined for sprout production has been evaluated. By using mung bean seeds as a model system, it was demonstrated that the sanitizer could be used to inactivate a five-strain cocktail of *Escherichia coli* O157:H7 or *Salmonella* introduced onto beans at 10^5 to 10^4 CFU/g. *Salmonella* was more tolerant to stabilized oxychloro than was *E. coli* O157:H7, with sanitizer levels of ≥150 and ≥50 ppm, respectively, being required to ensure pathogen-free sprouts. The decontamination efficacy was also found to be dependent on treatment time (≥8 h optimal) and the seed-to-sanitizer ratio (≥1:4 optimal). Stabilized oxychloro treatment did not exhibit phytotoxic effects, as germination and sprout yields were not significantly ($P \geq 0.05$) different as compared with untreated controls. Although human pathogens could be effectively eliminated from mung beans, the aerobic plate count of native microflora on sprouts grown from treated seed was not significantly ($P \geq 0.05$) different from the controls. The diversity of microbial populations (determined through 16S rRNA denaturing gradient gel electrophoresis analysis) associated with bean sprouts was not significantly affected by the sanitizer treatment. However, it was noted that *Klebsiella* and *Herbaspirillum* (both common plant endophytes) were absent in sprouts derived from decontaminated seed but were present in control sprouts. When a further range of seed types was evaluated, it was found that alfalfa, cress, flax, and soybean could be decontaminated with the stabilized oxychloro sanitizer. However, the decontamination efficacy with other seed types was less consistent. It appears that the rate of seed germination and putative activity of sanitizer sequestering system(s), in addition to other factors, may limit the efficacy of the decontamination method.

Sprouted seeds have a high nutritive value, in addition to anticholesterol and anticarcinogenic constituents *(11)*. However, sprouted seeds continue to be implicated in outbreaks of foodborne illness and hence are considered a significant food safety risk *(16)*. One of the largest outbreaks recorded occurred within Ontario, Canada, in 2005. Here over 600 reported cases of salmonellosis were traced to contaminated mung bean sprouts *(7)*. To date, the majority of cases have been linked to alfalfa and bean sprouts *(15)*, although other seed types have been identified as potential vehicles for human pathogens *(4, 5, 32)*. *Salmonella* is the most frequently isolated human pathogen on sprouts, although cases involving *Escherichia coli* O157:H7 have also been reported *(2, 30, 36, 37)*.

In the majority of foodborne illness outbreaks, the seed used for sprout production has been shown to be the most significant source of human pathogens *(2)*. Therefore, the U.S. Food and Drug Administration issued guidelines recommending that all seed destined for sprout production should be decontaminated with calcium hypochlorite treatment (20,000 ppm for at least 15 min) *(2, 14, 39)*. Although seed decontamination has improved the safety of sprouted seeds, a number of sporadic outbreaks of foodborne illness and product recalls associated with sprouts continue to occur *(16)*.

The limitation of calcium hypochlorite has led to a sustained effort to find alternative seed decontamination methods. However, the majority of interventions (chemical and physical) evaluated to date can reduce, but not eliminate, pathogens on seeds *(15, 21, 42)*. Yet, successful mung bean decontamination has been achieved by applying acetic acid vapor for 12 h at 45°C *(10)*. Mung beans held at 55°C for 4 to 7 days has also been reported to inactivate pathogens inoculated onto mung beans *(24)*. Dry heat (50°C for 1 h) in combination with irradiation (2.5 kGy) has been shown to eliminate *E. coli* O157:H7 on alfalfa, mung bean, and radish seeds, with negligible impact on germination and yield *(3)*. However, given the need to heat seeds, together with the expense of irradiation, the actual feasibility of applying the method in commercial practice is questionable.

The difficulty encountered with decontaminating seeds can be based on multiple factors. For example, human pathogens can be located within shielded sites on seed coats (crevices, damaged areas), thereby being protected from antimicrobial treatments *(9)*. Any residual human pathogens (even at levels <0.1 CFU/g) surviving the seed decontamination treatment can grow to high levels during the sub-

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TABLE 1. Escherichia coli *O157:H7 and* Salmonella *strains/serovars used in the study*

Escherichia coli O157:H7 strain	Source	*Salmonella enterica* serovar	Source
E. coli O157:H7-C1033[a]	Water sediment	Meleagridis E1[b]	Alfalfa sprouts
E. coli O157:H7-C1032[a]	Soil	Oranienburg C1[b]	Alfalfa sprouts
E. coli O157:H7-C652[a]	Clinical	Newport C2[b]	Alfalfa sprouts
E. coli O157:H7-C476[a]	Clinical	Senftenburg[b]	Alfalfa sprouts
E. coli O157:H7-C477[a]	Clinical	Montevideo[a]	Tomatoes

[a] Strains obtained from Canadian Research Institute for Food Safety, University of Guelph, Guelph, Ontario, Canada.
[b] Strains donated by Dr. C. Poppe, Health Canada, Guelph, Ontario, Canada.

sequent sprouting process *(22)*. Therefore, seed decontamination treatments applied must ensure complete inactivation of human pathogens while at the same time maintaining the viability and vigor of the seed.

In the following study, a stabilized oxychloro (SOC)-based sanitizer (commercially known as Germin-8-or) to decontaminated seeds has been evaluated. The sanitizer is essentially composed of a stabilizing agent and traces of chlorate, with chlorite constituting the primary antimicrobial agent. Chlorite is typically used within the food industry in its acidic form to generate chlorine dioxide *(19)*. In its nonacidified form, chlorite is not considered a suitable biocide because of its relative instability (typically stable in water at neutral pH for 48 h) and low antimicrobial activity over short contact times *(20, 25, 26)*. However, over extended contact times (≥6 h), chlorite has been shown to exhibit bactericidal properties without causing significant cytotoxic affects against mammalian cells or fungi *(25)*.

In the course of sprout production, it is standard practice to soak seeds for 3 to 16 h to stimulate the germination process *(2)*. Therefore, by the inclusion of SOC at the seed soak stage, it is possible to expose human pathogens to the bactericidal agent over long periods, without any adverse affects on sprout development. The objectives of the current study were to optimize and determine the efficacy of SOC treatment to inactivate human pathogens on a range of different seed types used in sprout production.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. *E. coli* O157:H7 and *Salmonella* used in the study were composed of environmental, clinical, tomato, or sprout isolates (Table 1). An aliquot (1 ml) of an overnight culture of the individual *E. coli* O157:H7 or *Salmonella* strains was transferred into 50 ml of tryptic soy broth (Difco, Becton Dickson, Sparks, Md.) and incubated for 24 h at 37°C. Bacterial cells were harvested by centrifugation (5,500 × *g* for 10 min at 4°C) and washed once in 0.8% saline. The final cell pellet was resuspended in saline to a cell density of 10^6 CFU/ml (ca. OD_{600} of 0.2). Equal volumes of the five different *E. coli* O157:H7 or *Salmonella* suspensions were then combined to produce a cocktail that was subsequently used to inoculate seeds.

Inoculation of seeds. Mung bean, alfalfa, broccoli, buckwheat, clover, chickpeas, cress, flax, mustard, onion, radish, soybean, sesame, and sunflower seeds were obtained from Mumms Seeds, Ltd. (Parkside, Saskatchewan, Canada). At least two separate batches of the different seed types were used during the course of the study.

Seeds (250 g) were soaked in 250 ml of the five-strain cocktail of *E. coli* O157:H7 or *Salmonella* for 20 min. The seeds were then transferred to sterile filter paper within a biological safety cabinet and allowed to dry at ambient temperature for 48 to 60 h. The inoculated seeds were then used immediately or stored at 4°C until required (maximum of 5 days).

Seed decontamination and sprouting. Contaminated seeds (25 g) were soaked for defined periods in 250 ml of the appropriate concentration (25 to 200 ppm) of SOC solution (Vernagene, Ltd., Bolton, Lancashire, UK) at 28°C. The seeds were then removed, rinsed with distilled water, and germinated for up to 4 days at 28°C in 1.5-liter plastic containers placed in an environmental chamber (Percival Scientific, Perry, Iowa). The sprouting seeds were watered daily with a 5-min soak in 500 ml of sterile distilled water. In parallel, control inoculated mung bean batches were soaked for 20 min in 20,000 ppm calcium hypochlorite (Fisher, Ottawa, Ontario, Canada) prepared in phosphate buffer (50 mM, pH 6). Verification of the free chlorine concentration was performed with a commercially available chlorine test kit (Fisher). The beans were rinsed with distilled water and sprouted as described above.

Microbiological analysis. Microbiological analysis was performed on both the inoculated seeds and sprouts. Duplicate samples (1 g) of inoculated seed were suspended in 9 ml of buffered peptone water (0.1%; Oxoid, Basingstoke, UK) and vortexed for 1 min. For sprouts, duplicate 25-g samples were suspended in 225 ml of buffered peptone water and stomached for 90 s at 230 rpm (model 400, A. J. Steward and Co., London, UK). Dilution series were prepared from the seed wash and sprout homogenates in saline prior to plating onto the appropriate agar medium. Aerobic plate counts were enumerated on tryptic soy agar (Oxoid) incubated at 30°C for 48 h. *Salmonella* was enumerated on either xylose lysine deoxychlorate (Oxoid) or brilliant green (Oxoid) agar incubated at 42°C for 24 h. *E. coli* O157:H7 was enumerated on sorbitol MacConkey agar containing cefixime and tellurite (Difco, Becton Dickinson) incubated at 37°C for 24 h.

Sprout samples (25 g) were tested for the presence of *Salmonella* with the method described in the Canadian Compendium of Analytical Methods, MFHPB 20 *(6)*. Sprouts were suspended in 225 ml of buffered peptone water and incubated at 42°C for 24 h. An aliquot (0.1 ml) of the enriched culture was then inoculated into the center of a semisolid Rappaport-Vassiliadis plate (Oxoid) that was subsequently incubated at 37°C for 24 h. Cells from the outer perimeter of the growth halo (presumptive motile *Salmonella*) were streaked onto brilliant green agar (Oxoid) and incubated at 37°C overnight. The Oxoid *Salmonella* Latex Test FT0203 was used for serological confirmation of typical colonies (i.e., red colonies surrounded by brilliant red zones).

Sprouts were enriched for *E. coli* O157:H7 with buffered

peptone water containing 0.5% (wt/vol) sodium thioglycolate incubated at 37°C for 24 h *(36)*. Aliquots (10 μl) of the enriched culture were then streaked onto cefixime and tellurite agar that was subsequently incubated at 37°C for 24 h. Typical colonies (colorless) were confirmed as *E. coli* O157 with the Oxoid *E. coli* O157 Latex Test DR0620M.

DNA typing of *E. coli* O157:H7 and *Salmonella*. Enterobacterial repetitive intergenic consensus (ERIC)–PCR *(40)* was used to identify which of the five strains of *E. coli* O157:H7 or serovars of *Salmonella* were present on sprouts upon completion of the sprouting process. Up to five colonies from the positive selective agar plates were picked onto Luria-Bertani agar (Difco, Becton Dickinson) that was subsequently incubated at 37°C for 24 h. Single colonies were then suspended in 0.2 ml of Tris-EDTA buffer heated at 100°C to lyse cells. Cell debris was removed by centrifugation (13,000 × *g*) and the supernatant, containing DNA, decanted into a new Eppendorf tube.

The PCR reactions were carried out with a final volume of 25 μl, containing 24 μl of master mix and 1 μl of the bacterial cell supernatant (DNA template). In all, the primers used were ERIC 1 (forward) 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC 2 (reverse) 5′-AAGTAAGTGACTGGGGTGAGCG-3′. The final PCR reaction contained 100 pM of each primer, 1 U of *Taq* DNA polymerase (New England BioLabs, Inc., Ipswich, Mass.), 0.2 mM each of the deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP; Sigma, St. Louis, Mo.), 2.0 mM $MgCl_2$ (Promega, Madison, Wis.), and Buffer III (1× concentration; Boehringer Mannheim, Ltd., Burlington, Ontario, Canada). PCR was performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, Calif.). Amplification parameters included 1 cycle at 94°C for 3 min, and 35 cycles at 94°C for 30 s, 36°C for 90 s, and 72°C for 4 min. The final cycle was 8 min at 72°C. PCR products were separated on 2% (wt/vol) agarose gels (Fisher) prepared in Tris-borate-EDTA buffer supplemented with ethidium bromide (0.5 μg/ml). All amplified DNA fragments were separated at 75 V for 2.5 h with a 100-bp DNA ladder (Sigma) acting as a molecular weight marker. After electrophoresis the gels were visualized and digital images captured with an image analyzer (Bio-Rad Laboratories, Mississauga, Ontario, Canada). DNA patterns were analyzed, and dice similarity coefficients calculated with Molecular Analyst Software, Bio-Rad Fingerprinting II, version 3.0 (Bio-Rad Laboratories, Hercules, Calif.).

DGGE. Denaturing gradient gel electrophoresis (DGGE) of native microflora was performed to determine if decontaminating seeds with SOC affected the native sprout microbiota. Noninoculated mung beans were soaked in 200 ppm SOC for 24 h at 28°C. The beans were then rinsed with distilled water and sprouted for a further 3 days with periodic watering. Sprouts samples (10 g) were collected from each batch and suspended in 90 ml of buffered peptone water (0.1%) prior to stomaching. DNA was extracted directly from the sprout homogenates with a commercial kit (DNeasy, Qiagen, Inc., Mississauga, Ontario, Canada) according to the manufacturer's instructions.

For the PCR-based DGGE analysis, a fragment of the ribosomal 16S rRNA gene containing the V2–V3 region (position 339 to 539 in *E. coli* gene) was targeted. This region was amplified from genomic DNA of the sprout microflora using primers HDA1-GC (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG** GAC TCC TAC GGG AGG CAG CAG T-3′; the GC clamp is in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) as previously described *(41)*. PCR amplification was carried out in a total volume of 50 μl containing reaction buffer (10 mM Tris-HCl, pH 8.3, containing 2.5 mM $MgCl_2$ and 50 mM KCl), 0.2 mM of each deoxynucleoside triphosphate, 20 pmol of each primer, 500 ng of bacterial DNA, and 2.5 U of *Taq* DNA polymerase. The amplification program was 94°C for 4 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 60 s; and finally, 68°C for 7 min.

Amplicons were separated by DGGE with the Bio-Rad DCode system (Bio-Rad Laboratories). The polyacrylamide gels consisted of 10% polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) and contained a 35 to 65% gradient of urea and formamide, which increased in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7 M urea. Electrophoresis was performed for 16 h at 70 V in Tris-acetate-EDTA buffer (pH 8.0) at a constant temperature of 60°C. The gels were then silver stained until sufficient band intensity had been attained.

Bands of interest were cut from the gel, and DNA was extracted using QIAquick Gel Extraction Kit (Qiagen). DNA samples were amplified using PCR and sequenced by Laboratory Services (University of Guelph, Guelph, Ontario, Canada). The obtained sequences were cross-matched with those contained within the GenBank, European Molecular Biology Laboratory and DNA Data Bank of Japan databases by using the BLAST algorithm.

SOC and protein concentration in spent soak water. SOC was measured as $NaOCl_2$ with the method described by Ingram et al. *(26)*. Seeds were soaked in 200 ppm SOC solution for 24 h as previously described. Aliquots (50 μl) of spent soak water were added to 50 μl of 2% (wt/vol) KI and 1.5 ml of 50 mM HCl, with the formation of iodine being detected spectrophotometrically at 350 nm. Residual chlorite in sprouts derived from seeds treated with SOC was also tested. Batches of sprouts (10 g) were suspended in 90 ml of distilled water and homogenized. The extract was centrifuged (8,000 × *g* for 20 min) to remove sprout debris and chlorite within the supernatant quantified by using the assay described above. Calibration curves were prepared with $NaOCl_2$ solutions ranging from 1 to 200 ppm.

The protein content of spent soak water samples (5 ml) was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

Statistical analysis. All experiments were performed at least three times with duplicate samples being tested on each occasion. Enumeration data was transformed into log values prior to analysis by analysis of variance and Tukey's test (S-Plus, Insightful Corp., New York). In all cases the significance level was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Optimization of mung bean decontamination treatment. SOC treatment did not significantly ($P > 0.05$) affect the extent of germination (>97%), sprout yield, or appearance when applied within the range of 0 to 200 ppm, compared with nontreated controls (Table 2). However, at >2,000 ppm SOC, there was a decrease in seed germination to <25%, and sprout development was stunted.

The aerobic plate counts recovered from mung beans were high but comparable to values reported by others *(33)*. *E. coli* O157:H7 was relatively sensitive to SOC with seed treatment using 100 ppm, resulting in pathogen-free sprouts (Table 3). In contrast, a higher level of 200 ppm SOC was required to inactivate *Salmonella* (Table 3). Because of the

TABLE 2. *Bean sprout yield from mung beans decontaminated with SOC sanitizer*

SOC (ppm)	Bean sprout yield (g)[a]
0	421 ± 12 A
25	444 ± 28 A
100	447 ± 8 A
125	408 ± 3 A
150	419 ± 20 A
175	421 ± 15 A
200	426 ± 23 A

[a] Mung bean batches (10 g) were soaked for 24 h at 28°C in distilled water (control) or SOC solution, and the weight of sprouts determined following a 4-day sprouting period. Means followed by the same letter are not significantly ($P > 0.05$) different.

higher tolerance of *Salmonella* to SOC, a concentration of 200 ppm was selected for subsequent studies.

Treatment of seeds with 20,000 ppm free chlorine from calcium hypochlorite did not lead to the elimination of the two pathogens from mung beans, with high levels of both *Salmonella* and *E. coli* O157:H7 being detected in sprouts grown from the treated seed (Table 3). This result is in agreement with the studies performed by Fett *(14)*, who also reported that calcium hypochlorite applied at the recommended levels was insufficient to eliminate pathogens introduced onto mung beans. Therefore, the results confirm that SOC is a more effective sanitizer for decontaminating seeds as compared with calcium hypochlorite.

Through DNA fingerprinting, *Salmonella enterica* serovar Meleagridis was the only serovar recovered from sprouts derived from mung beans treated with an ineffective concentration of SOC (150 ppm; Table 3). The dominance of Meleagridis was not associated with the inability of the other *Salmonella* to grow on sprouting seeds, as individually inoculated serovars attained comparable counts on bean sprouts at day 4 (results not shown). Howard and Hutcheson *(23)* also reported that the growth of *Salmonella* on sprouting alfalfa seed was serovar independent. However, the same workers did note that a *Salmonella* Cubana strain, originally isolated from alfalfa sprouts, had a higher growth rate as compared with isolates derived from meat or clinical sources *(23)*. The authors concluded that, although different serovars of *Salmonella* can grow on sprouting seeds, the actual growth rates vary, which ultimately affects which strain becomes dominant on alfalfa sprouts. This may be the underlying reason for the dominance of Meleagridis in bean sprouts encountered in the current study. It is also possible that Meleagridis had enhanced tolerance to SOC treatment. This is unlikely, as individual serovars introduced onto beans were equally sensitive to SOC treatment (results not shown).

Corresponding ERIC-PCR DNA fingerprint studies were performed with *E. coli* O157:H7 isolates recovered from bean sprouts derived from beans treated with 25 ppm SOC. However, the banding patterns obtained for the five strains of *E. coli* O157:H7 all exhibited close similarity, making differentiation problematic. Although ERIC-PCR has previously been applied to differentiate *E. coli* O157:H7, the technique has relatively low resolving power, compared with pulsed-field gel electrophoresis or phage typing *(18, 29)*. Therefore, in the present study it was not possible to establish which of the *E. coli* O157:H7 introduced on mung beans became dominant on the subsequent bean sprouts. However, the relative sensitivity of *E. coli* O157:H7 to SOC would suggest that no significant intrastrain resistance to the sanitizer exists.

Although *E. coli* O157:H7 and *Salmonella* were effectively inactivated on mung beans by SOC treatment at ≥50 and ≥150 ppm, respectively, the aerobic plate counts did not significantly ($P > 0.05$) differ between treated and nontreated sprout batches (Table 3). This would suggest that the endogenous microflora was unaltered by the SOC treatment. Indeed, through 16S rRNA analysis it was demonstrated that the microbial populations associated with bean sprouts derived from SOC-treated seeds (noninoculated) were similar to nontreated controls. However, it was noted that bands corresponding to *Klebsiella* and *Herbaspirillum* were missing in sprouts derived from decontaminated beans (Fig. 1). Both bacteria are common endophytes and frequently recovered from plants including sprouted seeds *(12,*

TABLE 3. *Effect of SOC concentration on the efficacy of mung bean decontamination and subsequent aerobic plate and pathogen counts on sprouted seed*[a]

Sanitizer	Concn (ppm)	Log CFU/g sprouts[b]		
		APC[c]	*Salmonella*	*E. coli* O157:H7
Control	0	9.44 ± 0.38 A	9.10 ± 0.26 A	9.12 ± 0.66 A
SOC	50	NT[d]	8.87 ± 0.43 A	9.09 ± 0.60 A
	100	9.52 ± 0.52 A	5.30 ± 0.20 B	ND[e] B
	150	9.25 ± 0.36 A	4.90 ± 0.32 B	ND B
	200	9.46 ± 0.58 A	ND C	ND B
Calcium hypochlorite	20,000	9.45 ± 0.33 A	7.96 ± 0.86 A	8.59 ± 0.40 A

[a] Initial loading on mung beans was 10^3 to 10^4 CFU/g in all cases.
[b] Means within columns followed by the same letter are not significantly ($P > 0.05$) different.
[c] APC, aerobic plate count. Values are from sprouts derived from treated beans inoculated with *E. coli* O157:H7.
[d] NT, not tested.
[e] ND, not detected (<1 CFU/25 g).

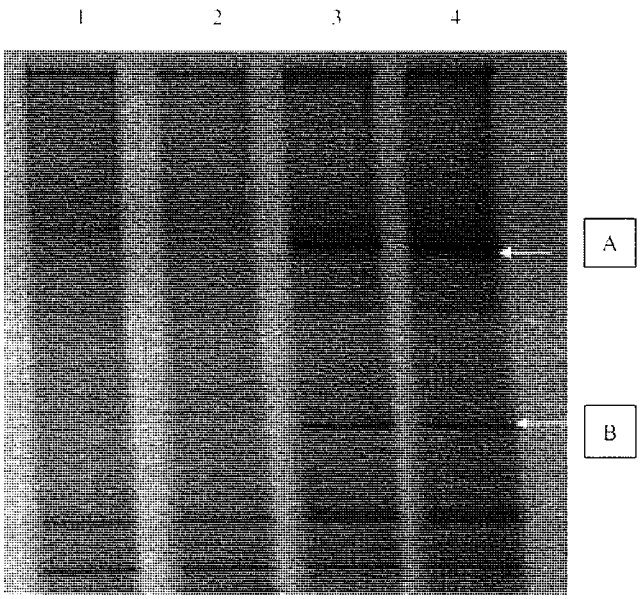

FIGURE 1. *DGGE analysis showing profiles of 16S rRNA amplified from the microbiota of bean sprouts derived from SOC-treated (lanes 1 and 2) and nontreated (lanes 3 and 4) mung beans. Arrowheads correspond to* Klebsiella *(a) and* Herbaspirillum *(b).*

13, 34), so their presence was not unexpected. However, it is unclear why the other microbial populations associated with bean sprouts were apparently insensitive to SOC. It could be that a proportion of the populations was present deep within the seed or alternatively, the bacterial populations represent posttreatment contamination derived from the environment and/or irrigation water. From the limited studies on the microbial ecology of sprouts, it is believed that seedborne contamination is of greater significance than the sprouting environment *(28)*. However, this may not apply to decontaminated seed, where it can be assumed the endogenous microflora would have been reduced thereby enabling environmental microbiota to become established on the developing sprouts.

It has been previously reported that human pathogens can potentially undergo recovery after sublethal exposure to sanitizers *(24)*. In the present study, sprouts testing negative at 48 h did not yield a positive reaction for either *E. coli* O157:H7 or *Salmonella* when additional samples were taken 96 h into the sprouting process. Therefore, no evidence of posttreatment recovery of either pathogen was observed. In this respect, SOC treatment is compatible with the current recommendation to screen for human pathogens in spent irrigation water 48 h into the sprouting process *(2)*.

Through further optimization studies, it was found that the minimum mung bean-to-SOC ratio was 1:4 (wt/vol) to achieve consistent inactivation of *E. coli* O157:H7 or *Salmonella* (Table 4). Currently, there are no specific recommendations on the seed-to-sanitizer ratio that should be applied during seed decontamination *(2, 38)*. However, the results obtained in this study clearly illustrate that for SOC at least, a defined seed-to-sanitizer ratio should be employed.

The contact time required to ensure elimination of human pathogens on seeds was between 8 and 19 h (Table 5). The reasons for the extended soaking time to ensure the effective decontamination of mung beans are unclear. However, it can be assumed that the sanitizer absorption rates vary among seeds within the same batch. Therefore, a long contact time is required to ensure that all pathogens are released from protective sites on the seed coat and subsequently inactivated. This is especially relevant considering that even low levels of surviving human pathogens can grow to high levels during the sprouting process *(17, 23)*.

TABLE 4. *Effect of mung bean to SOC sanitizer ratio on the efficacy of seed decontamination to eliminate* Escherichia coli *O157:H7 or* Salmonella

Bean-to-sanitizer ratio (wt/vol)	Treatment	Sprouts positive by enrichment (positive/no. tested)[a]	
		E. coli O157:H7	*Salmonella*
1:1	Nontreated[b]	4/4	4/4
	Treated[c]	4/4	4/4
1:2	Nontreated	4/4	4/4
	Treated	4/4	4/4
1:4	Nontreated	4/4	4/4
	Treated	0/4	0/4
1:6	Nontreated	4/4	4/4
	Treated	0/4	0/4
1:8	Nontreated	4/4	4/4
	Treated	0/4	0/4
1:10	Nontreated	4/4	4/4
	Treated	0/4	0/4

[a] Inoculated mung beans (25 g) were soaked in SOC solutions at different seed-to-sanitizer ratios. After 24 h the seeds were removed and sprouted for a further 48 h at 28°C. The sprouts were then enriched for either *Escherichia coli* O157:H7 or *Salmonella*.

[b] Nontreated: inoculated mung beans soaked in distilled water for 24 h.

[c] Treated: inoculated mung beans soaked in 200 ppm SOC sanitizer for 24 h.

Decontamination of different seed types. The efficacy of the optimized SOC treatment to decontaminate a broad range of seed types was evaluated. It was noted that

TABLE 5. *Effect of SOC sanitizer (200 ppm) contact time on mung bean decontamination efficacy (n = 4)*

Contact time (h)	No. of positive sprouts by enrichment/no. tested[a]	
	E. coli O157:H7	*Salmonella*
4	4/4	4/4
8	4/4	4/4
19	0/4	0/4
24	0/4	0/4

[a] Inoculated mung beans (25 g) were soaked in SOC solutions for different periods. The seeds were removed and sprouted for a further 48 h at 28°C prior to enrichment for either *Escherichia coli* O157:H7 or *Salmonella*.

TABLE 6. *Decontamination of a range of seed types inoculated with* Salmonella *or* Escherichia coli *O157:H7 with SOC sanitizer*

Seed type	Treatment	No. of sprouts positive by enrichment/no. tested	
		E. coli O157:H7	*Salmonella*
Soybeans	Nontreated[a]	4/4	4/4
	Treated (400 ppm)[b]	0/4	0/4
Alfalfa	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	0/4
Cress	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	0/4
Flax	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	0/4
Clover	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	2/4
Mustard	Nontreated	4/4	4/4
	Treated (400 ppm)	2/4	1/4
Radish	Nontreated	3/4	3/4
	Treated (200 ppm)	2/4	3/4
Chick pea	Nontreated	4/4	4/4
	Treated (200 ppm)	4/4	4/4
Broccoli	Nontreated	4/4	4/4
	Treated (200 ppm)	4/4	4/4
Sunflower (dehulled)	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Sunflower (with hulls)	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Buckwheat	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Sesame	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Onion	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3

[a] Nontreated: inoculated seeds soaked in distilled water for 24 h.
[b] Treated: inoculated seeds soaked in 200 or 400 ppm SOC sanitizer for 24 h.

with all the seed types tested, both *Salmonella* and *E. coli* O157:H7 became established on the subsequent sprouts (Table 6). This would suggest that although the majority of foodborne illness outbreaks are associated with alfalfa and mung bean, most, if not all, sprouted seed could potential act as vehicles for human pathogens.

Alfalfa, soybean, flax, and cress seed inoculated with either a five-strain or five-serovar cocktail of *E. coli* O157:H7 or *Salmonella* could be consistently decontaminated (Table 6). However, with clover, mustard, and radish, variable results were obtained. Although increasing the SOC concentration to 400 ppm enhanced the decontaminating efficacy for mustard without decreasing sprout yield, this was found to significantly decrease the yield with clover, radish, broccoli, and chickpea. SOC treatment of other seed types was unsuccessful even when applied at 400 ppm over longer (48 h) contact times (results not shown).

The effect of seed type on the efficacy of SOC treatment was unexpected. However, it was noted that those seeds that were not successfully decontaminated with the sanitizer germinated at a slower rate as compared with mung beans or alfalfa. Therefore, it is possible that the protective sites within the seed shielded human pathogens from the antimicrobial action of the sanitizer. An additional possibility is that the SOC acted synergistically with exudates released by the seed to inactivate pathogens. Indeed, the extracts from several seed types have found to be potent antimicrobial agents (*1, 8, 31*). Therefore, it is possible the antimicrobials released by seeds such as mung beans were higher as compared with those seeds that could not be decontaminated. This hypothesis was tested by treating a mixture of mung beans and radish seed with SOC prior to sprouting (Table 7). However, even a low proportion of radish seed added to a batch of mung beans was sufficient to reduce the efficacy of the SOC decontamination treatment, with *Salmonella* being recovered from the subsequent sprouts. This would suggest that SOC was being sequestered by germinating radish seeds as opposed to an antimicrobial being released by mung beans. It has been reported that chlorite can be readily sequestered by protein (*27*). However, when the protein content in spent soak solutions was assayed, content derived from mung bean (132 μg/ml) was significantly ($P < 0.05$) higher when compared with either radish (57 μg/ml) or soybean (37 μg/ml). Therefore, protein content cannot explain the low efficacy of SOC to decontaminate the different seed types.

TABLE 7. *Efficacy of SOC sanitizer in decontaminating* Salmonella-*inoculated radish seed and mung bean mixtures*

Ratio of mung beans to radish seeds[a]	No. of sprouts positive by enrichment/no. tested
100% mung bean	0/4
1:1	4/4
2:1	4/4
1:2	4/4
100% radish	4/4

[a] Mung beans were mixed with varying quantities of radish seeds and soaked in SOC (200 ppm) for 24 h at 28°C. The seed mixtures were then sprouted for 48 h with daily watering, and sprouts were screened for the presence of *Salmonella*.

When the residual SOC present in the spent soak solution was determined, it was noteworthy that the levels in samples taken from mung beans or soybean (72 to 74 ppm) soak water were significantly ($P < 0.05$) higher as compared with radish (32 ppm) or broccoli (28 ppm). This may indicate that with the latter seed types, the sanitizer concentration was reduced to below biocidal levels during the treatment period. However, it was noted that the residual level of spent soak water from alfalfa seeds after the 24 h treatment period was only 7 ppm despite the subsequent sprouts testing negative for *Salmonella*. This apparent discrepancy can be explained by the relatively rapid inactivation of human pathogens on alfalfa. Here the inactivation of *Salmonella* on alfalfa could be achieved in <8 h, compared with 8 to 19 h found with mung beans (results not shown). Therefore, it can be proposed that although the levels of active SOC decrease during the course of alfalfa

seed soaking, sufficient sanitizer is present at the critical part of the germinating stage to inactivate human pathogens.

As with all chemical-based sanitizers, there is a risk of chemical residues being associated with the sprouts at the end of the sprouting process. However, in the present case all sprout samples tested did not contain residual SOC (<1 ppm chlorite). This can be attributed to the endogenous SOC-sequestering systems associated with the sprouts and the multiple washing (irrigation) steps applied during sprouting process.

In conclusion, a seed decontamination treatment based on supplementing soak solutions with SOC has been evaluated. Although SOC was ineffective in decontaminating all seed types, several of major commercial importance (e.g., mung bean, alfalfa, and soybean) could be successfully treated. Therefore, SOC represents an alternative to the currently recommended calcium hypochlorite treatment. Future work will evaluate the efficacy of SOC to decontaminate naturally contaminated seed, in addition to evaluating sanitizer performance on a commercial scale.

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REFERENCES

1. Ahn, J., I. U. Grun, and A. Mustapha. 2004. Antimicrobial and antioxidant activities of natural extracts in vitro and in ground beef. *J. Food Prot.* 67:148–155.
2. Anonymous. 1999. Microbiological safety evaluations and recommendations on sprouted seeds. National Advisory Committee on Microbiological Criteria for Foods. *Int. J. Food Microbiol.* 52:123–153.
3. Bari, M. L., E. Nazuka, Y. Sabina, S. Todoriki, and K. Isshiki. 2003. Chemical and irradiation treatments for killing *Escherichia coli* O157:H7 on alfalfa, radish, and mung bean seeds. *J. Food Prot.* 66:767–774.
4. Brocknann, S. O., I. Piechotowski, and P. Kimmig. 2004. *Salmonella* in sesame seed products. *J. Food Prot.* 67:178–180.
5. Brooks, J. T., S. Y. Rowe, P. Shillam, D. M. Heltzel, S. B. Hunter, L. Slutsker, R. M. Hoekstra, and S. P. Luby. 2001. *Salmonella* Typhimurium infections transmitted by chlorine-pretreated clover sprout seeds. *Am. J. Epidemiol.* 154:1020–1028.
6. Canadian Compendium of Analytical Methods. 2003. Health Canada, Ottawa. Available at: http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/e_index.html. Accessed 9 March 2006.
7. Canadian Food Inspection Agency. 2005. Health hazard alert: mung bean sprouts manufactured by Toronto Sun Wah Trading may contain *Salmonella* bacteria. Available at http://www.inspection.gc.ca/english/corpaffr/recarapp/2005/20051224e.shtml. Accessed 7 March 2006.
8. Chandrasekaran, M., and V. Venkatesalu. 2004. Antibacterial and antifungal activity of *Syzygium jambolanum* seeds. *J. Ethnopharmacol.* 91:105–108.
9. Charkowski, A. O., C. Z. Sarreal, and R. E. Mandrell. 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. *J. Food Prot.* 64:1292–1298.
10. Defaquis, P. J., P. L. Sholberg, and K. Stanich. 1999. Disinfection of mung bean seed with gaseous acetic acid. *J. Food Prot.* 62:953–957.
11. Donaldson, M. S. 2004. Nutrition and cancer: a review of the evidence for an anti-cancer diet. *Nutr. J.* 3:19.
12. Dong, Y., A. L. Iniguez, B. M. Ahmer, and E. W. Triplett. 2003. Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa* and *Medicago truncatula*. *Appl. Environ. Microbiol.* 69:1783–1790.
13. Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui, and K. Minamisawa. 2001. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. *Appl. Environ. Microbiol.* 67:5285–5293.
14. Fett, W. F. 2002. Reduction of *Escherichia coli* O157:H7 and *Salmonella* spp. on laboratory-inoculated mung bean seed by chlorine treatment. *J. Food Prot.* 65:848–852.
15. Fett, W. F. 2005. Interventions to ensure the microbiological safety of sprouts, p. 187–210. *In* Sapers, G. M., J.R. Gorny, and A.E. Yousef (ed.), Microbiology of fruit and vegetables. CRC Press, Boca Raton, Fla.
16. Fett, W. F., M. Tortorello, and T. Fu. 2005. Seed sprouts: the state of microbiological safety, p. 167–219. *In* Matthews, K.R. (ed.), Microbiology of fresh produce. ASM Press, Washington, D.C.
17. Fu, T., D. Stewart, K. Reineke, J. Ulaszek, J. Schlosser, and M. Tortorello. 2001. Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J. Food Prot.* 64:802–806.
18. Giammanco, G. M., S. Pignato, F. Grimont, P. A. Grimont, A. Caprioli, S. Morabito, and G. Giammanco. 2002. Characterization of Shiga toxin-producing *Escherichia coli* O157:H7 isolated in Italy and in France. *J. Clin. Microbiol.* 40:4619–4624.
19. Gill, C. O., and M. Badoni. 2004. Effects of peroxyacetic acid, acidified sodium chlorite or lactic acid solutions on the microflora of chilled beef carcasses. *Int. J. Food Microbiol.* 91:43–50.
20. Hautman, D. P., and M. Bolyard. 1992. Analysis of oxyhalide disinfection by-products and other anions of interest in drinking water by ion chromatography. *J. Chromatogr.* 602:65–74.
21. Himathongkham, S., S. Nuanualsuwan, H. Riemann, and D. O. Cliver. 2001. Reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in artificially contaminated alfalfa seeds and mung beans by fumigation with ammonia. *J. Food Prot.* 64:1817–1819.
22. Holliday, S. L., A. J. Scouten, and L. R. Beuchat. 2001. Efficacy of chemical treatments in eliminating *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. *J. Food Prot.* 64:1489–1495.
23. Howard, M. B., and S. W. Hutcheson. 2003. Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Appl. Environ. Microbiol.* 69:548–553.
24. Hu, H., J. J. Churey, and R. W. Worobo. 2004. Heat treatments to enhance the safety of mung bean seeds. *J. Food Prot.* 67:1257–1260.
25. Ingram, P. R., N. Z. Homer, R. A. Smith, A. R. Pitt, C. G. Wilson, O. Olejnik, and C. M. Spickett. 2003. The interaction of sodium chlorite with phospholipids and glutathione: a comparison of effects in vitro, in mammalian and in microbial cells. *Arch. Biochem. Biophys.* 410:121–133.
26. Ingram, P. R., A. R. Pitt, C. G. Wilson, O. Olejnik, and C. M. Spickett. 2004. A comparison of the effects of ocular preservatives on mammalian and microbial ATP and glutathione levels. *Free Radic. Res.* 38:739–750.
27. Kemp, G. K., and K. R. Schneider. 2000. Validation of thiosulfate for neutralization of acidified sodium chlorite in microbiological testing. *Poult. Sci.* 79:1857–1860.
28. Matos, A., J. L. Garland, and W. F. Fett. 2002. Composition and physiological profiling of sprout-associated microbial communities. *J. Food Prot.* 65:1903–1908.
29. Milch, H., J. Paszti, I. Gado, K. Glatz, and M. Jakab. 2003. Comparison of traditional and molecular typing methods of *Escherichia coli* O157. *Acta Microbiol. Immunol. Hung.* 50:349–367.
30. Mohle-Boetani, J. C., J. A. Farrar, S. B. Werner, D. Minassian, R. Bryant, S. Abbott, L. Slutsker, D. J. Vugia, and Investigation Team. 2001. *Escherichia coli* O157 and *Salmonella* infections associated with sprouts in California, 1996–1998. *Ann. Intern. Med.* 135:239–247.
31. Rajkowski, K. T. 2004. Simplified qualitative method for canavanine in seeds and sprouts. *J. Food Prot.* 67:212–214.
32. Rajkowski, K. T., G. Boyd, and D. W. Thayer. 2003. Irradiation D-values for *Escherichia coli* O157:H7 and *Salmonella* sp. on inoculated

broccoli seeds and effects of irradiation on broccoli sprout keeping quality and seed viability. *J. Food Prot.* 66:760–766.
33. Rasch, M., J. B. Andersen, K. F. Nielsen, L. R. Flodgaard, H. Christensen, M. Givskov, and L. Gram. 2005. Involvement of bacterial quorum-sensing signals in spoilage of bean sprouts. *Appl. Environ. Microbiol.* 71:3321–3330.
34. Robertson, L. J., G. S. Johannessen, B. K. Gjerde, and S. Loncarevic. 2002. Microbiological analysis of seed sprouts in Norway. *Int. J. Food Microbiol.* 75:119–126.
35. Sata, S., T. Fujisawa, R. Osawa, A. Iguchi, S. Yamai, and T. Shimada. 2003. An improved enrichment broth for isolation of *Escherichia coli* O157, with specific reference to starved cells, from radish sprouts. *Appl. Environ. Microbiol.* 69:1858–1860.
36. Stewart, D. S., K. F. Reineke, J. M. Ulaszek, and M. L. Tortorello. 2001. Growth of *Salmonella* during sprouting of alfalfa seeds associated with salmonellosis outbreaks. *J. Food Prot.* 64:618–622.
37. Taormina, P. J., L. R. Beuchat, and L. Slutsker. 1999. Infections associated with eating seed sprouts: an international concern. *Emerg. Infect. Dis.* 5:626–634.
38. Thomas, J. L., M. S. Palumbo, J. A. Farrar, T. B. Farver, and D. O. Cliver. 2003. Industry practices and compliance with U.S. Food and Drug Administration guidelines among California sprout firms. *J. Food Prot.* 66:1253–1259.
39. U.S. Food and Drug Administration and the California Department of Health Services, Food and Drug Branch. 2000. Safer processing of sprouts. Information available at: http://www.cfsan.fda.gov/~dms/sprouvid.html. Accessed 9 March 2006.
40. Versalovic, J., T. Koeuth, and J. R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* 19:6823–6831.
41. Walter, J., G. W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D. M. Loach, K. Munro, and T. Alatossava. 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.* 66:297–303.
42. Weissinger, W. R., and L. R. Beuchat. 2000. Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. *J. Food Prot.* 63:1475–1482.

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ORIGINAL ARTICLE

Mode of *Salmonella* and *Escherichia coli* O157:H7 inactivation by a stabilized oxychloro-based sanitizer

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bacterial inactivation, chlorite, decontamination, *Escherichia coli* O157:H7, *Salmonella*, sprouted seed.

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Abstract

Aim: To determine the mechanisms by which a stabilized oxychloro (SOC)-based sanitizer, applied to decontaminate seeds destined for sprout production, inactivates *Escherichia coli* O157:H7 ph1 and *Salmonella* serotype Meleagridis.

Materials and Results: The action of SOC on the metabolism, membrane and DNA integrity of *Salmonella* and *E. coli* O157:H7 was studied. In both pathogens, there was an oxidative burst and depletion of intracellular glutathione (GSH) upon initial exposure to 200 ppm SOC. Metabolic activity, measured via bioluminescence, decreased over a 4-h period in *E. coli* O157:H7 ph1 cells exposed to SOC. Membrane integrity, assessed through viability staining, decreased progressively over 23 h when exposed to SOC. The appearance of auxotrophic mutants suggested that DNA damage had also occurred. Enzymes rich in disulfide bonds (alkaline phosphatase and protease) were sensitive to the chlorite-based sanitizer. Through challenging other microbial types, it was found that Gram positive had higher tolerance to SOC than Gram negatives with the exception of *Salmonella*. MS2 bacteriophage was highly sensitive; however, *Bacillus* endospores were not inactivated by SOC.

Conclusions: SOC inactivates *E. coli* O157:H7 and *Salmonella* through GSH oxidation and disruption of disulfide bonds. Ultimately, membrane damage resulting from prolonged exposure to SOC leads to the loss of cell viability.

Significance and Impact of the Study: The results provide a basis for understanding why extended treatment times are required to inactivate bacteria using SOC.

Introduction

A stabilized oxychloro (SOC)-based sanitizer has previously been shown to decontaminate seeds destined for sprout production (Kumar *et al.* 2006). The treatment is based on introducing SOC into the water used to soak seeds prior to the sprouting process. In validation studies, it was demonstrated that by applying 200 ppm SOC, it was possible to achieve consistent inactivation of *Salmonella* and *Escherichia coli* O157:H7 on seeds such as mung beans, without adversely affecting sprout development or yield. However, to achieve pathogen inactivation, it was necessary to soak the seeds in SOC for 8–19 h (Kumar *et al.* 2006). The underlying reasons for the relatively long exposure times are unclear as is the mode by which SOC inactivates pathogens.

SOC is a sanitizer principally composed of stabilized sodium chlorite with traces of chlorate. Within the food industry, it is a common practice to apply chlorite in an acidified form or as a precursor for chlorine dioxide (Gomez-Lopez *et al.* 2005; Inatsu *et al.* 2005). Acidified sodium chlorite or chlorine dioxide inactivates microbes through membrane disruption (via peroxidation) in combination with inhibition of protein and nucleic acid synthesis (Scatina *et al.* 1985). In the nonacidified form, chlorite has relatively low oxidizing power, and hence the mode of inactivation of cells is likely to differ. The most substantial studies performed to date with respect

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to elucidating the mode of inactivation elicited by non-acidified chlorite have been reported by Ingram *et al.* (2003,2004). Here, the workers were specifically interested in the effect of chlorite on lipid membranes and glutathione (GSH) levels in bacterial or mammalian cells. The main conclusion from the studies was that chlorite can directly oxidize GSH (stoichiometry of four GSH to one chlorite) to GSSH with H_2O and NaCl as side products of the reaction. The accumulated GSSH was then released from bacterial cells, thereby increasing the susceptibility of the cell to oxidative stress. No direct chemical modification, such as peroxidation, of membrane lipids was observed. The studies by Ingram *et al.* (2004) also noted the insensitivity of eukaryotic (mammalian and fungi) cells to chlorite.

The mode by which chlorite inactivates bacteria appears different from that of hypochlorite and hydrogen peroxide. The latter is considered to act as a nondiscriminating oxidizing agent that attacks DNA, proteins and lipids, although the membranes of bacteria remain intact (Luppens *et al.* 2003). Hypochlorite (chlorous acid), in addition to direct interaction with lipids, also oxidizes GSH. Unlike chlorite, oxidation of GSH via hypochlorite is slower and goes beyond the GSSH stage resulting in the formation of sulfonamides (Fu *et al.* 2002). In addition, the action of hypochlorite causes generation of toxic chloroamines, direct damage to lipids and disruption of nucleic acids (Hawkins and Davies 2002; Hawkins *et al.* 2002).

The aim of the following study was to further elucidate the mode by which nonacidified chlorite inactivates *E. coli* O157:H7 and *Salmonella*. Specifically, the effect of sodium chlorite on the intracellular GSH levels, activity of metabolic pathways, DNA and membrane integrity of the enteric pathogens were investigated.

Methods and materials

Microbial types and cultivation conditions

The different bacterial and bacteriophage types used in the study are listed in Table 1. The majority of bacteria was cultivated overnight within tryptic soy broth (TSB) or on tryptic soy agar (TSA) at either 30 or 37°C. *Lactococcus lactis* ssp. *lactis* was cultivated at 30°C in M17 media supplemented with 2% lactose. Stationary phase cultures were harvested by centrifugation (4000 *g* for 10 min at 4°C) and washed once in 0·8% w/v saline. The cell pellet was finally resuspended in saline to give a cell density of 10^8 CFU ml^{-1}. *Bacillus subtilis* endospore crops were prepared on 2× SG medium incubated for 14 days at 30°C. The spores were harvested and washed four times in distilled water as described by Nicholson and Setlow (1990). On the final centrifugation step (5500 *g*, 10 min at 4°C), the spore pellet was resuspended in sterile distilled water (SDW) to give a final density of 10^8 CFU ml^{-1}. MS2 bacteriophage was prepared using the double agar overlay method with *Salmonella* serotype Typhimurium WG 49 acting as host cell (Schaper and Jofre 2000). Plates were incubated for 24 h at 37°C and MS2 phage recovered in SM buffer. The bacteriophage suspension was then centrifuged (5500 *g* for 10 min at 4°C) and supernatant passed through a microporous filter (0·45 μm; Fisher Scientific, Ottawa, ON, Canada). MS2 numbers were determined using the single agar method as described in Method 1602 USEPA (United States Environmental Protection Agency) (2001).

Table 1 Bacteria and bacteriophage used in the study

Bacteria/Bacteriophage	Source
Aeromonas hydrophilia	NCTC 8049
Bacillus subtilis PS 346	Fairhead *et al.* (1993)
Escherichia coli O157:H7 ph1 *lux CDABE (Ph.1)*, Amp^R	Hora *et al.* (2005)
Escherichia coli O157:H7 C9490 *uspA::gfpuv*, Amp^R	Jablasone *et al.* (2005)
Escherichia coli P36 *gfp/luxCDABE*, Km^R	Warriner *et al.* (2003)
Lactococcus lactis ssp. *Lactis*	ATCC 19435
Listeria monocytogenes	NCTC 7973
Pseudomonas fluorescens P35 *gfp/luxCDABE*, Km^R	Warriner (unpublished data)
Salmonella serotype Meleagridis	E1*
Staphylococcus aureus	NCTC 8325
MS2 F+ coliphage	ATCC 15597-B1

NCTC, National collection of type cultures; ATTC, American type culture collection.
*Isolate from alfalfa sprouts donated by Dr C. Poppe, Health Canada, Guelph, ON, Canada.

Inactivation of bacteria, spores and bacteriophage by stabilized oxychloro sanitizer

SOC (commercial name Germin-8-or; Vernagene, Bolton, Lancashire, UK) was supplied as a 20 000 ppm concentrate and diluted in sterile saline as required. The final pH of 200 ppm SOC in saline was 7·2. The test bacteria, spore or bacteriophage stock suspensions were diluted using saline to give a final density of *c.* 10^7 CFU or PFU ml^{-1}. The suspensions were pre-equilibrated at 30°C for 10 min prior to the addition of SOC (25–200 ppm) and maintained at the same temperature for the duration of the 24-h treatment period. Samples (0·1 ml) were withdrawn after 24 h and transferred to 9·9 ml saline containing 0·1% w/v sodium thiosulfate to neutralize the SOC sanitizer (Kemp and Schneider 2000). A dilution series was prepared in

saline and survivors enumerated on TSA or M17 lactose agar in the case of *L. lactis*. Results were expressed as the log reduction in cell/phage numbers compared with suspensions containing no SOC.

The inactivation of *E. coli* O157:H7 ph1 and *Salmonella* serotype Meleagridis by SOC were also determined in 0·1% peptone or mung bean exudate. The latter was prepared by steeping 50 g of mung beans in 50 ml of SDW at 30°C for 24 h. The spent steep solution (exudates) was passed through a Whatman no. 2 filter (Fisher Scientific) to remove bean debris. The filtrate was sterilized by passing through a 0·45 μm filter membrane (Fisher Scientific) and stored at −20°C until required.

SOC was measured as $NaOCl_2$ using the method described by Ingram *et al.* (2003). Aliquots (1 ml) of bacterial suspension were withdrawn and cells were pelleted by centrifugation (5500 *g* for 10 min). The supernatant was transferred to a fresh tube and 50 μl aliquots mixed with an equal volume of 2% w/v KI prior to adding 1·5 ml of 50 mmol l^{-1} HCl. The formation of iodine was detected spectrophotometrically at 350 nm.

Glutathione measurement

Suspensions (20 ml) of *E. coli* O157:H7 ph1 and *Salmonella* (10^6 CFU ml^{-1}) were prepared in saline as described previously. At $t = 0$, SOC (200 ppm) was added to the suspension and aliquots (1 ml) were removed periodically at 0, 4, 16 and 24 h for GSH analysis. Here, the cell samples were harvested by centrifugation (13 000 *g* for 10 min at 4°C) and washed once in phosphate-buffered saline (PBS). The pellet was resuspended in 80 μl of 10 mmol l^{-1} HCl and cells lysed by two freeze/thaw cycles. Lysed cells were then resuspended in 20 μl of 5% w/v 5-sulfosalicylic acid and centrifuged at 8000 *g* for 10 min. The supernatant was then transferred to a fresh tube and GSH assay using quantification kit in accordance with the manufacturer's instructions (Dojindo Molecular Technologies, Inc., MD, USA). A standard curve was prepared using GSH in the range of 5–100 μmol l^{-1}.

Enzyme assays

Spectrophotometric assays were performed in 1 ml quartz cuvettes with absorbance measurements being recorded using a Beckman spectrophotometer (Beckman Coulter, Mississauga, ON, Canada). All enzymes were purchased from commercial sources (Sigma Chemical Co., MO, USA). The appropriate enzyme was diluted in PBS to give a final concentration of 40 U ml^{-1} unless otherwise stated. SOC was added to a final concentration of 200 ppm and tubes were incubated at room temperature for 20 min. The samples were then withdrawn and residual activity was compared with that of controls containing no SOC.

Catalase (EC 1.11.1.6 from *Aspergillus niger*) activity was determined at 37°C by adding 1–5 μl of sample into phosphate buffer (50 mmol l^{-1}, pH 7.2) containing 10 mmol l^{-1} hydrogen peroxide. The decomposition of hydrogen peroxide was followed by monitoring the absorbance at 240 nm (Aebi 1984). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49 from *Leuconostoc mesenteroides*) measurement was performed using the method described by DeMoss *et al.* (1953). The assay mixture (final volume 1 ml) consisted of 100 mmol l^{-1} Tris–HCl (pH 7.6), 0·5 mmol l^{-1} $NADP^+$, 5 mmol l^{-1} $MgCl_2{\cdot}6H_2O$ and sample (1–5 μl). The reaction was initiated by the addition of 20 μmol l^{-1} glucose-6-phosphate (monosodium salt) and the rate of increase in absorbance at 340 nm recorded. NAD-dependent L-lactic dehydrogenase (EC 1.1.1.27) was measured by the method described by Melville *et al.* (1988). The assay mixture (final volume 1 ml) consisted of 70 mmol l^{-1} potassium phosphate buffer (pH 6.2), 0·2 mmol l^{-1} NADH and sample (1–5 μl). The reaction was started by the addition of sodium pyruvate (5 mmol l^{-1}) and the decrease in A_{340nm} recorded.

Superoxide dismutase (SOD; EC 1.15.1.1 from *E. coli*) activity was measured using SOD assay kit in accordance to the manufacturer's instructions (Oxford Biomedical Research, Oxford, MI, USA). Alkaline phosphatase (EC 3.1.3.1 from *E. coli*) was measured in 2·8 ml phosphate buffer (50 mmol l^{-1}; pH 10·25) containing $MgCl_2{\cdot}6H_2O$ (20 mmol l^{-1}), 0·1 ml of 0·4 μmol l^{-1} *p*-nitrophenyl phosphate. The reaction was started by the addition of 0·1 ml of sample and the A_{405nm} measured after 20 min incubation at 37°C (Garen and Levinthal 1960).

Protease (EC 3.4.23.18 from *Aspergillus saitoi*), 2 U ml^{-1}, was prepared in 10 mmol l^{-1} sodium acetate buffer with 5 mmol l^{-1} calcium acetate (pH 7·5). Aliquots (1 ml) of SOC-treated enzyme sample was mixed with 5 ml of casein solution (0·65% w/v prepared in 50 mmol l^{-1} potassium phosphate buffer, pH 7·5) and incubated at 37°C for 10 min. Reaction was stopped by adding 100 mmol l^{-1} trichloroacetic acid reagent and samples were incubated for a further 30 min at 37°C. This solution was then passed through a Whatman no. 50 filter (Fisher Scientific) and the protein content of the filtrate determined using Folin & Ciocalteu (F-C) phenol reagent. Briefly, 2 ml of filtrate was added to 5 ml of 500 mmol l^{-1} sodium carbonate solution and 1 ml of 25% v/v F-C phenol reagent prior to incubating at 37°C for 30 min. The reaction mixture was filtered through Whatman no. 50 filter prior to determining the A_{660nm} (Anson 1938).

Bioluminescence and green fluorescence protein

Aliquots (180 μl) of filter-sterilized mung bean exudates were transferred to the wells of a microtitre plate. Each well was inoculated with 20 μl of an overnight culture of *E. coli* O157:H7 ph1 (*lux CDABE*, Amp^R) prepared in TSB supplemented with 200 μg ml^{-1} ampicillin. SOC (200 ppm) was introduced to half of the microtitre plate wells with the remaining acting as nontreated controls. The plate was then transferred to a Victor microtitre plate reader (Perkin Elmer, Wellesley, MA, USA), maintained at 37°C, with the bioluminescence and OD_{450nm} being recorded every 30 min for 23 h.

The effect of SOC on the stability of green fluorescent protein (GFP) was assessed using *E. coli* O157:H7 C9490 (*uspA::gfpuv*, Amp^R). Here, suspensions (10^6 CFU ml^{-1}) of the *E. coli* O157:H7 strain were prepared in saline and 180 μl was transferred to the wells of a microtitre plate. SOC (200 ppm final concentration) was added to ten of the wells with the remaining wells acting as controls. The plate was placed in a microtitre plate reader programmed to record the A_{600nm} and A_{380nm} at 30-min intervals for 23 h.

Oxidative burst

Suspensions (10^7 CFU ml^{-1}) of either *Salmonella* or *E. coli* O157:H7 ph1 were prepared in 0.8% saline containing 100 μmol l^{-1} lucigenin (Sigma-Aldrich). The suspensions were pre-equilibrated at 37°C prior to adding 200 ppm SOC. The increase in chemiluminescence was recorded every 30 s for 5 min using a Turner Luminometer (Turner BioSystems, Sunnyvale, CA, USA).

LIVE/DEAD® BacLight™ viability staining

LIVE/DEAD viability stains were obtained from Molecular Probes, Inc. (Invitrogen, Burlington, ON, Canada). Suspensions of *E. coli* O157:H7 ph1 or *Salm.* Meleagridis (10^6 CFU ml^{-1}) were prepared in 0·8% saline containing 200 ppm SOC. Aliquots (0·1 ml) were transferred periodically (t = 0, 4, 16 and 24 h) and mixed with 5 μl BacLight stain. After 10 min incubation in the dark at room temperature, 3 μl of the suspension was transferred to a glass slide and viewed under an epifluorescent microscope. Live cells appeared green while those with low or no viability stained orange/red.

Auxotrophic mutants

Escherichia coli O157:H7 ph1 and *Salm.* Meleagridis suspensions (10^6 CFU ml^{-1}) in saline were treated with sublethal concentrations of SOC. Samples (0.1 ml) were transferred periodically to 9·9 ml saline containing sodium thiosulfate and a dilution series was prepared. Survivors were then plated onto Luria–Bertani (LB) agar and incubated for 24 h at 37°C. Individual colonies were picked onto LB and minimal media agar (Neidhardt *et al.* 1974) plates prior to incubating for 24 h at 37°C.

Enhanced resistance of *Escherichia coli* O157:H7 and *Salmonella* exposed to sublethal doses of stabilized oxychloro sanitizer

Escherichia coli O157:H7 ph1 and *Salm.* Meleagridis (*c.* 10^6 CFU ml^{-1}) were inoculated onto the mung bean seeds using the method described by Kumar *et al.* (2006). Inoculated beans were soaked in 25–50 ppm SOC for 24 h and sprouted for 48 h at 30°C with periodic watering. Sprout samples (10 g) were collected and suspended in 90 ml saline prior to stomaching. A dilution series was prepared and plated onto either CT-SMac or brilliant green agar. The plates were incubated at 37°C for 24 h and a typical colony transferred to 10 ml TSB and incubated overnight at 37°C. The culture was used as an inoculum for a further batch of mung beans. This process was repeated seven times and the resistance of the final isolates to SOC was assessed as previously described.

Statistical analysis

All experiments were repeated in triplicate. Quantitative data were tested for significance using a combination of the Student's *t*-test, ANOVAR and Tukey's test.

Results

Suspensions of the different bacterial types exhibited a spectrum of sensitivities to SOC when contacted with the sanitizer over a 24-h period. *Escherichia coli* O157:H7 ph1 and generic *E. coli* P36 exhibited significantly (Tukey–Kramer *post hoc* analysis, $P < 0.05$) greater sensitivity to SOC compared with the other bacterial types tested. High log reductions of *Aeromonas hydrophilia* were also achieved when >100 ppm was applied. Of the Gram-negative bacteria, *Salmonella* had the highest tolerance to SOC with concentrations of 200 ppm being required to reduce counts below the limit of detection. In comparison, the tested Gram-positive bacteria exhibited significantly (Tukey–Kramer *post hoc* analysis, $P < 0.05$) higher resistance to SOC than Gram negatives (apart from *Salmonella*) with *Listeria monocytogenes* being the most tolerant (Table 2). No significant reduction in *B. subtilis* endospores was achieved,

Table 2. Log count reduction of bacteria, endospore and bacteriophage treated for 24 h in saline supplemented with varying levels of a stabilized oxychloro-based sanitizer

	Log count reduction SOC (ppm)				
Bacterium	25	50	100	150	200
Gram negative					
Escherichia coli O157:H7 ph1	$6{\cdot}4 \pm 0{\cdot}10^{A}$	MDL^{A}	MDL^{A}	MDL^{A}	MDL^{A}
E. coli P36	$6{\cdot}4 \pm 0{\cdot}12^{A}$	$6{\cdot}4 \pm 0{\cdot}11^{B}$	MDL^{A}	$5{\cdot}6 \pm 0{\cdot}14^{B}$	MDL^{A}
Salmonella serotype Meleagridis	$3{\cdot}8 \pm 0{\cdot}21^{B}$	$4{\cdot}2 \pm 0{\cdot}14^{E}$	$4{\cdot}0 \pm 0{\cdot}11^{B}$	$3{\cdot}6 \pm 0{\cdot}12^{C}$	MDL^{A}
Gram positive					
Lactococcus lactis	$2{\cdot}1 \pm 0{\cdot}12^{C}$	$2{\cdot}9 \pm 0{\cdot}13^{C}$	$3{\cdot}9 \pm 0{\cdot}12^{B}$	MDL^{A}	MDL^{A}
Staphylococcus aureus	$2{\cdot}8 \pm 0{\cdot}11^{D}$	$2{\cdot}6 \pm 0{\cdot}13^{C,D}$	$4{\cdot}4 \pm 0{\cdot}17^{B}$	$4{\cdot}8 \pm 0{\cdot}11^{D}$	MDL^{A}
Listeria monocytogenes	$1{\cdot}6 \pm 0{\cdot}10^{C}$	$2{\cdot}5 \pm 0{\cdot}15^{D}$	$1{\cdot}9 \pm 0{\cdot}12^{C}$	$1{\cdot}8 \pm 0{\cdot}10^{E}$	$5{\cdot}3 \pm 0{\cdot}30^{B}$
Aeromonas hydrophilia	$3{\cdot}0 \pm 0{\cdot}17^{D}$	$6{\cdot}5 \pm 0{\cdot}11^{B}$	MDL^{A}	MDL^{A}	MDL^{A}
Endospores					
Bacillus subtilis PS346	NT	NT	NT	NT	$0{\cdot}12 \pm 0{\cdot}10^{C}$
Bacteriophage					
MS2 F+ coliphage	NT	NT	NT	NT	MDL^{A}

NT, not tested; MDL (minimum detection limit; no survivors) <10 CFU or 100 PFU ml^{-1}.
Initial concentration was c. 7 log CFU/PFU ml^{-1} in all cases.
Values within each column with the same superscript letters are not significantly different.

suggesting that SOC has negligible sporocidal activity. However, it was noted that MS2 coliphage could be readily inactivated by SOC (Table 2) with populations decreasing below the level of detection (<100 PFU ml^{-1}) within the 24-h treatment period.

The inactivation kinetics of both *Salmonella* and *E. coli* O157:H7 ph1 by SOC was dependent on the suspension medium used. A relatively rapid reduction in *Salmonella* numbers was observed with cell suspensions prepared in saline (Fig. 1a). However, when *Salmonella* was suspended in mung bean exudates, there was a delay (shoulder) in the inactivation of the bacterium before a progressive decrease in cell numbers occurred. In contrast, *Salmonella* suspended in 0·1% peptone had a significantly higher (ANOVA, $P < 0{\cdot}05$) tolerance towards SOC with levels remaining stable over the 24-h treatment period (Fig. 1a).

Escherichia coli O157:H7 ph1 numbers decreased relatively and rapidly in saline or mung bean exudates supplemented with 200 ppm SOC (Fig. 1b). Although inactivation of *E. coli* O157:H7 occurred in 0·1% peptone, the rate was significantly (ANOVA, $P < 0{\cdot}05$) lower than that in either mung bean exudates or saline (Fig. 1b).

There was no significant (*t*-test, $P > 0{\cdot}05$) depletion of chlorite in cell suspensions prepared in saline, as levels at the start (200 ppm) were comparable to those measured at the end of the 24-h contact period. SOC was less stable in the presence of mung bean exudates and decreased by 42% ± 3% by the end of the 24-h incubation period. However, SOC was rapidly neutralized by 0·1% peptone with levels decreasing to <10% within 2 min. A comparable rate of SOC inactivation was also observed when the sanitizer was in contact with cysteine (10 mmol l^{-1}) or thiosulfate (0·1%) (results not shown).

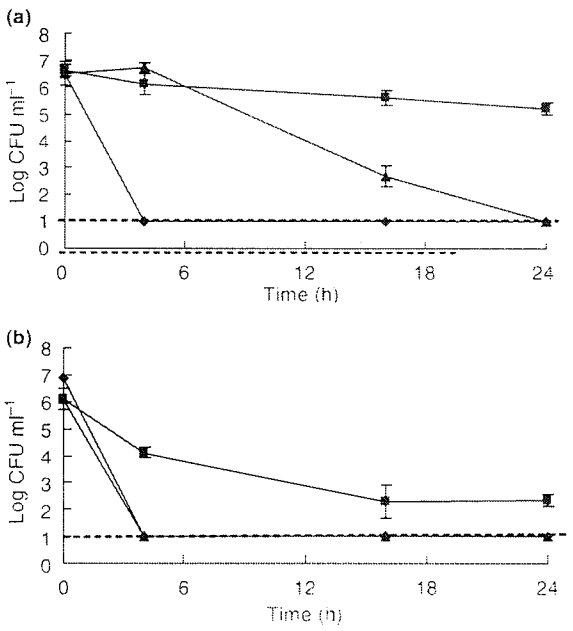

Figure 1 Inactivation of *Salmonella* serotype Meleagridis (a) and *Escherichia coli* O157:H7 ph1 (b) in suspensions supplemented with 200 ppm SOC. The resuspension medium was saline (◆), 0·1% peptone (■) or mung bean exudate (▲). Aliquots from suspensions were removed periodically and chlorite was neutralized with 0·1% w/v sodium thiosulfate prior to plating onto TSA and incubating for 24 h at 37°C. The dashed line denotes the limit of detection.

Table 3 Intracellular glutathione concentration in *Escherichia coli* O157:H7 ph1 or *Salmonella* serotype Meleagridis exposed to 200 ppm stabilized oxychloro-based sanitizer. Suspensions were prepared in saline supplemented with 200 ppm SOC with aliquots being removed periodically to determine intracellular glutathione concentration

Time of exposure to 200 ppm SOC (h)	Glutathione content (μmol l^{-1}) *E. coli* ph1 O157:H7	Glutathione content (μmol l^{-1}) *Salm.* Meleagridis
Control (0 ppm SOC)	135·0 ± 2·6^{A}	120·0 ± 1·3^{A}
0	1·5 ± 0·7^{B}	13·2 ± 1·0^{B}
4 h	1·4 ± 0·5^{B}	13·5 ± 1·0^{B}
16 h	1·5 ± 0·5^{B}	28·3 ± 1·2^{C}
24 h	25·0 ± 1·2^{C}	14·0 ± 1·3^{D}

Values within each column with the same superscript letters are not significantly different.

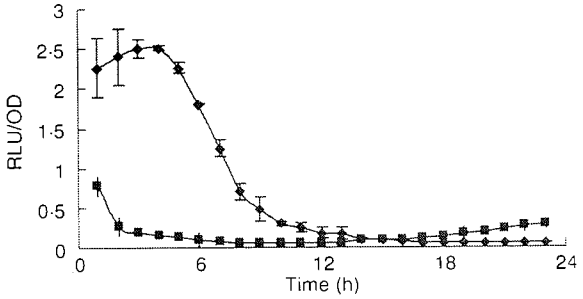

Figure 2 Bioluminescence of *Escherichia coli* O157:H7 ph1 in the presence (■) and absence (◆) of 200 ppm SOC. Filter-sterilized mung bean exudates were supplemented with SOC and bioluminescence monitored over a 23-h period. Values are presented as relative light units per OD_{600nm}.

Oxidative stress response of *Salmonella* and *Escherichia coli* O157:H7 exposed to stabilized oxychloro sanitizer

Using lucigenin as a reporter for the generation of reactive O^{-} ions, it was found that both *Salmonella* and *E. coli* O157:H7 ph1 experienced an oxidative burst within 5 min of exposure to 200 ppm SOC. The chemiluminescence peaked at $2{\cdot}03 \times 10^{3}$ RLU OD_{600nm} U^{-1} in *E. coli* O157:H7 compared with $2{\cdot}73 \times 10^{3}$ RLU OD_{600nm} U^{-1} for *Salmonella*. The chemiluminescence then progressively declined as the lucigenin returned to its ground state. However, no chemiluminescence was observed with SOC and lucigenin in the absence of bacterial cells. In a similar manner, no chemiluminescence was detected when lucigenin was added to cell suspensions containing no SOC. This would confirm that SOC elicited the oxidative response within the enteric pathogens and was not an artefact of the lucigenin reporter.

In addition, to an oxidative burst, there was a significant (ANOVA, $P < 0{\cdot}05$) depletion of intracellular GSH levels within both *E. coli* O157:H7 ph1 and *Salmonella* (Table 3). The rate of depletion was rapid in both bacterial types and occurred within minutes upon contact of cells with the SOC solution. However, a marginal recovery of intracellular GSH was observed towards the end of the 24-h treatment period (Table 3).

Effect of stabilized oxychloro sanitizer on the metabolism of growing *Escherichia coli* O157:H7 ph1

Bioluminescence can be used as a measure of intracellular ATP concentration, and hence provide an assessment on the metabolic activity of microbes (Chumkhunthod *et al.* 1998). When *E. coli* O157:H7 ph1 was cultured in filter-sterilized mung bean exudate supplemented with 200 ppm SOC, the bioluminescence emitted from the culture decreased progressively over the initial 4 h and reached a low level throughout the remaining incubation period (Fig. 2). In contrast, the bioluminescent profile of non-SOC-treated controls increased during the log phase. The bioluminescence then decreased as the suspension entered the stationary phase of growth (Fig. 2).

Interaction of stabilized oxychloro sanitizer with green fluorescent protein and selected enzymes

Monitoring changes in intracellular GFP provides a direct means of establishing whether SOC disrupts protein structures. The fluorescence of GFP is generated by the excitation by light, which results in a rapid cyclization between Ser65 and Gly67 to form an imidazolin-5-one intermediate, which is followed by a much slower rate-limiting oxygenation of the Tyr66 side chain by O_2 (March *et al.* 2003). Therefore, the fluorescence of GFP is not only dependent on conformation but also the presence of oxygen.

When GFP-tagged *E. coli* O157:H7 C9490 was exposed to 200 ppm SOC over a 24-h period, no loss of fluorescence was observed. Indeed, there was a progressive increase in GFP fluorescence, despite the decrease in OD of the cell suspension (Fig. 3).

When the stability of key enzymes (catalase, glucose-6-phosphate dehydrogenase, L-lactate dehydrogenase and SOD) was assayed following exposure to SOC (200 ppm for 20 min), no significant (*t*-test, $P > 0{\cdot}05$) loss in activity occurred (results not shown). However, the activity of protease was decreased by 27% ± 8% following exposure to 200 ppm SOC and that of alkaline phosphatase completely inactivated within 20 min contact with the sanitizer.

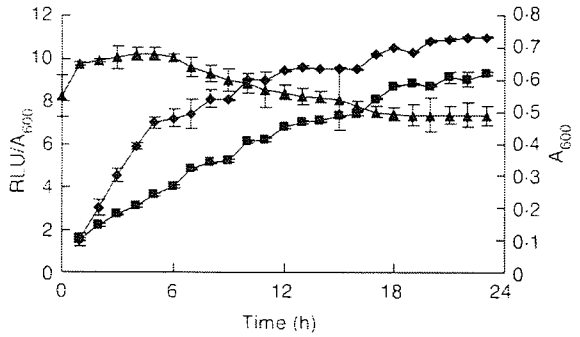

Figure 3 Green fluorescent protein stability within *Escherichia coli* O157:H7 C9490 in saline without (■) and with (◆) 200 ppm SOC. The A_{600nm} of the *E. coli* O157:H7 suspension supplemented with 200 ppm SOC (▲) is also illustrated.

Membrane damage resulting from exposure to stabilized oxychloro sanitizer

Suspensions of either *Salm.* Meleagridis or *E. coli* O157:H7 ph1 (prepared in saline) were treated with 200 ppm SOC and aliquots withdrawn periodically for viability staining. Using the BacLight viability stain, it was observed that a proportion of the population (*c.* 2%) of both *Salmonella* and *E. coli* O157:H7 had lost viability upon initial contact with SOC. However, the level of dead or low-viability cells increased to *c.* 50% by 4 h into the incubation period. By 19 h and beyond, all the cells within the population were stained orange/red, suggesting that the population had lost viability.

DNA damage in *Escherichia coli* O157:H7 ph1 and *Salmonella* by exposure to stabilized oxychloro sanitizer

The extent to which SOC damages DNA was assessed by screening for auxotrophic mutants in *E. coli* O157:H7 or *Salmonella* exposed to sublethal doses of the sanitizer. A significant (ANOVA, $P < 0{\cdot}05$) number of auxotrophic *E. coli* O157:H7 mutants were generated within 2 h exposure to 25 or 200 ppm SOC (Table 4). In a similar manner, exposure of *Salmonella* to sublethal concentrations of SOC also resulted in a high proportion of auxotrophic mutants (Table 4). The results would strongly suggest that SOC either directly or indirectly induces DNA damage.

Table 4 Auxotrophic mutants of *Escherichia coli* O157:H7 ph1 and *Salmonella* serotype Meleagridis exposed to sublethal concentrations of SOC

Bacterium	SOC concentration/ contact time (ppm/h)	Auxotrophic mutants/ total colonies screened
E. coli O157:H7	0	5/144
	25/1	5/144
	25/2	74/144
	200/1	6/144
	200/2	52/144
Salm. Meleagridis	0	7/144
	25/24	60/144

A potential consequence of the mutagenic effects of SOC is the generation of resistant mutants to the sanitizer. However, neither *E. coli* O157:H7 ph1 nor *Salmonella* acquired enhanced resistance to SOC when repeatedly subjected to sublethal concentrations of the sanitizer.

Discussion

The inactivation of both *Salmonella* and *E. coli* O157:H7 by SOC follows a similar route. In the initial period following exposure to SOC, there is clear evidence for the accumulation of superoxide ions (O^-) with a subsequent loss of intracellular GSH. Within this early phase, damage to the genomic DNA occurred as assessed through the generation of auxotrophic mutants. However, there was a slower loss of metabolic activity as observed through the gradual reduction in the level of bioluminescence produced by *E. coli* O157:H7 ph1. Cell membrane disruption was also slow and there was a clear variation in the rate at which individual members of the population were inactivated. This probably was the underlying reason for the relatively long contact periods required to inactivate pathogens by SOC *in vitro* and when applied into decontaminating seeds destined for sprout production (Kumar *et al.* 2006).

In plants and macrophages, an oxidative burst of free radicals forms the basis of defence systems against microbial invaders (Gozzo 2003; Klebanoff 2005). However, within bacterial cells, the cause of the oxidative burst is less well understood. It has been proposed that the rapid accumulation of oxidative ions occurs when the anabolic and catabolic pathways of metabolism become uncoupled through a cessation of growth (Aldsworth *et al.* 1998; Dodd and Aldsworth 2002). Here, the cell attempts to dissipate ATP and reducing equivalents to nongrowth functions, thereby leading to a surge in free radicals. Typically, the SOD would catalyse the conversion of the superoxide ions to hydrogen peroxide that would subsequently be acted upon by catalase. Therefore, in the present case, the oxidative burst observed may have been because of metabolic uncoupling and/or the inactivation of SOD. The latter is unlikely because of the fact that the activity of SOD was unaffected by SOC. An alternative mechanism would be the depletion of intracellular GSH caused by the direct interaction with the chlorite component of SOC. This hypothesis is supported by the work of Ingram *et al.* (2003), who demonstrated the direct oxidation of GSH by sodium chlorite. Once in the oxidized

form GSH can react with DNA or other reactive species, thereby leading to significant intracellular damage (Ingram *et al.* 2003). In the present study, it was noted that in the initial period of SOC exposure there was a significant number of auxotrophic mutants generated both within *Salmonella* and *E. coli* O157:H7 ph1. This was likely because of the accumulated GSSH that interacted directly with the genomic DNA leading to gene disruption (Akerboom and Sies 1989). The cell counters the damaging effect of GSSH by transporting the reactive thiol into the outside environment (Akerboom and Sies 1989). This occurred with both *E. coli* O157:H7 and *Salmonella* where the intracellular GSH concentrations remained low throughout the incubation period.

The contribution of DNA damage to the loss of cell viability is unclear. If SOC was indeed highly mutagenic then it would be expected that exposure to sublethal doses would eventually lead to the generation of resistant mutants. This has been previously found with *E. coli* O157:H7 exposed to sublethal concentrations of chlorate (Callaway *et al.* 2001). Here, mutants with reduced nitrite reductase activity were less sensitive to chlorate because of the negligible accumulation of chlorite (Callaway *et al.* 2001). Clearly, in the present case where chlorite is applied directly, mutations in the nitrate reductase gene would be less significant. Indeed, no evidence of enhanced resistance in *E. coli* O157:H7 or *Salmonella* exposed to sublethal concentrations over successive generations was found. However, given the variation in the sensitivity of different bacterial types to SOC, it is possible that mechanisms exist, which enhance resistance to the sanitizer. The nature of such mechanisms remains unclear but is worthy of further study.

Although the initial contact of bacterial cells to SOC provoked a cellular response, the actual rate of inactivation was slow. It was noted that the loss of bioluminescence within *E. coli* O157:H7 upon exposure to SOC occurred over a 4-h period. This would strongly suggest that catabolic pathways that supply the energy and reducing equivalents to sustain bioluminesence were functional upon initial exposure to SOC, despite the cessation of bacterial growth. A possible explanation for the stability of enzymes in the presence of chlorite can be attributed to the disulfide bond content. It is noteworthy that enzymes present in the cytosol of bacterial cells have a low abundance of disulfide bonds compared with exoenzymes or periplasmic proteins (Gilbert 1990; Hiniker and Bardwell 2004). In addition, GFP is devoid of disulfide bonds and correspondingly stable in the presence of chlorite. In contrast, the apparent high sensitivity of MS2 bacteriophage may be the result of disruption of disulfide bonds essential for capsid stability (Golmohammadi *et al.* 1996). The direct oxidation of cysteine by chlorite along with the sensitivity of alkaline phosphatase (contains two disulfide bonds) and protease (a single disulfide bond) would also support the view that disulfide-bond-rich proteins are more susceptible to attack by chlorite.

Through viability staining, it was evident that pore formation in the cell membrane progressively occurred over the 24-h treatment period. This would strongly suggest that the final loss of cell viability was likely because of the collapse of membrane functionality as opposed to direct attack on enzymes or nucleic acids. This is in contradiction to the results of Ingram *et al.* (2004), who suggested that negligible interaction of chlorite with lipid membranes occurs. However, it is important to note that Ingram *et al.* (2004) studied the effect of chlorite on lipids *in vitro* and not membrane proteins. Therefore, it is possible that membrane disruption was caused by the action of chlorite on the disulfide-rich membrane proteins as opposed to lipids directly.

Although *Salmonella* and *E. coli* O157:H7 were sensitive to SOC in saline, the tolerances of both pathogens were enhanced in the presence of 0.1% peptone. This can be attributed to the neutralization of SOC by interaction with proteins contained within peptone. Nevertheless, it was interesting to note that although SOC was rapidly neutralized by peptone, a significant reduction in *E. coli* O157:H7 levels were observed and the growth of *Salmonella* inhibited. This may suggest that the brief exposure of bacterial cells to chlorite initiated a chain reaction that ultimately resulted in loss of cell viability. However, *Salmonella* and, to some extent, *E. coli* O157:H7 were still able to grow on agar plates to form visible colonies. This in turn would suggest that the transient exposure to chlorite causes cell injury that can be repaired. Indeed, Ingram *et al.* (2003) noted that counts of *Pseudomonas aeruginosa* or *Staphylococcus aureus* treated with 50 ppm chlorite increased towards the end of the 24-h exposure period. In this respect, the long treatment time (>19 h) required to inactivate pathogens on seeds may be due to the need to exceed this critical exposure time. It was also interesting to note that although chlorite inactivated both pathogens in saline, there was no decrease in the sanitizer levels throughout the 24-h incubation period. The same result was obtained by Ingram *et al.* (2004) with *Ps. aeruginosa* and *Staph. aureus*. This would suggest that relatively small concentrations of chlorite react with cellular constituents to cause cell inactivation. It is possible that the dose dependency of chlorite in inactivating bacteria is a result of the concentration gradients required to facilitate the uptake of chlorite into the cell. Very few studies have been performed to determine how chlorite is transported into the cell, although specific transporters of the oxychloro compound have been identified in fungi (Zhou

et al. 2000). Whether bacteria also possess an equivalent chlorite transporter is not known.

Based on plate count data, it was evident that *E. coli* O157:H7 was significantly more sensitive to SOC compared with *Salmonella*. This was especially emphasized with the relative inactivation of both bacterial types in mung bean exudates. Here, the inactivation of *Salmonella* by SOC was delayed and occurred at a decreased rate compared with *E. coli* O157:H7 ph1. A possible explanation for the enhanced tolerance of *Salmonella* to SOC may be associated with the ability of the bacterium to utilize protective constituents within the mung bean exudates. In addition, it should also be noted that as an intracellular pathogen, *Salmonella* has evolved protective mechanisms to avoid being inactivated by the oxidative stress incurred during interaction with macrophages (Sly *et al.* 2002). Therefore, it is plausible that the *Salmonella* could resist the early oxidative stress response but subsequently was inactivated through continuous exposure to SOC.

The higher tolerance of Gram-positive bacteria to chlorite has been previously reported (Ingram *et al.* 2003). This has been attributed to the absence of GSH within Gram-positive bacteria (Ingram *et al.* 2003). Therefore, it is likely that the mode of inactivation of Gram-positive bacteria differ from that of Gram negatives. The resistance of endospores to SOC was most likely because of the poor penetration of active chlorite through the protective spore coat. It has previously been found that *Bacillus* spores are highly resistant to both hypochlorite and chlorine dioxide unless the spore coat is removed (Young and Setlow 2003).

In conclusion, the evidence obtained in this study suggests that inactivation of *Salmonella* and *E. coli* O157:H7 occurs through the direct action of SOC with disulfide bonds of proteins and indirectly via reduced thiol-containing compounds. This subsequently leads to DNA damage and finally disruption of the membrane integrity. Killing by SOC is different from that observed for hydrogen peroxide and hypochlorite but similar to that of chlorine dioxide (Scatina *et al.* 1985; Fu *et al.* 2002; Hawkins *et al.* 2002; Luppens *et al.* 2003). However, the action of chlorite appears to be more discriminating and selective compared with other oxidizing agents, including chlorine dioxide, which may explain the insensitivity of mammalian cells and sprouting seeds to the sanitizer (Ingram *et al.* 2004; Kumar *et al.* 2006).

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References

Aebi, H. (1984) Catalase in vitro. *Meth Enzymol* **105**, 121–126.

Akerboom, T.P. and Sies, H. (1989) Transport of glutathione, glutathione disulfide, and glutathione conjugates across the hepatocyte plasma membrane. *Meth Enzymol* **173**, 523–534.

Aldsworth, T.G., Sharman, R.L., Dodd, C.E. and Stewart, G.S. (1998) A competitive microflora increases the resistance of *Salmonella typhimurium* to inimical processes: evidence for a suicide response. *Appl Environ Microbiol* **64**, 1323–1327.

Anson, M.L. (1938) The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J Gen Physiol* **22**, 79–89.

Callaway, T.R., Anderson, R.C., Anderson, T.J., Poole, T.L., Bischoff, K.M., Kubena, L.F. and Nisbet, D.J. (2001) *Escherichia coli* O157:H7 becomes resistant to sodium chlorate in pure culture, but not in mixed culture or in vivo. *J Appl Microbiol* **91**, 427–434.

Chumkhunthod, P., Schraft, H. and Griffiths, M.W. (1998) Rapid monitoring method to assess efficacy of sanitizers against *Pseudomonas putida* biofilms. *J Food Prot* **61**, 1043–1046.

DeMoss, R.D., Gunsalus, I.C. and Bard, R.C. (1953) A glucose-6-phosphate dehydrogenase in *Leuconostoc mesenteroides*. *J Bacteriol* **66**, 10–16.

Dodd, C.E. and Aldsworth, T.G. (2002) The importance of RpoS in the survival of bacteria through food processing. *Int J Food Microbiol* **74**, 189–194.

Fu, X., Mueller, D.M. and Heinecke, J.W. (2002) Generation of intramolecular and intermolecular sulfenamides, sulfinamides, and sulfonamides by hypochlorous acid: a potential pathway for oxidative cross-linking of low-density lipoprotein by myeloperoxidase. *Biochemistry* **41**, 1293–1301.

Garen, A. and Levinthal, C. (1960) A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. *Biochim Biophys Acta* **38**, 470–483.

Gilbert, H.F. (1990) Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol Relat Areas Mol Biol* **63**, 69–172.

Golmohammadi, R., Fridborg, K., Bundule, M., Valegard, K. and Liljas, L. (1996) The crystal structure of bacteriophage Q beta at 3.5 A resolution. *Structure* **4**, 543–554.

Gomez-Lopez, V.M., Devlieghere, F. and Debevere, J. (2005) Decontamination of minimally processed vegetables by chlorine dioxide gas. *Commun Agric Appl Biol Sci* **70**, 143–146.

Gozzo, F. (2003) Systemic acquired resistance in crop protection: from nature to a chemical approach. *J Agric Food Chem* **51**, 4487–4503.

Hawkins, C.L. and Davies, M.J. (2002) Hypochlorite-induced damage to DNA, RNA, and polynucleotides: formation of

chloramines and nitrogen-centered radicals. *Chem Res Toxicol* **15**, 83–92.

Hawkins, C.L., Rees, M.D. and Davies, M.J. (2002) Superoxide radicals can act synergistically with hypochlorite to induce damage to proteins. *FEBS lett* **510**, 41–44.

Hiniker, A. and Bardwell, J.C. (2004) In vivo substrate specificity of periplasmic disulfide oxidoreductases. *J Biol Chem* **279**, 12967–12973.

Hora, R., Warriner, K., Shelp, B.J. and Griffiths, M.W. (2005) Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. *J Food Prot* **68**, 2506–2509.

Inatsu, Y., Bari, M.L., Kawasaki, S., Isshiki, K. and Kawamoto, S. (2005) Efficacy of acidified sodium chlorite treatments in reducing *Escherichia coli* O157:H7 on Chinese cabbage. *J Food Prot* **68**, 251–255.

Ingram, P.R., Homer, N.Z., Smith, R.A., Pitt, A.R., Wilson, C.G., Olejnik, O. and Spickett, C.M. (2003) The interaction of sodium chlorite with phospholipids and glutathione: a comparison of effects in vitro, in mammalian and in microbial cells. *Arch Biochem Biophys* **410**, 121–133.

Ingram, P.R., Pitt, A.R., Wilson, C.G., Olejnik, O. and Spickett, C.M. (2004) A comparison of the effects of ocular preservatives on mammalian and microbial ATP and glutathione levels. *Free Radic Res* **38**, 739–750.

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

Kemp, G.K. and Schneider, K.R. (2000) Validation of thiosulfate for neutralization of acidified sodium chlorite in microbiological testing. *Poult Sci* **79**, 1857–1860.

Klebanoff, S.J. (2005) Myeloperoxidase: friend and foe. *J Leukoc Biol* **??** ???–???.

Kumar, M., Hora, R., Kostrzynska, M., Waites, W.M. and Warriner, K. (2006) Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on mung beans, alfalfa and other seed types destined for sprout production by using an oxychloro-based sanitizer. *J Food Prot* **69**, 1571–1578.

Luppens, S.B., Barbaras, B., Breeuwer, P., Rombouts, F.M. and Abee, T. (2003) Selection of fluorescent probes for flow cytometric viability assessment of *Listeria monocytogenes* exposed to membrane-active and oxidizing disinfectants. *J Food Prot* **66**, 1393–1401.

March, J.C., Rao, G. and Bentley, W.E. (2003) Biotechnological applications of green fluorescent protein. *Appl Microbiol Biotechnol* **62**, 303–315.

Melville, S.B., Michel, T.A. and Macy, J.M. (1988) Regulation of carbon flow in *Selenomonas ruminantium* grown in glucose-limited continuous culture. *J Bacteriol* **170**, 5305–5311.

Neidhardt, F., Bloch, P. and Smith, D. (1974). Culture medium for enterobacteria. *J Bacteriol* **119**, 736–747.

Nicholson, W.L. and Setlow, P. (1990) Dramatic increase in negative superhelicity of plasmid DNA in the forespore compartment of sporulating cells of *Bacillus subtilis*. *J Bacteriol* **172**, 7–14.

Scatina, J., Abdel-Rahman, M.S. and Goldman, E. (1985) The inhibitory effect of Alcide, an antimicrobial drug, on protein synthesis in Escherichia coli. *J Appl Toxicol* **5**, 388–394.

Schaper, M. and Jofre, J. (2000) Comparison of methods for detecting genotypes of F-specific RNA bacteriophages and fingerprinting the origin of faecal pollution in water samples. *J Virol Methods* **89**, 1–10.

Sly, L.M., Guiney, D.G. and Reiner, N.E. (2002) *Salmonella enterica* serovar Typhimurium periplasmic superoxide dismutases SodCI and SodCII are required for protection against the phagocyte oxidative burst. *Infect Immun* **70**, 5312–5315.

USEPA (United States Environmental Protection Agency) (2001) *Method 1602: Male-specific (F') and somatic coliphage in water by single agar layer procedure.* (http://www.epa.gov/nerlcwww/1602ap01.pdf). Accessed April 2006.

Warriner, K., Spaniolas, S., Dickinson, M., Wright, C. and Waites, W.M. (2003) Internalization of bioluminescent *Escherichia coli* and *Salmonella* Montevideo in growing bean sprouts. *J Appl Microbiol* **95**, 719–727.

Young, S.B. and Setlow, P. (2003) Mechanisms of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. *J Appl Microbiol* **95**, 54–67.

Zhou, J.J., Trueman, L.J., Boorer, K.J., Theodoulou, F.L., Forde, B.G. and Miller, A.J. (2000) A high affinity fungal nitrate carrier with two transport mechanisms. *J Biol Chem* **275**, 39894–39899.

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ORIGINAL ARTICLE

Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on artificially or naturally contaminated mung beans (*Vigna radiata* L) using a stabilized oxychloro-based sanitizer

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Abstract

Aims: To evaluate the efficacy of a stabilized oxychloro-based (SOC) sanitizer to decontaminate mung beans artificially or naturally contaminated with *Escherichia coli* O157:H7 or *Salmonella*.

Methods and Results: Naturally contaminated beans were produced by introducing a five-strain cocktail of *E. coli* O157:H7 or *Salmonella* onto the flowers of growing mung bean plants. *Escherichia coli* O157:H7 was only sporadically recovered from sprout lots (three testing positive from 10 tested) derived from harvested beans. In contrast, *Salmonella* was recovered from 18 of 20 lots screened. Pathogens present on naturally contaminated seed could be successfully inactivated with SOC applied at 200 ppm for 24 h at 28°C. SOC treatment could also decontaminate artificially inoculated mung bean batches containing different levels of contaminated seed. SOC inactivated *E. coli* O157:H7, but not *Salmonella* introduced onto damaged (scarified) beans.

Conclusions: SOC sanitizer could inactivate *Salmonella* or *E. coli* O157:H7 naturally or artificially introduced onto mung beans. However, the SOC treatment failed to inactivate *Salmonella* introduced onto damaged mung beans.

Significance and Impact of the Study: SOC sanitizer represents an effective method for decontaminating undamaged mung beans.

Introduction

Over the last decade there has been numerous food-borne illness outbreaks linked to sprouted seeds such as alfalfa and bean sprouts (Fett *et al.* 2005). *Salmonella* is the most frequently encountered human pathogen on sprouts although cases involving *Escherichia coli* O157:H7 have also been reported (Mohle-Boetani *et al.* 2001; Fett *et al.* 2005). In the majority of outbreaks the seed used for sprout production has been shown to be the source of human pathogens (Anonymous 1999). Therefore, it was recommended that seeds should be treated with 20 000 ppm calcium hypochlorite prior to soaking and subsequently sprouting (Anonymous 1999). However, it is widely acknowledged that treatments based on hypochlorite or alternative sanitizing agents (e.g. peroxyacetic acid, hydrogen peroxide) can only reduce pathogen levels on seeds but cannot ensure complete elimination (Anonymous 1999; Wessinger and Beuchat 2000; Brooks *et al.* 2001). This is relevant considering that even relatively low levels of surviving pathogens (<0·1 CFU g^{-1}) can grow to densities in excess of 6 log CFU g^{-1} within 48 h into the sprouting process (Holliday *et al.* 2001).

A successful decontamination method has been devised based on supplementing the soak water used in the early stages of sprout production with a stabilized oxychloro-based sanitizer (SOC) (Kumar *et al.* 2006). A previous study has demonstrated that SOC applied at 200 ppm for >19 h can consistently inactivate either *E. coli* O157:H7 or *Salmonella* introduced onto mung beans (Kumar *et al.* 2006). However, the efficacy of SOC to decontaminate naturally contaminated or damaged seed has yet to be

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determined. In both cases it is possible that low levels of pathogens can become located in protective sites within the seed inaccessible to sanitizing agents and significantly reduce the efficacy of decontamination treatments (Brooks *et al.* 2001; Charkowski *et al.* 2001; Fu *et al.* 2001; Howard and Hutcheson 2003; Winthrop *et al.* 2003; Montville and Schaffner 2005).

In the following study the efficacy of SOC sanitizer to inactivate pathogens (*Salmonella* and *E. coli* O157:H7) when introduced at low levels or on damaged mung beans was evaluated. In addition, batches of naturally contaminated mung beans were produced by introducing the pathogens onto the flowers of growing plants from which the seed pods emerged. The choice of flowers as the inoculation site was selected to mimic the effect of contaminated irrigation water which is considered to be an important source of human pathogens present on seeds (Anonymous 1999).

Materials and methods

Bacterial strains and preparation of inocula

Escherichia coli O157:H7 and *Salmonella* used in the study were composed of environmental, clinical, tomato or sprout isolates (Table 1). An aliquot (1 ml) of an overnight culture of the individual *E. coli* O157:H7 or *Salmonella* strains was transferred into 50 ml of tryptic soy broth (Difco, Sparks, MD, USA) and incubated for 24 h at 37°C. Bacterial cells were harvested by centrifugation (5500 *g* for 10 min, 4°C) and washed once in 0·8% saline. The final cell pellet was re-suspended in saline to a final cell density of 10^6 CFU ml^{-1}. Equal volumes of the five different *E. coli* O157:H7 or *Salmonella* suspensions were then combined to produce a cocktail that was subsequently used to inoculate beans or flowers of mung bean plants.

Inoculation of undamaged and damaged mung beans

Mung beans (*Vigna radiata*) were purchased from Mumms Seeds Ltd (Parkside, SK, Canada). Beans (250 g) were soaked in 250 ml of the five-strain cocktail of *E. coli* O157:H7 or *Salmonella* for 20 min. The seeds were then transferred to sterile filter paper within a biological safety cabinet and allowed to dry at ambient temperature for 48–60 h. The inoculated mung beans were then introduced into 100-g batches of uninoculated beans at different levels (1–50%, w/w) and manually mixed prior to sprouting. For example, 1 g of inoculated seeds was mixed with 99 g of non-inoculated seed to give an inoculation level of 1% (w/w).

For damaged seed, mung beans (in 5-g lots) were placed between sheets of abrasive paper and rubbed with moderate pressure for *c.* 10 s to produce visible scarification. The damaged mung beans (100 g) were then soaked in bacterial suspension as described above and sprouted directly without mixing with non-inoculated seed.

Cultivation and inoculation of mung bean plants

Mung beans (non-inoculated) were soaked in distilled water at 30°C overnight to stimulate germination prior to transplanting into plug trays containing commercial grade PGX soil (Professional plug and germination growing medium; Premier Horticulture Ltd, ????, QC, Canada). The trays were transferred to a sealed growth room maintained at 25°C with a relative humidity set at 90%. After 2 weeks the seedlings were transplanted into 2-l soil microcosms containing commercial grade BX soil (Professional general purpose growing medium, Premier Horticulture Ltd). The relative humidity within the growth room was maintained at 90% with 12/12 h day/night at 25/10°C respectively. Flowers started to appear on plants 7–8 weeks into the cultivation period. The calyx of individual flowers was inoculated with a single 0·1 ml aliquot of *E. coli* O157:H7 or *Salmonella* (10^6 CFU ml^{-1}). The plants (12 inoculated with *E. coli* O157 and 24 with *Salmonella*) were cultivated for a further period (6–8 weeks) to enable seed pod development and ripening. Seed pods were manually harvested using sterile gloved hands and transferred to the laboratory in sterile bags. The beans were extracted from pods using a sterile scalpel blade and pooled prior to subdividing into 0·7–1·2 g lots (40 lots

Table 1 *Escherichia coli* O157:H7 and *Salmonella* strains/serovars used in the study

Escherichia coli O157:H7 strain*	Source	*Salmonella enterica* serovar	Source
E. coli O157:H7 C1033	Water sediment	Meleagridis E1†	Alfalfa sprouts
E. coli O157:H7 C1032	Soil	Oranienburg C1†	Alfalfa sprouts
E. coli O157:H7 C652	Clinical	Newport C2†	Alfalfa sprouts
E. coli O157:H7 C476	Clinical	Senftenburg†	Alfalfa sprouts
E. coli O157:H7 C477	Clinical	Montevideo*	Tomatoes

*Strains obtained from Canadian Research Institute for Food Safety, University of Guelph, Guelph, ON, Canada.
†Strains donated by Dr C. Poppe, Health Canada, Guelph, ON, Canada.

for *Salmonella* and 20 for *E. coli* O157:H7). Half the seed lots (for each pathogen type) were treated using SOC sanitizer with the remaining acting as non-treated controls.

SOC treatment and sprouting of mung beans

The different mung bean batches (i.e. naturally contaminated, damaged or those containing inoculated seed introduced at different levels) were soaked for 24 h at 28°C in 200 ppm SOC (commercial name Germin-8-or; Vernagene Ltd, Bolton, Lancashire, UK) using a ratio of 1 part beans (dry weight) to 5 parts sanitizer (Kumar *et al.* 2006). The seeds were then removed, rinsed with distilled water prior to germinating for a further 4 days at 28°C. The developing sprouts were watered daily by a 5-min soak in 500 ml of sterile distilled water. Control (non-decontaminated) mung bean batches were sprouted using the same method except the SOC solution was replaced with distilled water.

Microbiological analysis

For artificially inoculated mung beans, microbiological analysis was performed on both the inoculated seeds and sprouts. Duplicate samples (1 g) of inoculated seed were suspended in 9 ml of buffered peptone water (0·1%, Oxoid, Basingstoke, UK) and vortexed for 1 min. *Salmonella* was enumerated on Brilliant Green agar (BG; Oxoid) incubated at 42°C for 24 h. *Escherichia coli* O157:H7 was enumerated on Sorbitol Maconkey agar containing cefixime and tellurite (CT-SMAC; Difco) incubated at 37°C for 24 h.

For sprouts, duplicate 25 g samples were suspended in 225 ml buffered peptone water and stomached for 90 s at 230 rev min^{-1} (Model 400; A.J. Steward and Co., London, UK). The sprout homogenates were tested for the presence of *Salmonella* using the method described in the Canadian Compendium of Analytical Method, MFHPB 20 (Health Canada 2003). Sprouts were suspended in 225 ml buffered peptone water and incubated at 42°C for 24 h. An aliquot (0·1 ml) of the enriched culture was then inoculated into the centre of a semi-solid Rappaport–Vassiliadis plate (Oxoid) that was subsequently incubated at 37°C for 24 h. Cells from the outer perimeter of the growth halo (presumptive motile *Salmonella*) were streaked onto BG agar (Oxoid) and incubated at 37°C overnight. The Oxoid *Salmonella* Latex Test FT0203 was used for serological confirmation of typical colonies (i.e. red colonies surrounded by brilliant red zones).

Sprouts were enriched for *E. coli* O157:H7 using buffered peptone water containing 0·5% (w/v) sodium thioglycolate incubated at 37°C for 24 h (Sata *et al.* 2003). Aliquots (10 μl) of the enriched culture were then streaked onto CT-SMAC that was subsequently incubated at 37°C for 24 h. Typical colonies (colourless) were confirmed as *E. coli* O157:H7 using the Oxoid *E. coli* O157 Latex Test DR0620M.

Sprouts from mung beans derived from inoculated plants were screened for the presence of pathogens using the same methods highlighted above. However, due to the small bean lots sprouted the entire batch of sprouts (8–13 g) was analysed.

DNA typing of *Salmonella*

Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR (Versalovic *et al.* 1991) was used to identify which of the five strains of *Salmonella* serovar present on sprouts upon completion of the sprouting process. Up to five colonies from the positive selective agar plates were picked onto Luria–Bertani (LB; Difco) agar that was subsequently incubated at 37°C for 24 h. Single colonies were then suspended in 0·2 ml of TE buffer heated at 100°C to lyse cells. Cell debris were removed by centrifugation (13 000 ***g***) and the supernatant, containing DNA, decanted into a new Eppendorf tube. ERIC-PCR was performed using the method described by Kumar *et al.* (2006). DNA patterns were analysed and dice similarity coefficients calculated using Molecular Analyst Software, Bio-Rad Fingerprinting II version 3·0 (Bio-Rad Laboratories, Hercules, CA, USA).

Bioassay for antimicrobial metabolites produced by *Salmonella*

A bioassay was performed (as described by Fett 2006) to determine the antagonistic activity exhibited by individual *Salmonella* serovars used in the study. Briefly, aliquots (20 μl) of an overnight culture of the test *Salmonella* was spotted onto LB agar and incubated at 30°C for 24 h. The agar plate was then exposed to chloroform vapour for 1 h at room temperature within a fume hood (Protector, VWR, Mississauga, ON, Canada). Each plate was then overlaid with 6 ml molten water agar (6 g agar l^{-1}) containing 60 μl of an overnight suspension of the appropriate *Salmonella* serovar. The agar was allowed to solidify prior to incubating plates at 30°C for 24 h. The plates were then removed and visually inspected for zones of inhibition.

Data analysis

Qualitative data were statistically analysed using 2×2 contingency tables (S-PLUS, Insightful Corp., ????, NY, USA). In all cases the significance level was set at $P \leq 0{\cdot}05$.

Results

Inactivation of *E. coli* O157:H7 and *Salmonella* inoculated onto undamaged or damaged mung beans

When the inoculated beans were introduced into batches of non-inoculated beans and sprouted the subsequent bean sprouts tested positive for *Salmonella* or *E. coli* O157:H7 (Table 2). However, no pathogens were recovered from sprouts derived from SOC-treated beans (Table 2). *Escherichia coli* O157:H7 introduced onto damaged beans could also be successfully inactivated by treating with 200 ppm SOC. However, SOC treatment failed to decontamination damaged seeds inoculated with *Salmonella* with all the subsequent sprout samples testing positive for the enteric pathogen (Table 2).

Decontamination of naturally contaminated seed

Mung beans derived from plants inoculated with *E. coli* O157:H7 were sporadically contaminated with the pathogen. When the beans was sprouted without applying SOC sanitizer, only three of the 10 lots were found to be contaminated with *E. coli* O157:H7 (Table 3). However, none of the sprouts derived from mung beans subjected to SOC treatment tested positive for the pathogen (Table 3).

The majority of mung bean lots (18 of 20) derived from *Salmonella*-inoculated plants tested positive for the bacterium when sprouted over a 4-day period (Table 3). However, no *Salmonella* was recovered from sprouts produced from beans treated with SOC (Table 3).

Through DNA typing, *Salmonella enterica* serovar Meleagridis was found to be the only serovar of the five introduced that was recovered from sprouts derived from naturally contaminated, non-sanitized, mung beans. A bioassay was performed to determine if serovar Meleagridis may have produced an antimicrobial compound to inhibit the growth of the other *Salmonella* within the cocktail. However, no zones of inhibition were noted for any of the combinations of *Salmonella* applied (results not shown).

Table 3 *Salmonella* or *Escherichia coli* O157:H7 recovered from bean sprouts produced from mung beans derived from plants in which the human pathogens were introduced onto flowers

	Mung bean treatment*	Sprouts positive by enrichment (positive/number tested)§
Escherichia coli O157:H7	Non-treated†	3/10
	SOC treated‡	0/10
Salmonella	Non-treated	18/20
	SOC treated	0/20

*Mung beans derived from inoculated plants were pooled and segregated into equal lots.
†Mung beans were soaked in distilled water for 24 h prior to sprouting.
‡Mung bean batches ($n = 3$) soaked in 200 ppm SOC for 24 h prior to sprouting.
§At the end of the 4-day sprouting process, bean sprouts (8–13 g) were enriched for either *E. coli* O157:H7 or *Salmonella*.

Table 2 The effect of introducing contaminated mung beans at different levels on the decontaminating efficacy of stabilized oxychloro-based sanitizer treatment

Level of contaminated mung beans introduced†	Treatment	*Escherichia coli* O157:H7 (positive/number tested)¶	*Salmonella* (positive/number tested)¶
1%	Not treated‡	3/3*	3/3*
	SOC treated§	0/3	0/3
10%	Not treated	3/3	3/3
	SOC treated	0/3	0/3
50%	Not treated	3/3	3/3
	SOC treated	0/3	0/3
100%	Not treated	3/3	3/3
	SOC treated	0/3	0/3
Damaged seed†† 100%	Not treated	8/8	8/8
	SOC treated	0/8	8/8

*Significantly ($P < 0{\cdot}05$) greater number of sprout batches derived from non-treated mung beans tested positive for pathogens compared with those receiving SOC treatment.
†Mung beans were inoculated with a five-strain cocktail of either *Salmonella* or *E. coli* O157:H7. The inoculated mung beans (containing 10^3–10^4 CFU g^{-1}) were introduced to batches of non-inoculated beans (1–50%, w/w) prior to treatment.
‡Mung bean batches ($n = 3$) soaked in distilled water for 24 h prior to sprouting.
§Mung bean batches ($n = 3$) soaked in 200 ppm SOC for 24 h prior to sprouting.
¶At the end of the 4-day sprouting process, bean sprouts (25 g) were enriched for either *E. coli* O157:H7 or *Salmonella*.
††Bean sprouts derived from scarified mung beans inoculated with *Salmonella* or *E. coli* O157:H7 and sprouted over a 4-day period.

Discussion

The study has demonstrated that the efficacy of SOC to inactivate *Salmonella* or *E. coli* O157:H7 was independent of pathogen levels introduced into batches of undamaged beans. It has been reported that human pathogens present at low levels in the presence of a high level of endogenous microflora express stress proteins that provide enhanced tolerance to inimical processes (Dodd and Aldsworth 2002; Komitopoulou *et al.* 2004). Obviously, in the present case even if stress responses were induced within *Salmonella* and *E. coli* O157:H7 this did not affect the efficacy of SOC treatment.

Stabilized oxychloro-based treatment was less effective in inactivating *Salmonella* introduced onto damaged mung beans. This is in agreement with other works that have reported on the difficulty in decontaminating scarified or damaged seed (Charkowski *et al.* 2001). It is likely that by being located within crevices deep within the seed the *Salmonella* were protected from the antimicrobial effects of SOC. The greater susceptibility of *E. coli* O157:H7 to SOC relates to the lower tolerance of the pathogen to the sanitizer. In previous studies it was noted that SOC sanitizer applied at 100 ppm could ensure inactivation of *E. coli* O157:H7 on mung beans compared with 200 ppm required to inactivate *Salmonella* (Kumar *et al.* 2006). Therefore, in the current study it was likely that SOC could penetrate into the bean interior but at an insufficient concentration to inactivate *Salmonella*. Regardless of this fact, the results support the view that scarification or using damaged seed in sprout production should be avoided (Anonymous 1999).

From studies using naturally contaminated seed it was clearly evident that *Salmonella* could become established on beans to a greater extent compared with *E. coli* O157:H7.

The result would suggest that *E. coli* O157:H7 has a lower level of persistence on plants or unable to become associated with developing beans. This may explain why food-borne illness outbreaks linked to sprouts are more commonly associated with *Salmonella* as opposed to *E. coli* O157:H7 (Barak *et al.* 2002). However, it should be noted that in a previous study performed by Cooley *et al.* (2003) it was reported that *E. coli* O157:H7 could contaminate *Arabidopsis* seed to a greater extent compared with *S. enterica* serovar Newport when introduced onto growing plants. Therefore, it is likely that the ability of human pathogens to become established on seeds is plant and strain dependent. In this respect it was interesting to note that from the five *Salmonella* serovars introduced onto the flowers of plants only serovar Meleagridis was recovered from the subsequent sprouts. In a previous study, the same serovar was recovered from sprouts derived from mung beans inoculated with the same *Salmonella* serovar combination (Kumar *et al.* 2006). This may suggest that Meleagridis has physiological attributes that enhance interaction with sprouted seeds or elicits antagonistic effects against other *Salmonella*. The latter seems unlikely as none of the *Salmonella* used in the current study exhibited antagonistic affects in the agar plate bioassay. Therefore, it is possible that Meleagridis could exhibit greater tolerance to environmental stress, higher attachment strength and/or greater growth rates on sprouting seeds. Similar attributes have been implicated in the establishment of *Salmonella* on sprouting alfalfa (Howard and Hutcheson 2003; Barak *et al.* 2005). Whether such factors are associated with Meleagridis is unclear at present but is worthy of further study.

In the current study it was found that SOC treatment could be used to decontaminate naturally contaminated seed. Despite the sporadic occurrence of *E. coli* O157:H7 on mung beans it is questionable whether the pathogen was present on the seed prior to decontamination. However, the fact that no *Salmonella* was recovered from control sprouts strongly indicates that the SOC treatment was a success. In previous studies with naturally contaminated seeds it was reported that 1800–2000 ppm calcium hypochlorite was insufficient to ensure the elimination of *Salmonella* (Fett 2002). Given that the SOC sanitizer could achieve complete elimination of *Salmonella* when applied at 200 ppm underlines the effectiveness of the treatment compared with the recommended hypochlorite-based method. Nevertheless, it is acknowledged that seeds can be contaminated via various routes other than irrigation water. In this respect further studies are warranted on the efficacy of SOC treatment to decontaminate seeds derived from sprout-related outbreaks where the levels and spatial distribution of human pathogens may differ.

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References

Anonymous (1999) Microbiological safety evaluations and recommendations on sprouted seeds. National Advisory Committee on Microbiological Criteria for Foods. *Int J Food Microbiol* **52**, 123–153.

Barak, J.D., Whitehand, L.C. and Charkowski, A.O. (2002) Differences in attachment of *Salmonella enterica* serovars

and *Escherichia coli* O157:H7 to alfalfa sprouts. *Appl Environ Microbiol* **68**, 4758–4763.

Barak, J.D., Gorski, L., Naraghi-Arani, P. and Charkowski, A.O. (2005) *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. *Appl Environ Microbiol* **71**, 5685–5691.

Brooks, J.T., Rowe, S.Y., Shillam, P., Heltzel, D.M., Hunter, S.B., Slutsker, L., Hoekstra, R.M. and Luby, S.P. (2001) *Salmonella* Typhimurium infections transmitted by chlorine-pretreated clover sprout seeds. *Am J Epidemiol* **154**, 1020–1028.

Charkowski, A.O., Sarreal, C.Z. and Mandrell, R.E. (2001) Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. *J Food Prot* **64**, 1292–1298.

Cooley, M.B., Miller, W.G. and Mandrell, R.E. (2003) Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. *Appl Environ Microbiol* **69**, 4915–4926.

Dodd, C.E. and Aldsworth, T.G. (2002) The importance of RpoS in the survival of bacteria through food processing. *Int J Food Microbiol* **74**, 189–194.

Fett, W.F. (2002) Factors affecting the efficacy of chlorine against *Escherichia coli* O157:H7 and *Salmonella* on alfalfa seed. *Food Microbiol* **19**, 135–149.

Fett, W.F. (2006) Inhibition of *Salmonella enterica* by plant-associated pseudomonads *in vitro* and on sprouting alfalfa seed. *J Food Prot* **69**, 719–728.

Fett, W.F., Tortorello, M. and Fu, T. (2005) Seed sprouts: the state of microbiological safety. In *Microbiology of Fresh Produce* ed. Matthews, K.R. pp. 167–219. Washington, DC: ASM Press.

Fu, T., Stewart, D., Reineke, K., Ulaszek, J., Schlesser, J. and Tortorello, M. (2001) Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J Food Prot* **64**, 802–806.

Health Canada (2003) *Health Canada Compendium of Analytical Methods*. Health Canada, Ottawa [WWW document]. URL http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/e_index.html [accessed on 9 March 2006].

Holliday, S.L., Scouten, A.J. and Beuchat, L.R. (2001) Efficacy of chemical treatments in eliminating *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. *J Food Prot* **64**, 1489–1495.

Howard, M.B. and Hutcheson, S.W. (2003) Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Appl Environ Microbiol* **69**, 548–553.

Komitopoulou, E., Bainton, N.J. and Adams, M.R. (2004) Premature *Salmonella* Typhimurium growth inhibition in competition with other Gram-negative organisms is redox potential regulated via RpoS induction. *J Appl Microbiol* **97**, 964–972.

Kumar, M., Hora, R., Kostrzynska, M., Waites, W.M. and Warriner, K. (2006) Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on mung beans, alfalfa and other seed types destined for sprout production by using an oxychloro-based sanitizer. *J Food Prot* **69**, 1571–1578.

Mohle-Boetani, J.C., Farrar, J.A. *et al.* (2001) *Escherichia coli* O157 and *Salmonella* infections associated with sprouts in California, 1996–1998. *Ann Intern Med* **135**, 239–247.

Montville, R. and Schaffner, D. (2005) Monte Carlo simulation of pathogen behaviour during the sprout production process. *Appl Environ Microbiol* **71**, 746–753.

Sata, S., Fujisawa, T., Osawa, R., Iguchi, A., Yamai, S. and Shimada, T. (2003) An improved enrichment broth for isolation of *Escherichia coli* O157, with specific reference to starved cells, from radish sprouts. *Appl Environ Microbiol* **69**, 1858–1860.

Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.

Wessinger, W.R. and Beuchat, L.R. (2000) Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. *J Food Prot* **63**, 1475–1482.

Winthrop, K.L., Palumbo, M.S., Farrar, J.A., Mohle-Boetani, J.C., Abbott, S., Beatty, M.E., Inami, G. and Werner, S. B. (2003) Alfalfa sprouts and *Salmonella* Kottbus infection: a multistate outbreak following inadequate seed disinfection with heat and chlorine. *J Food Prot* **66**, 13–17.

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ORIGINAL ARTICLE

Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on artificially or naturally contaminated mung beans (*Vigna radiata* L) using a stabilized oxychloro-based sanitizer

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Abstract

Aims: To evaluate the efficacy of a stabilized oxychloro-based (SOC) sanitizer to decontaminate mung beans artificially or naturally contaminated with *Escherichia coli* O157:H7 or *Salmonella*.

Methods and Results: Naturally contaminated beans were produced by introducing a five-strain cocktail of *E. coli* O157:H7 or *Salmonella* onto the flowers of growing mung bean plants. *Escherichia coli* O157:H7 was only sporadically recovered from sprout lots (three testing positive from 10 tested) derived from harvested beans. In contrast, *Salmonella* was recovered from 18 of 20 lots screened. Pathogens present on naturally contaminated seed could be successfully inactivated with SOC applied at 200 ppm for 24 h at 28°C. SOC treatment could also decontaminate artificially inoculated mung bean batches containing different levels of contaminated seed. SOC inactivated *E. coli* O157:H7, but not *Salmonella* introduced onto damaged (scarified) beans.

Conclusions: SOC sanitizer could inactivate *Salmonella* or *E. coli* O157:H7 naturally or artificially introduced onto mung beans. However, the SOC treatment failed to inactivate *Salmonella* introduced onto damaged mung beans.

Significance and Impact of the Study: SOC sanitizer represents an effective method for decontaminating undamaged mung beans.

Introduction

Over the last decade there has been numerous food-borne illness outbreaks linked to sprouted seeds such as alfalfa and bean sprouts (Fett *et al.* 2005). *Salmonella* is the most frequently encountered human pathogen on sprouts although cases involving *Escherichia coli* O157:H7 have also been reported (Mohle-Boetani *et al.* 2001; Fett *et al.* 2005). In the majority of outbreaks the seed used for sprout production has been shown to be the source of human pathogens (Anonymous 1999). Therefore, it was recommended that seeds should be treated with 20 000 ppm calcium hypochlorite prior to soaking and subsequently sprouting (Anonymous 1999). However, it is widely acknowledged that treatments based on hypochlorite or alternative sanitizing agents (e.g. peroxyacetic acid, hydrogen peroxide) can only reduce pathogen levels on seeds but cannot ensure complete elimination (Anonymous 1999; Wessinger and Beuchat 2000; Brooks *et al.* 2001). This is relevant considering that even relatively low levels of surviving pathogens (<0·1 CFU g^{-1}) can grow to densities in excess of 6 log CFU g^{-1} within 48 h into the sprouting process (Holliday *et al.* 2001).

A successful decontamination method has been devised based on supplementing the soak water used in the early stages of sprout production with a stabilized oxychloro-based sanitizer (SOC) (Kumar *et al.* 2006). A previous study has demonstrated that SOC applied at 200 ppm for >19 h can consistently inactivate either *E. coli* O157:H7 or *Salmonella* introduced onto mung beans (Kumar *et al.* 2006). However, the efficacy of SOC to decontaminate naturally contaminated or damaged seed has yet to be

determined. In both cases it is possible that low levels of pathogens can become located in protective sites within the seed inaccessible to sanitizing agents and significantly reduce the efficacy of decontamination treatments (Brooks *et al.* 2001; Charkowski *et al.* 2001; Fu *et al.* 2001; Howard and Hutcheson 2003; Winthrop *et al.* 2003; Montville and Schaffner 2005).

In the following study the efficacy of SOC sanitizer to inactivate pathogens (*Salmonella* and *E. coli* O157:H7) when introduced at low levels or on damaged mung beans was evaluated. In addition, batches of naturally contaminated mung beans were produced by introducing the pathogens onto the flowers of growing plants from which the seed pods emerged. The choice of flowers as the inoculation site was selected to mimic the effect of contaminated irrigation water which is considered to be an important source of human pathogens present on seeds (Anonymous 1999).

Materials and methods

Bacterial strains and preparation of inocula

Escherichia coli O157:H7 and *Salmonella* used in the study were composed of environmental, clinical, tomato or sprout isolates (Table 1). An aliquot (1 ml) of an overnight culture of the individual *E. coli* O157:H7 or *Salmonella* strains was transferred into 50 ml of tryptic soy broth (Difco, Sparks, MD, USA) and incubated for 24 h at 37°C. Bacterial cells were harvested by centrifugation (5500 *g* for 10 min, 4°C) and washed once in 0·8% saline. The final cell pellet was re-suspended in saline to a final cell density of 10^6 CFU ml^{-1}. Equal volumes of the five different *E. coli* O157:H7 or *Salmonella* suspensions were then combined to produce a cocktail that was subsequently used to inoculate beans or flowers of mung bean plants.

Inoculation of undamaged and damaged mung beans

Mung beans (*Vigna radiata*) were purchased from Mumms Seeds Ltd (Parkside, SK, Canada). Beans (250 g) were soaked in 250 ml of the five-strain cocktail of *E. coli* O157:H7 or *Salmonella* for 20 min. The seeds were then transferred to sterile filter paper within a biological safety cabinet and allowed to dry at ambient temperature for 48–60 h. The inoculated mung beans were then introduced into 100-g batches of uninoculated beans at different levels (1–50%, w/w) and manually mixed prior to sprouting. For example, 1 g of inoculated seeds was mixed with 99 g of non-inoculated seed to give an inoculation level of 1% (w/w).

For damaged seed, mung beans (in 5-g lots) were placed between sheets of abrasive paper and rubbed with moderate pressure for *c.* 10 s to produce visible scarification. The damaged mung beans (100 g) were then soaked in bacterial suspension as described above and sprouted directly without mixing with non-inoculated seed.

Cultivation and inoculation of mung bean plants

Mung beans (non-inoculated) were soaked in distilled water at 30°C overnight to stimulate germination prior to transplanting into plug trays containing commercial grade PGX soil (Professional plug and germination growing medium; Premier Horticulture Ltd, Dorval, QC, Canada). The trays were transferred to a sealed growth room maintained at 25°C with a relative humidity set at 90%. After 2 weeks the seedlings were transplanted into 2-l soil microcosms containing commercial grade BX soil (Professional general purpose growing medium, Premier Horticulture Ltd). The relative humidity within the growth room was maintained at 90% with 12/12 h day/night at 25/10°C respectively. Flowers started to appear on plants 7–8 weeks into the cultivation period. The calyx of individual flowers was inoculated with a single 0·1 ml aliquot of *E. coli* O157:H7 or *Salmonella* (10^6 CFU ml^{-1}). The plants (12 inoculated with *E. coli* O157 and 24 with *Salmonella*) were cultivated for a further period (6–8 weeks) to enable seed pod development and ripening. Seed pods were manually harvested using sterile gloved hands and transferred to the laboratory in sterile bags. The beans were extracted from pods using a sterile scalpel blade and pooled prior to subdividing into 0·7–1·2 g lots (40 lots

Table 1 *Escherichia coli* O157:H7 and *Salmonella* strains/serovars used in the study

Escherichia coli O157:H7 strain*	Source	*Salmonella enterica* serovar	Source
E. coli O157:H7 C1033	Water sediment	Meleagridis E1†	Alfalfa sprouts
E. coli O157:H7 C1032	Soil	Oranienburg C1†	Alfalfa sprouts
E. coli O157:H7 C652	Clinical	Newport C2†	Alfalfa sprouts
E. coli O157:H7 C476	Clinical	Senftenburg†	Alfalfa sprouts
E. coli O157:H7 C477	Clinical	Montevideo*	Tomatoes

*Strains obtained from Canadian Research Institute for Food Safety, University of Guelph, Guelph, ON, Canada.
†Strains donated by Dr C. Poppe, Health Canada, Guelph, ON, Canada.

for *Salmonella* and 20 for *E. coli* O157:H7). Half the seed lots (for each pathogen type) were treated using SOC sanitizer with the remaining acting as non-treated controls.

SOC treatment and sprouting of mung beans

The different mung bean batches (i.e. naturally contaminated, damaged or those containing inoculated seed introduced at different levels) were soaked for 24 h at 28°C in 200 ppm SOC (commercial name Germin-8-or; Vernagene Ltd, Bolton, Lancashire, UK) using a ratio of 1 part beans (dry weight) to 5 parts sanitizer (Kumar *et al.* 2006). The seeds were then removed, rinsed with distilled water prior to germinating for a further 4 days at 28°C. The developing sprouts were watered daily by a 5-min soak in 500 ml of sterile distilled water. Control (non-decontaminated) mung bean batches were sprouted using the same method except the SOC solution was replaced with distilled water.

Microbiological analysis

For artificially inoculated mung beans, microbiological analysis was performed on both the inoculated seeds and sprouts. Duplicate samples (1 g) of inoculated seed were suspended in 9 ml of buffered peptone water (0·1%, Oxoid, Basingstoke, UK) and vortexed for 1 min. *Salmonella* was enumerated on Brilliant Green agar (BG; Oxoid) incubated at 42°C for 24 h. *Escherichia coli* O157:H7 was enumerated on Sorbitol Maconkey agar containing cefixime and tellurite (CT-SMAC; Difco) incubated at 37°C for 24 h.

For sprouts, duplicate 25 g samples were suspended in 225 ml buffered peptone water and stomached for 90 s at 230 rev min^{-1} (Model 400; A.J. Steward and Co., London, UK). The sprout homogenates were tested for the presence of *Salmonella* using the method described in the Canadian Compendium of Analytical Method, MFHPB 20 (Health Canada 2003). Sprouts were suspended in 225 ml buffered peptone water and incubated at 42°C for 24 h. An aliquot (0·1 ml) of the enriched culture was then inoculated into the centre of a semi-solid Rappaport–Vassiliadis plate (Oxoid) that was subsequently incubated at 37°C for 24 h. Cells from the outer perimeter of the growth halo (presumptive motile *Salmonella*) were streaked onto BG agar (Oxoid) and incubated at 37°C overnight. The Oxoid *Salmonella* Latex Test FT0203 was used for serological confirmation of typical colonies (i.e. red colonies surrounded by brilliant red zones).

Sprouts were enriched for *E. coli* O157:H7 using buffered peptone water containing 0·5% (w/v) sodium thioglycolate incubated at 37°C for 24 h (Sata *et al.* 2003). Aliquots (10 μl) of the enriched culture were then streaked onto CT-SMAC that was subsequently incubated at 37°C for 24 h. Typical colonies (colourless) were confirmed as *E. coli* O157:H7 using the Oxoid *E. coli* O157 Latex Test DR0620M.

Sprouts from mung beans derived from inoculated plants were screened for the presence of pathogens using the same methods highlighted above. However, because of sprouting of the small bean lots the entire batch of sprouts (8–13 g) was analysed.

DNA typing of *Salmonella*

Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR (Versalovic *et al.* 1991) was used to identify which of the five strains of *Salmonella* serovar present on sprouts upon completion of the sprouting process. Up to five colonies from the positive selective agar plates were picked onto Luria–Bertani (LB; Difco) agar that was subsequently incubated at 37°C for 24 h. Single colonies were then suspended in 0·2 ml of TE buffer heated at 100°C to lyse cells. Cell debris were removed by centrifugation (13 000 *g*) and the supernatant, containing DNA, decanted into a new Eppendorf tube. ERIC-PCR was performed using the method described by Kumar *et al.* (2006). DNA patterns were analysed and dice similarity coefficients calculated using Molecular Analyst Software, Bio-Rad Fingerprinting II version 3·0 (Bio-Rad Laboratories, Hercules, CA, USA).

Bioassay for antimicrobial metabolites produced by *Salmonella*

A bioassay was performed (as described by Fett 2006) to determine the antagonistic activity exhibited by individual *Salmonella* serovars used in the study. Briefly, aliquots (20 μl) of an overnight culture of the test *Salmonella* was spotted onto LB agar and incubated at 30°C for 24 h. The agar plate was then exposed to chloroform vapour for 1 h at room temperature within a fume hood (Protector, VWR, Mississauga, ON, Canada). Each plate was then overlaid with 6 ml molten water agar (6 g agar l^{-1}) containing 60 μl of an overnight suspension of the appropriate *Salmonella* serovar. The agar was allowed to solidify prior to incubating plates at 30°C for 24 h. The plates were then removed and visually inspected for zones of inhibition.

Data analysis

Qualitative data were statistically analysed using 2×2 contingency tables (S-PLUS, Insightful Corp., New York, NY, USA). In all cases the significance level was set at $P \leq 0{\cdot}05$.

Results

Inactivation of *E. coli* O157:H7 and *Salmonella* inoculated onto undamaged or damaged mung beans

When the inoculated beans were introduced into batches of non-inoculated beans and sprouted the subsequent bean sprouts tested positive for *Salmonella* or *E. coli* O157:H7 (Table 2). However, no pathogens were recovered from sprouts derived from SOC-treated beans (Table 2). *Escherichia coli* O157:H7 introduced onto damaged beans could also be successfully inactivated by treating with 200 ppm SOC. However, SOC treatment failed to decontaminate damaged seeds inoculated with *Salmonella*, with all the subsequent sprout samples testing positive for the enteric pathogen (Table 2).

Decontamination of naturally contaminated seed

Mung beans derived from plants inoculated with *E. coli* O157:H7 were sporadically contaminated with the pathogen. When the beans was sprouted without applying SOC sanitizer, only three of the 10 lots were found to be contaminated with *E. coli* O157:H7 (Table 3). However, none of the sprouts derived from mung beans subjected to SOC treatment tested positive for the pathogen (Table 3).

The majority of mung bean lots (18 of 20) derived from *Salmonella*-inoculated plants tested positive for the bacterium when sprouted over a 4-day period (Table 3). However, no *Salmonella* was recovered from sprouts produced from beans treated with SOC (Table 3).

Through DNA typing, *Salmonella enterica* serovar Meleagridis was found to be the only serovar of the five introduced that was recovered from sprouts derived from naturally contaminated, non-sanitized, mung beans. A bioassay was performed to determine if serovar Meleagridis may have produced an antimicrobial compound to inhibit the growth of the other *Salmonella* within the cocktail. However, no zones of inhibition were noted for any of the combinations of *Salmonella* applied (results not shown).

Table 3 *Salmonella* or *Escherichia coli* O157:H7 recovered from bean sprouts produced from mung beans derived from plants in which the human pathogens were introduced onto flowers

	Mung bean treatment*	Sprouts positive by enrichment (positive/number tested)§
Escherichia coli O157:H7	Non-treated†	3/10
	SOC treated‡	0/10
Salmonella	Non-treated	18/20
	SOC treated	0/20

*Mung beans derived from inoculated plants were pooled and segregated into equal lots.

†Mung beans were soaked in distilled water for 24 h prior to sprouting.

‡Mung bean batches ($n = 3$) soaked in 200 ppm SOC for 24 h prior to sprouting.

§At the end of the 4-day sprouting process, bean sprouts (8–13 g) were enriched for either *E. coli* O157:H7 or *Salmonella*.

Table 2 The effect of introducing contaminated mung beans at different levels on the decontaminating efficacy of stabilized oxychloro-based sanitizer treatment

Level of contaminated mung beans introduced†	Treatment	*Escherichia coli* O157:H7 (positive/number tested)¶	*Salmonella* (positive/number tested)¶
1%	Not treated‡	3/3*	3/3*
	SOC treated§	0/3	0/3
10%	Not treated	3/3	3/3
	SOC treated	0/3	0/3
50%	Not treated	3/3	3/3
	SOC treated	0/3	0/3
100%	Not treated	3/3	3/3
	SOC treated	0/3	0/3
Damaged seed†† 100%	Not treated	8/8	8/8
	SOC treated	0/8	8/8

*Significantly ($P < 0{\cdot}05$) greater number of sprout batches derived from non-treated mung beans tested positive for pathogens compared with those receiving SOC treatment.

†Mung beans were inoculated with a five-strain cocktail of either *Salmonella* or *E. coli* O157:H7. The inoculated mung beans (containing 10^3–10^4 CFU g^{-1}) were introduced to batches of non-inoculated beans (1–50%, w/w) prior to treatment.

‡Mung bean batches ($n = 3$) soaked in distilled water for 24 h prior to sprouting.

§Mung bean batches ($n = 3$) soaked in 200 ppm SOC for 24 h prior to sprouting.

¶At the end of the 4-day sprouting process, bean sprouts (25 g) were enriched for either *E. coli* O157:H7 or *Salmonella*.

††Bean sprouts derived from scarified mung beans inoculated with *Salmonella* or *E. coli* O157:H7 and sprouted over a 4-day period.

Discussion

The study has demonstrated that the efficacy of SOC to inactivate *Salmonella* or *E. coli* O157:H7 was independent of pathogen levels introduced into batches of undamaged beans. It has been reported that human pathogens present at low levels in the presence of a high level of endogenous microflora express stress proteins that provide enhanced tolerance to inimical processes (Dodd and Aldsworth 2002; Komitopoulou *et al.* 2004). Obviously, in the present case even if stress responses were induced within *Salmonella* and *E. coli* O157:H7 this did not affect the efficacy of SOC treatment.

Stabilized oxychloro-based treatment was less effective in inactivating *Salmonella* introduced onto damaged mung beans. This is in agreement with other works that have reported on the difficulty in decontaminating scarified or damaged seed (Charkowski *et al.* 2001). It is likely that by being located within crevices deep within the seed the *Salmonella* were protected from the antimicrobial effects of SOC. The greater susceptibility of *E. coli* O157:H7 to SOC relates to the lower tolerance of the pathogen to the sanitizer. In previous studies it was noted that SOC sanitizer applied at 100 ppm could ensure inactivation of *E. coli* O157:H7 on mung beans compared with 200 ppm required to inactivate *Salmonella* (Kumar *et al.* 2006). Therefore, in the current study it was likely that SOC could penetrate into the bean interior but at an insufficient concentration to inactivate *Salmonella*. Regardless of this fact, the results support the view that scarification or using damaged seed in sprout production should be avoided (Anonymous 1999).

From studies using naturally contaminated seed it was clearly evident that *Salmonella* could become established on beans to a greater extent compared with *E. coli* O157:H7. The result would suggest that *E. coli* O157:H7 has a lower level of persistence on plants or unable to become associated with developing beans. This may explain why food-borne illness outbreaks linked to sprouts are more commonly associated with *Salmonella* as opposed to *E. coli* O157:H7 (Barak *et al.* 2002). However, it should be noted that in a previous study performed by Cooley *et al.* (2003) it was reported that *E. coli* O157:H7 could contaminate *Arabidopsis* seed to a greater extent compared with *S. enterica* serovar Newport when introduced onto growing plants. Therefore, it is likely that the ability of human pathogens to become established on seeds is plant and strain dependent. In this respect it was interesting to note that from the five *Salmonella* serovars introduced onto the flowers of plants only serovar Meleagridis was recovered from the subsequent sprouts. In a previous study, the same serovar was recovered from sprouts derived from mung beans inoculated with the same *Salmonella* serovar combination (Kumar *et al.* 2006). This may suggest that Meleagridis has physiological attributes that enhance interaction with sprouted seeds or elicits antagonistic effects against other *Salmonella*. The latter seems unlikely as none of the *Salmonella* used in the current study exhibited antagonistic affects in the agar plate bioassay. Therefore, it is possible that Meleagridis could exhibit greater tolerance to environmental stress, higher attachment strength and/or greater growth rates on sprouting seeds. Similar attributes have been implicated in the establishment of *Salmonella* on sprouting alfalfa (Howard and Hutcheson 2003; Barak *et al.* 2005). Whether such factors are associated with Meleagridis is unclear at present but is worthy of further study.

In the current study it was found that SOC treatment could be used to decontaminate naturally contaminated seed. Despite the sporadic occurrence of *E. coli* O157:H7 on mung beans it is questionable whether the pathogen was present on the seed prior to decontamination. However, the fact that no *Salmonella* was recovered from control sprouts strongly indicates that the SOC treatment was a success. In previous studies with naturally contaminated seeds it was reported that 1800–2000 ppm calcium hypochlorite was insufficient to ensure the elimination of *Salmonella* (Fett 2002). Given that the SOC sanitizer could achieve complete elimination of *Salmonella* when applied at 200 ppm underlines the effectiveness of the treatment compared with the recommended hypochlorite-based method. Nevertheless, it is acknowledged that seeds can be contaminated via various routes other than irrigation water. In this respect further studies are warranted on the efficacy of SOC treatment to decontaminate seeds derived from sprout-related outbreaks where the levels and spatial distribution of human pathogens may differ.

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References

Anonymous (1999) Microbiological safety evaluations and recommendations on sprouted seeds. National Advisory Committee on Microbiological Criteria for Foods. *Int J Food Microbiol* **52**, 123–153.

Barak, J.D., Whitehand, L.C. and Charkowski, A.O. (2002) Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. *Appl Environ Microbiol* **68**, 4758–4763.

Barak, J.D., Gorski, L., Naraghi-Arani, P. and Charkowski, A.O. (2005) *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. *Appl Environ Microbiol* **71**, 5685–5691.

Brooks, J.T., Rowe, S.Y., Shillam, P., Heltzel, D.M., Hunter, S.B., Slutsker, L., Hoekstra, R.M. and Luby, S.P. (2001) *Salmonella* Typhimurium infections transmitted by chlorine-pretreated clover sprout seeds. *Am J Epidemiol* **154**, 1020–1028.

Charkowski, A.O., Sarreal, C.Z. and Mandrell, R.E. (2001) Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. *J Food Prot* **64**, 1292–1298.

Cooley, M.B., Miller, W.G. and Mandrell, R.E. (2003) Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. *Appl Environ Microbiol* **69**, 4915–4926.

Dodd, C.E. and Aldsworth, T.G. (2002) The importance of RpoS in the survival of bacteria through food processing. *Int J Food Microbiol* **74**, 189–194.

Fett, W.F. (2002) Factors affecting the efficacy of chlorine against *Escherichia coli* O157:H7 and *Salmonella* on alfalfa seed. *Food Microbiol* **19**, 135–149.

Fett, W.F. (2006) Inhibition of *Salmonella enterica* by plant-associated pseudomonads *in vitro* and on sprouting alfalfa seed. *J Food Prot* **69**, 719–728.

Fett, W.F., Tortorello, M. and Fu, T. (2005) Seed sprouts: the state of microbiological safety. In *Microbiology of Fresh Produce* ed. Matthews, K.R. pp. 167–219. Washington, DC: ASM Press.

Fu, T., Stewart, D., Reineke, K., Ulaszek, J., Schlesser, J. and Tortorello, M. (2001) Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J Food Prot* **64**, 802–806.

Health Canada (2003) *Health Canada Compendium of Analytical Methods*. Health Canada, Ottawa [WWW document]. URL http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/e_index.html [accessed on 9 March 2006].

Holliday, S.L., Scouten, A.J. and Beuchat, L.R. (2001) Efficacy of chemical treatments in eliminating *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. *J Food Prot* **64**, 1489–1495.

Howard, M.B. and Hutcheson, S.W. (2003) Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Appl Environ Microbiol* **69**, 548–553.

Komitopoulou, E., Bainton, N.J. and Adams, M.R. (2004) Premature *Salmonella* Typhimurium growth inhibition in competition with other Gram-negative organisms is redox potential regulated via RpoS induction. *J Appl Microbiol* **97**, 964–972.

Kumar, M., Hora, R., Kostrzynska, M., Waites, W.M. and Warriner, K. (2006) Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on mung beans, alfalfa and other seed types destined for sprout production by using an oxychloro-based sanitizer. *J Food Prot* **69**, 1571–1578.

Mohle-Boetani, J.C., Farrar, J.A., Werner, S.B., Minassian, D., Bryant, R., Abbott, S., Slutsker, L. and Vugia, D.J. (2001) *Escherichia coli* O157 and *Salmonella* infections associated with sprouts in California, 1996–1998. *Ann Intern Med* **135**, 239–247.

Montville, R. and Schaffner, D. (2005) Monte Carlo simulation of pathogen behaviour during the sprout production process. *Appl Environ Microbiol* **71**, 746–753.

Sata, S., Fujisawa, T., Osawa, R., Iguchi, A., Yamai, S. and Shimada, T. (2003) An improved enrichment broth for isolation of *Escherichia coli* O157, with specific reference to starved cells, from radish sprouts. *Appl Environ Microbiol* **69**, 1858–1860.

Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.

Wessinger, W.R. and Beuchat, L.R. (2000) Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. *J Food Prot* **63**, 1475–1482.

Winthrop, K.L., Palumbo, M.S., Farrar, J.A., Mohle-Boetani, J.C., Abbott, S., Beatty, M.E., Inami, G. and Werner, S. B. (2003) Alfalfa sprouts and *Salmonella* Kottbus infection: a multistate outbreak following inadequate seed disinfection with heat and chlorine. *J Food Prot* **66**, 13–17.

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Composite *versus* single sampling of spent irrigation water to assess the microbiological status of sprouting mung bean beds

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Abstract

BACKGROUND: Spent irrigation water testing has been recommended in sprouted seed production to detect the presence of pathogens. However, because of the heterogeneous distribution of contamination within batches of sprouted seed, taking single samples of spent irrigation water may return false-negative results. The following evaluated whether spent irrigation water collected from multiple points provided a more representative assessment of the microbiological status of the sprouting mung bean bed compared to when single samples were taken.

RESULTS: Generic *Escherichia coli* or *Aeromonas* was recovered in one and 10 of the 160 sprout samples taken from 32 sprouting mung bean batches, respectively. Composite spent irrigation water samples tested positive for generic *E. coli* on 19 occasions compared to 12 when single samples were taken. Mesophilic *Aeromonas* was detected in 13 composite spent irrigation water samples which compared to eight single samples. The prevalence of either target bacterium in composite spent irrigation water samples was not significantly ($P > 0.05$) different compared to when a single sample was collected.

CONCLUSIONS: Sampling spent irrigation water from multiple points under sprouting mung bean beds does not significantly increase the probability of detecting contamination, if present. The findings of the study should be considered when devising sampling plans for spent irrigation water testing in bean sprout production.

Keywords: mung bean sprouts; spent irrigation water; testing; *Salmonella*; *Escherichia coli*; *Aeromonas*

INTRODUCTION

Sprouted seeds, such as bean sprouts, have been implicated in numerous outbreaks of foodborne illness.[1,2] The largest outbreak of foodborne illness linked to bean sprouts occurred within Ontario in 2005 and resulted in over 600 clinical cases of salmonellosis (Canadian Food Inspection Agency, http://www.inspection.gc.ca, 7 March 2007). To address the increasing number of foodborne illness outbreaks associated with sprouts, the US Food and Drug Administration issued guidelines to enhance food safety standards.[3] Amongst other measures, the guidelines recommend screening spent irrigation water collected 48 h into the sprouting process to screen for the presence of pathogens such as *Salmonella* and *Escherichia coli* O157:H7.[4] The screening of spent irrigation water is preferred over testing sprouts directly due to uniformity, ease of collection and analysis.[3] More significantly, because irrigation water runs over the sprouts it is considered to provide a more representative assessment of the microbiological status of the sprouting seedbed compared to when individual sprout samples are screened.[3]

Current recommendations state that a single spent irrigation water sample should be taken from individual sprouted seed batches.[3] Screening single spent irrigation water samples has been considered satisfactory for sprouts (e.g., alfalfa) produced within rotating drums where the seeds are distributed as a monolayer and irrigation water intimately mixed with the sprouting seeds.[4,5] However, a study performed by Liu and Schaffner[6] illustrated that spent irrigation water sampling for alfalfa sprouted on trays is more problematic owing to the heterogeneous distribution of contamination. Here, contamination introduced via seed at a single point within a batch of sprouting alfalfa seed fails to disseminate through the entire bed and hence is restricted to localized sites. Consequently, when spent irrigation water is taken from a distance (>20 cm in the reported study)[6] from the original contamination site

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the probability of detecting the target bacteria is significantly reduced. The problems associated with the heterogeneous distribution of contamination are more significant when attempting to devise sampling plans for screening spent irrigation water derived from mung bean sprout production.[7] Unlike alfalfa, mung beans are sprouted in large quantities (25–75 kg) within deep bins and hence the distribution of microbial populations are more heterogeneous. This was confirmed by the findings of Hora *et al.*,[7] who reported on the distribution of opportunistic contaminants (generic *Escherichia coli* and mesophilic *Aeromonas*) within 25 kg batches of sprouting mung bean sprouts. The selection of generic *E. coli* and *Aeromonas* to assess the distribution of contamination within sprouting seed batches was based on the fact that both bacterial types are occasionally recovered from sprout batches.[7] Therefore, similar to human pathogens the bacteria would be present on a proportion of the seed and spread though the bed during the course of the sprouting process. Screening for pathogens, such as *Salmonella*, directly would have been problematic owing to the sporadic nature in which they are encountered and also the ethical issues associated with detecting a positive sample. In the study by Hora *et al.*,[7] three spent irrigation water samples were collected (left, center and right side of the bed) in addition to corresponding sprout samples at different depths over the sampling point. It was found that contamination was localized within the sprouting mung bean bed and consequently screening a single sample of irrigation water for pathogens would be unreliable in terms of assessing the microbiological status of the bed.[7] Laboratory-based trials using beans inoculated with either *Salmonella* or *E. coli* O157:H7 confirmed that contamination introduced at a specific point in the bed remains localized and does not spread throughout the sprout batch within 48 h into the sprouting process.[7]

A potential approach to address the heterogeneous distribution of contamination within sprouting mung bean batches is to take multiple spent irrigation water samples. However, increasing the number of samples will significantly add to the cost of testing, which would be commercially unfeasible in the majority of sprout operations. A more practical approach is to form a composite from multiple samples, thereby enabling a single test to be performed. Although composite sampling in microbiological analysis can reduce the cost of testing, there are disadvantages in terms of diluting contamination to below the level of detection.[8]

The objective of the following study was to compare composite *versus* single sampling of spent irrigation water derived from sprouting mung bean beds. To address the issues relating to dilution of contamination by composite sampling the individual samples were enriched prior to preparing composites. Although this approach increased the number of steps in sample analysis the enrichment step is relatively less costly than selective detection and hence would not significantly add to the expense of testing.

MATERIALS AND METHODS

Description of the commercial sprout house and sprouting process

The commercial sprout facility sprouted 75 kg lots of mung beans within stainless steel bins (1.5 m^3) with narrow slits on the base of the bed to permit drainage of excess water. The mung beans were prepared for sprouting by an initial rinse in municipal water prior to loading into the sprouting bins. No seed sanitation step was included in the preparation of beans prior to sprouting. Irrigation water was delivered from a moving overhead shower every 3 h. The temperature of the growth room was maintained at 23 °C with filtered fresh air being constantly introduced. Between batches of sprouts, the growth rooms and bins were sanitized in accordance with Good Manufacturing Practice.

Spent irrigation water and sprout sample collection

Spent irrigation water for preparing composite samples was collected 48 h into the sprouting period using 10 × 100 mL sterile bottles (Pharm-Fisher, Ottawa, ON, Canada) positioned at random locations under the bed. In parallel, a 500 mL sample was collected from under the bed at a randomly selected location. In all cases the sampling bottles were temporally fixed in place using denture adhesive purchased from a local supermarket. Samples (500 mL) of incoming municipal water were also collected directly from the overhead shower. Five 100 g sprout samples were collected at random locations on and within the sprout bed and placed in sterile bags. All the samples were transferred to a portable cooler and processed within 24 h.

Microbiological analysis

Composite samples were prepared by subdividing the 100 mL water samples into two 45 mL volumes. Peptone water (Oxoid, Basingstoke, UK; 5mL, 10× concentration) was added to one set of samples and incubated overnight at 42 °C to enrich for generic *E. coli*. Alkaline peptone water (5 mL, pH 8, 10× concentration containing 100 $\mu g\,mL^{-1}$ ampicillin) was added to the second set of subsamples and incubated overnight at 37 °C to enrich for mesophilic *Aeromonas*. Upon completion of the incubation period the respective enriched cultures were combined to form a 500 mL composite sample. The single spent irrigation water sample was subdivided into two 225 mL samples and supplemented with either 25 mL of 10× concentration peptone water or alkaline peptone as described above.

Sprout samples (25 g) were suspended in 225 mL peptone water prior to incubating at 42 °C to enrich for generic *E. coli*. or *Aeromonas*, 225 mL alkaline peptone water containing 100 $\mu g\,mL^{-1}$ ampicillin was added to 25 g sprouts and incubated at 37 °C for 24 h.

Aeromonas in the enriched sprout or spent irrigation water samples was isolated by preparing a dilution series of the enriched samples in Butterfield's

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phosphate diluent (BPD; Oxoid). Aliquots (0.1 mL) of the 10^{-4} to 10^{-6} dilutions were spread plated onto the surface of starch ampicillin agar (Oxoid) plates prior to incubating at 37 °C for 18–24 h. Confirmation of *Aeromonas* isolates was performed using triple sugar iron agar slants and amino acids, and oxidase-positive reaction.[9]

Generic *E. coli* was detected in the enriched samples by preparing a dilution series in BPD that were subsequently dispensed on *E. coli*/coliform Petri films (3M, London, ON, Canada). The Petri films were subsequently incubated at 37 °C for 24 h and examined for typical colonies (purple/black with gas).

Statistical analysis

Sampling trials at a commercial sprout grower were performed two or three times a week over an 11-week period (32 sprout beds sampled in total). On each visit, sprouts and spent irrigation water samples were taken from two randomly selected bins contained within the same growth room. Microbiological data were reported as presence or absence in duplicate enriched sprouts. The composite and single spent irrigation water samples were also reported as presence/absence for generic *E. coli* or mesophilic *Aeromonas*. The qualitative data derived from the commercial sprout sampling trial were analyzed using contingency table analysis (S-Plus, Insightful Corp., NY, USA).

RESULTS

Generic *E. coli* and mesophilic *Aeromonas* were consistently recovered from spent irrigation water over the 11-week sampling period. From composite sampling 19 of the 32 beds screened tested positive for generic *E. coli* (Table 1). In comparison, 12 single spent irrigation water samples tested positive for generic *E. coli*, which is not significantly ($P = 0.08$) different compared to when composite sampling was performed (Table 1). On six occasions the composite sample tested positive for generic *E. coli* and the single spent irrigation water negative despite being derived from the same sprout bin. However, in one sample set the single spent irrigation water tested positive but the composite negative (Table 1). Only one sprout sample from a total of 160 screened tested positive for generic *E. coli*, which was significantly ($P < 0.05$) lower compared to the prevalence of the indicator bacterium in spent irrigation water.

Mesophilic *Aeromonas* was recovered from 13 beds sampled when performing composite sampling, which is not significantly ($P = 0.183$) different compared to when single samples were screened (Table 1). By using composite sampling, the spent irrigation water from nine beds tested positive for *Aeromonas* but not when single samples were screened. In contrast, four single spent irrigation water samples tested positive for the bacterium with the composite sampling returning negative results.

Sprout samples taken from 10 sprouting mung bean beds tested positive for *Aeromonas*. In two beds, *Aeromonas* was recovered in all sprout samples tested but was only recovered in one to three samples in the other sprouting seed beds testing positive. This would confirm that the distribution of *Aeromonas* was heterogeneous. In two mung bean beds *Aeromonas* was detected in the sprout samples screened but not in either the single or composite spent irrigation water samples. However, overall there was no significant ($P > 0.05$) difference between the number of positive *Aeromonas* in sprout and spent irrigation water samples.

DISCUSSION

In total, generic *E. coli* was recovered from 20 (63%) of the spent irrigation water derived from 32 individual sprouting mung bean beds sampled, which compares with 57% found in a previous study from a commercial sprout producer.[7] The prevalence of generic *E. coli* associated with sprouts was 0.63%, which compares to 4% reported for sprouts sampled at retail[10] or 5% for sprouts sampled 48 h into the sprouting process.[7]

Mesophilic *Aeromonas* was recovered in 41% of spent irrigation water and, collectively, 15% of sprout samples taken from sprouting mung bean beds. In comparison, the prevalence of mesophilic *Aeromonas* in spent irrigation water and sprouts in a previous study was 79% and 39% respectively.[7]

The origins of *E. coli* and *Aeromonas* were not identified in the current study. The incoming water used to irrigate sprouts tested negative for both bacterial types, although it is possible that environmental sources contributed to the microflora of sprout beds. However, it is well established that the seed used for sprouting is a common source of contamination recovered from sprouts.[3] Regardless of the origins, the prevalence of generic *E. coli* and mesophilic *Aeromonas* it can be confirmed that spent irrigation water provides a more reliable index of the microbiological status of sprouting mung bean beds compared to sprouts.[7] Nevertheless, it was interesting to note that on several occasions sprouts tested positive for mesophilic *Aeromonas* but negative in spent irrigation water. Therefore, although spent irrigation water provides a more reliable assessment of the microbiological status of sprouting mung bean beds it should not be used as a sole intervention to prevent contaminated products reaching the market.

The main objective of the study was to evaluate whether composite spent irrigation water sampling provided a more representative assessment of the microbiological status of sprouting mung bean beds compared to when single samples of spent irrigation water were taken. Although numerically, composite sampling identified more *Aeromonas* and generic *E. coli* positive sprout beds compared to when single samples were screened, such differences were found to

Table 1. Prevalence of generic *Escherichia coli* and mesophilic *Aeromonas* in sprouting mung bean bins as assessed by screening sprouts, composite or single spent irrigation water samples

	Generic *E. coli*			Mesophilic *Aeromonas*		
	Spent irrigation water[a]			Spent irrigation water[a]		
Mung bean bed	Composite	Single	Sprouts[b]	Composite	Single	Sprouts[b]
1	−	−	−	−	−	1/5
2	+	−	−	+	−	−
3	−	−	−	+	+	−
4	−	−	−	−	−	−
5	−	−	−	−	−	−
6	+	−	−	−	−	−
7	+	+	−	−	−	−
8	+	+	−	+	−	2/5
9	−	−	−	+	+	1/5
10	+	−	−	−	−	−
11	+	−	−	+	−	−
12	+	+	−	+	−	−
13	−	+	−	+	−	3/5
14	−	−	−	+	−	3/5
15	+	+	−	−	−	−
16	+	+	−	−	−	−
17	+	+	−	+	+	−
18	−	−	−	−	−	−
19	+	−	−	−	+	−
20	−	−	−	+	−	−
21	−	−	−	+	−	−
22	+	+	−	−	−	−
23	+	−	−	+	+	5/5
24	+	−	−	+	−	5/5
25	−	−	−	−	−	−
26	+	+	1/5	−	−	−
27	+	+	−	−	−	−
28	−	−	−	−	−	−
29	+	+	−	−	+	1/5
30	+	+	−	−	+	−
31	−	−	−	−	+	2/5
32	+	−	−	−	−	1/5
Total positive beds	19A	12A	1B	13A	8A	10A

+, positive; −, negative.

[a] Spent irrigation water was collected from the same 32 sprouting mung bean bins.

[b] Five sprout samples per bin.

Values for total positive beds for individual bacterial types followed by the same letter are not significantly different ($P > 0.05$).

be insignificant. This may have been unexpected given that sampling at more sites would have increased the probability of capturing contamination, if present. It is possible that the target bacteria were homogeneously distributed within the sprouting mung bean beds, thereby negating the benefits of composite sampling. This is unlikely given that the *Aeromonas* and especially generic *E. coli* were sporadically recovered from sprout samples. A more probable explanation is that even though multiple samples were taken from under the sprouting mung bean bins, this only represented a relatively small area of the entire production bed. It is also possible that collecting a single 500 mL sample from a single site, compared to 10 × 100 mL from multiple locations, increased the probability of capturing contamination from a specific area under the bed. The increased probability of detected contamination in 500 mL single samples, compared to 10 × 100 mL, was supported by the finding that a number of single spent irrigation water samples tested positive for target bacteria, with the composite samples testing negative. The results of the study are in agreement with those of Liu and Schaffner,[6] who reported that testing single larger volumes (100 mL *versus* 0.1 mL) significantly increased the probability of detecting contamination in sprouting alfalfa seed batches.

In conclusion, the study has demonstrated that composite sampling does not provide a significantly greater probability in detecting contamination in sprouting mung bean beds compared to screening single spent irrigation water samples.

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REFERENCES

1 Fett WF and Cooke PH, Reduction of *Escherichia coli* O157:H7 and *Salmonella* on laboratory-inoculated alfalfa seed with commercial citrus-related products. *J Food Prot* **66**:1158–1165 (2003).
2 Fett WF, Interventions to ensure the microbiological safety of sprouts, in *Microbiology of Fruit and Vegetables*, ed. by Sapers GM, Gorny JR and Yousef AE. CRC Press, Boca Raton, FL, pp. 187–210 (2005).
3 Anonymous, Microbiological safety evaluations and recommendations on sprouted seeds. National Advisory Committee on Microbiological Criteria for Foods. *Int J Food Microbiol* **52**:123–153 (1999).
4 Fu T, Stewart D, Reineke K, Ulaszek J, Schlesser J and Tortorello M, Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J Food Prot* **64**:802–806 (2001).
5 Howard MD and Hutcheson SW, Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Appl Environ Microbiol* **69**:548–553 (2003).
6 Liu B and Schaffner DW, Mathematical modeling and assessment of microbial migration during the sprouting of alfalfa in trays in a nonuniformly contaminated seed batch using *Enterobacter aerogenes* as a surrogate for *Salmonella* Stanley. *J Food Prot* **70**:2602–2605 (2007).
7 Hora R, Kumar M, Garcia L, Schumacher B, Odumeru J and Warriner K, Spatial distribution of *Salmonella, Escherichia coli* O157:H7, and other bacterial populations in commercial and laboratory-scale sprouting mung bean beds. *J Food Prot* **68**:2510–2518 (2005).
8 Pearce RA and Bolton DJ, Excision vs sponge swabbing: a comparison of methods for the microbiological sampling of beef, pork and lamb carcasses. *J Appl Microbiol* **98**:896–900 (2005).
9 Neyts K, Huys G, Uyttendaele M, Swings J and Debevere J, Incidence and identification of mesophilic *Aeromonas* spp. from retail foods. *Lett Appl Microbiol* **31**:359–363 (2000).
10 Little CL and Mitchell RT, Microbiological quality of pre-cut fruit, sprouted seeds, and unpasteurised fruit and vegetable juices from retail and production premises in the UK, and the application of HAACP. *Commun Dis Public Health* **7**:184–190 (2004).

Concentration and Detection of *Salmonella* in Mung Bean Sprout Spent Irrigation Water using Tangential Flow Filtration Coupled with an Amperometric Flow-Through ELISA

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ABSTRACT

The following reports on the development of a culture-free method for *Salmonella* screening of spent irrigation water derived from sprouting mung bean beds. The system used Tangential Flow Filtration (TFF) to non-specifically concentrate cells from large (2 – 10 l) sample volumes. The retentate (100 ml) from the TFF was then flowed over an anti-*Salmonella* antibody modified cellulose acetate membrane. The captured *Salmonella* was detected by reacting with a secondary anti-*Salmonella* and goat anti-rabbit biotin labelled antibody, followed by avidin-tagged glucose oxidase. The hydrogen peroxide generated from the enzymic oxidation of glucose was amperometrically detected at an underlying platinum electrode. It was found that 10 l of 2 log CFU/ml *Salmonella* suspensions could be concentrated to 4 log CFU/ml with 60% recovery regardless of the flow rate (112 – 511 ml/min) or transmembrane pressure (0-20 Ib in^2) applied. The solids content of spent irrigation water negatively affected the filtration rate of TFF. This was most evident in spent irrigation water collected in the initial 24 h of the sprouting period where the solids content was high (4170 mg/l) compared to samples collected at 96 h (560 mg/l). Trials were performed using mung bean beds inoculated with different *Salmonella* levels (1.3 – 3.3 log CFU/g). By using the optimized TFF and flow-through immunoassay it was possible to detect *Salmonella* in spent irrigation water at levels of 2.43 log CFU/ml within 4 h. The integrated concentration and detection system will provide a useful tool for sprout producers to perform in-house pathogen screening of spent irrigation water.

Concentration and detection of *Salmonella*

Sprouted seeds have been implicated in numerous outbreaks of foodborne illness (*1*). *Salmonella* remains the most common human pathogen associated with sprouts although outbreaks linked to *Escherichia coli* O157:H7 have also been reported (*27, 34, 43*). To address the increasing number of foodborne illness outbreaks, the U.S. Food and Drug Administration issued guidelines in 1999 to enhance food safety standards within the sprout industry (*1*). Amongst other measures, the guidelines recommend testing the spent irrigation water, 48 h into the sprouting process, for presence of pathogens with specific reference to *Salmonella* (*1*). Screening spent irrigation water is preferred over testing sprouts directly due to uniformity, ease of collection and simple analysis (*1*). More significantly, because irrigation water runs over the sprouts, it is considered to provide a better assessment on the microbiological status of the sprouting seed bed compared to when individual sprout samples are screened (*1*).

The protocols for sampling spent irrigation water were validated through studies using inoculated and non-inoculated alfalfa (*11, 33*). The results from the studies indicated that the growth of *Salmonella* on sprouting alfalfa occurred early in the sprouting period and was homogenously distributed throughout the sprout bed (*11, 33*). However, a study performed by Hora et al (*15*) illustrated that contamination within sprouting mung bean beds was heterogeneously distributed and the spread of pathogens (*Salmonella* and *Escherichia coli* O157:H7) delayed when introduced at low levels (1 g inoculated beans per 500 g) (*16*). Consequently, screening spent irrigation water from a single point early in the sprouting period would not provide a reliable measure of the microbiological safety of mung bean batches (*15*). A more effective screening method would be to screen large volumes of spent irrigation water late in the sprouting period.

However, large sample volumes are incompatible with standard microbiological techniques which typically involve pre-enrichment followed by selective plating. In addition, because of the time required to perform pathogen screening, spent irrigation samples need to be collected within the initial 48 h into sprouting to ensure that the results are known prior to product release (*1*). To address such time constraints, there has been a sustained interest in rapid detection methods for screening spent irrigation water. To date the majority of studies have focused on pathogen detection methods based on immuno (latex test) or molecular methods (real-time PCR) (*22, 27, 32*). Although such techniques are both rapid and sensitive, the sample size applied to the sensor is restricted to µl volumes thereby requiring a pre-enrichment step to increase pathogen levels to the limit of detection. In addition, culture based techniques are unsuitable for on-site testing due to the need for laboratory facilities and incurred time delay *(22)*.

A non-culture based method has been described for screening spent irrigation water derived from alfalfa beds. The method described was based on centrifugation of spent irrigation water (400 ml) to concentrate cells followed by selective detection using PCR (*20*). The researchers reported 99% recovery of *Salmonella* or *E. coli* O157:H7 with a lower detection limit of 2 log CFU/ml. Although sensitive, it can be considered that centrifugation and molecular based techniques are too expensive for sprout producers to adopt.

In the following an integrated system has been developed based on Tangential Flow Filtration (TFF) in combination with a flow-through electrochemical ELISA to enable sensitive, culture-free, approach to screening spent irrigation water derived from mung bean sprout production (Figure 1). TFF is a cross-flow system that enables high filtration rates of large volumes of sample without excessive pore blocking as experienced with dead-end filtration techniques (*26*).

Concentration and detection of *Salmonella*

TFF has been used extensively in the biotechnology industry to recover proteins or metabolic products from fermentations (*38*) with less attention being placed on recovering microbial biomass (*13, 26*). However, Fu (*12*) developed a TFF sampling system for testing spent irrigation water derived from alfalfa sprout production. Here, the spent irrigation water sample collected 48 h into the sprouting period was circulated within a TFF system achieving 100-fold concentration of inoculated *Salmonella* or *E. coli* O157:H7 within 2 h. (*12*).

In the current study, the selective detection of *Salmonella* was achieved using a flow-through electrochemical ELISA-sensor. Flow-through immune-sensors have the distinct advantage over conventional ELISA in terms of greater sample volumes that can be analyzed thereby enhancing sensitivity (*4, 36, 44, 45*). Here, the sample is allowed to flow over a layer of antibodies to capture the target analyte and subsequently reacted with an enzyme conjugate. The enzymatic product of the conjugate can then be detected either calorimetrically or electrochemically. Electrochemical transduction has the advantage over optical based methods with respect to low cost hardware, robustness and relatively simple design, in addition to multi-analyte sensing using microarrays (*25*). The two basic formats available for electrochemical based sensors are chromatographic (immune-columns) or antibody modified electrodes. In the chromatographic format, the cells are captured within a column, reacted with an enzyme conjugate along with substrate. The electroactive product is then eluted and detected at a down-stream electrode (*25*). The alternative approach is to directly or indirectly immobilize antibodies onto the electrode surface thereby enabling low amounts of enzyme conjugate to be used in addition to enhanced signals due to the lower dilution of enzyme product (*25*). However, a potential limitation of this approach is passivation of the electrode by deposition of proteins and other organic constituents

from the sample matrix (*6, 28 ,35*). Therefore, a protective membrane is typically overlaid onto the electrode to prevent fouling of the electrode whilst at the same time allowing diffusion of the enzyme product (*21, 30*). This is the main reason for selecting a low molecular weight enzymatic product, such as hydrogen peroxide, that readily diffuses across membrane films to the underlying electrode (*41*).

The objective of the following study was to develop a robust system for spent irrigation water screening that could be readily adopted by sprout producers. To mimic the worse-case-scenario, the integrated system was verified using spent irrigation water derived from sprouting mung bean beds inoculated with low levels of *Salmonella* (1.3 log CFU/g) which may be encountered naturally in contaminated seeds (*23*)

MATERIALS AND METHODS

Preparation of *Salmonella* suspensions

The *Salmonella enterica* serovars selected to study have either been implicated in sprout related foodborne illness outbreaks or are of clinical significance. The *Salmonella* serovars used (obtained from the culture collection of the Canadian Research Institute for Food Safety, Guelph, Canada) in the study were Montevideo P2 (kanamycin resistant (*42*)), Heidelberg, Senftenberg, Typhimurium DT104, Newport, Meleagridis and Oranienburg. The *Salmonella* were cultivated aerobically in Luria-Bertani (LB) broth (Oxoid, Basingstoke, UK) at 37°C for 24 h. The cells were harvested by centrifugation (5000 x g for 10 min at 4°C) and washed once in sterile saline.

The pellet was finally resuspended in saline to give a final cell density of 7.0 log CFU/ml (OD_{600} = 0.2).

Preparation of spent mung bean irrigation water

Batches (500 g) of mung beans (donated by a local sprout producer) were placed into a 10 l container with a perforated base to allow drainage of irrigation water. The container was placed within a secondary vessel to collect the spent irrigation water. The mung beans were soaked overnight in 2 l of water at 25°C. The water was removed and sprouting continued for a further 96 h with daily irrigation using 2 l volumes of sterile distilled water. On each occasion the water was collected and transferred to a sterile bottle and maintained at 4°C until required.
Solids content of spent irrigation water was determined using a conductivity meter (Thermo-Fisher, Whitby, Ontario, Canada).

Enumeration of *Salmonella* in spent irrigation water derived from sprouting mung bean beds

Suspensions (500 ml) of *Salmonella* Montevideo P2 were prepared as described above. Mung beans (100 g) were steep inoculated in the bacterial suspension (7 log CFU/ml) for 20 min and subsequently transferred to blotting paper and allowed to dry overnight at room temperature (*ca.* 23°C). Initial loading of the mung beans was determined by placing 1 g of inoculated beans into 9 ml 0.1% peptone and vortexed for 60 s to release the attached cells. Serial dilutions were prepared in 0.1% peptone and plated onto the LB_{KAN} agar (LB containing 30 µg/ml kanamycin).

Concentration and detection of *Salmonella*

Different weights (1, 10 or 100 g) of inoculated beans were introduced into non-inoculated mung bean batches to give a total weight of 500 g. The mung beans were soaked in 2 l water for 24 h at 25°C to stimulate germination. The beans were transferred to 10 l containers as described above and sprouting continued for up to 96 h with daily irrigation using 2 l volumes of water. *Salmonella* levels within the spent irrigation water samples were determined by preparing a dilution series and plating onto LB_{KAN} that was subsequently incubated at 37°C for 24 h. When no counts were recovered on plates, 100 ml volumes of spent irrigation water were passed through a sterile filter (47 mm, 0.45μm pore size; Thermo-Fisher, Whitby, ON, Canada) and overlaid onto an LB_{KAN} agar prior to incubating at 37°C for 24 h.

Presence/absence tests for *Salmonella* were performed by adding 200 ml sample with 25 ml 0.1% peptone water and incubating at 37°C for 24 h. Aliquots (0.1 ml) was spotted onto semi-solid RV agar (Oxoid) and incubated at 42°C for 20 h. Presumptive positive samples were plated onto XLD agar and incubated for 24 h at 37°C. The Oxoid *Salmonella* Latex Test FT0203 was used for serological confirmation of typical colonies (i.e. red colonies surrounded by brilliant red zones).

Tangential flow filtration

The Tangential Flow Filtration (TFF) system, developed by Fu et al. (*12*), was adopted. The systems uses a MiniKros module composed of hollow fiber polysulfone membranes with a 0.72 m^2 working area and 0.2 μm pore size (Spectrum Labs, CA, USA). Other components of the TFF system were a 10 l capacity holding chamber, Polycap 36HD pre-filter (10 μm pore size, Spectrum Labs), peristaltic pump (Masterflex Economy Pump, Cole-Parmer, Vernon Hills, IL,

USA), pressure gauges at the in- and out-let of the TFF filter (Figure 1). The sample to be concentrated was placed in the 10 l holding chamber and re-circulated in a closed loop system through the TFF filtration unit. The flow rate was adjusted by the speed of the pump with the inlet and outlet pressure being controlled by tightening screw clamps (Thermo-Fisher) on the retentate side of the TFF.

The transmembrane pressure and flux rate were calculated using the following equations:-

Transmembrane Pressure = [(feed pressure + retentate pressure) / 2] – filtrate pressure

Flux Rate = filtrate rate (h) / area of the membrane (m)

The filtrate was collected in a secondary container and filtration continued until the dead-volume of the system (100 ml) had been attained. The percent capture was calculated using the equation

% Capture = *Salmonella* CFU in Retentate/ *Salmonella* CFU original suspension x 100

Clean-In-Place of TFF filter cartridge

Three sanitizers were evaluated for Clean-In-Place regimes for regenerating the TFF system. The sanitizers tested were sodium hypochlorite (200 mg/l); acidified sodium chlorite (200 mg/l); and sodium hydroxide (200 mg/l). For each test the filter was used to concentrate a 2 l *Salmonella* cell suspension (5 log CFU/ml). Upon completion of the concentration run the TFF was back-flushed using 5 l sterile distilled water followed by a 20 min circulation of 1 l volumes of the selected sanitizer. Residual sanitizer was then removed by back-flushing with a further 5 l of sterile distilled water. To verify the efficacy of sanitation, a 2 l volume of saline was

circulated through the TFF until the 100 ml dead-volume had been attained. The feed, retentate, permeate, final back flush, and second retentate were screened for the presence of *Salmonella* by plating onto LB_{KAN} agar as previously described.

Construction of flow through immuno-assay

The electrochemical cell consisted of a 3 mm diameter platinum electrode with a Ag/AgCl outer electrode that served as the counter and reference (Rank Brothers, Cambridge, UK). The electrode was linked to a Solartron 1260 potentiostat (London Scientific, London Ontario, Canada) and polarised at 0.65V vs Ag/AgCl. The current response was recorded and data processed using CorrWare (Scribner, Southern Pines, North Carolina, USA). The retentate was placed in a holding chamber as fed into the electrochemical cell via a peristaltic pump (Thermo-Fisher) operating at 0.89 ml/min. The volume above the membrane was set at 0.5 cm via a plunger which was placed into the electrochemical cell.

The working electrode was overlaid with a membrane that served as a surface to immobilize the capture antibody and semi-permeable interface to permit diffusion of hydrogen peroxide (product of glucose oxidase enzyme conjugate), as well as a means of minimizing electrode fouling. The membranes evaluated for this purpose were plasticized polyvinyl chloride (PVC), cellulose acetate and poly phenyl ether sulphone (PES). PVC was prepared by dissolving 60 mg of PVC (MW 43000, Sigma-Aldrich, Oakville, ON, Canada) in 10 ml tetrahydrofuran (THF) containing 100 µl Tween 20. The solution was cast into a glass petri-dish (100 mm diameter) and solvent evaporated over-night at room temperature (*ca.* 23°C) within a fume-hood.

Poly(phenyl ether sulphone) (60 mg, Sigma-Aldrich, Oakville, ON, Canada) was dissolved in 10 ml chloroform and cast into a glass petri-dish (100 mm diameter). Cellulose acetate (0.1 g) (MW 30000, Sigma-Aldrich) was dissolved in 10 ml acetone and cast into a glass Petri dish. In each case the solvent was allowed to evaporate at room temperature within a fume-hood. The permeability of the membranes to hydrogen peroxide was assessed by overlaying the working electrode with 1.5 cm^2 sections of the test films. An aliquot (2 ml) of KCl (0.5 M) was added to the electrochemical cell that was stirred via a magnetic stirrer. The working electrode was polarized by applying 0.65 V vs Ag/AgCl and the current allowed to attain a steady baseline. A step-calibration curve was constructed by the sequential addition of aliquots (0.1 ml) of hydrogen peroxide (1 mM, Thermo-Fisher). The response was determined by subtracting the steady-state current from the baseline.

To assess the anti-fouling properties of the different membranes, step-calibration curves were performed in the presence of bovine serum albumin (10 mg/ml; Sigma-Aldrich) or a 7.0 log CFU/ml heat killed *Salmonella* suspension.

Capture antibody immobilization

A mouse monoclonal anti-*Salmonella* IgG antibody, with specificity to the LPS core of Groups A - E, (Abcam, Cambridge, MA, USA; ab20949) was immobilized onto cellulose acetate membranes using a thiolation reaction. The reactive gold layer was deposited onto cellulose acetate membranes by sputter coating at 15 mV for 2 min. The monoclonal anti-*Salmonella* antibody was diluted to 0.5 mg /ml in PBS. Three cross-linker solutions (all obtained from Sigma-Aldrich) were used to covalently attach a thiol group to the antibody; specifically: 20 mM

dimethyl-3,3'-dithiobispropionimidate· 2HCl (DTBP) (20 mM bicarbonate buffer, pH 9.0); 20 mM 3,3'-dithiobis (sulfosuccinimidylpropionate; DTSSP) (5 mM sodium citrate buffer, pH 5.0); and 20 mM each of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido] hexanoate (sulfo-LC-SPDP) in sterile water. Aliquots (3 µl) of each of the antibody and the three cross-linker solutions were gently mixed and incubated at room temperature for 1 h. To reduce the disulfide bond of the now thiololated antibody, 2 µl of dithiothreitol (DTT) (0.1 M sodium acetate buffer, 0.1 M NaCl, pH 4.5) was added to the mixture and reacted for 30 min. The solution was then spread onto the gold layered cellulose acetate membrane and allowed to dry at room temperature for 1 h. The antibody-coated cellulose acetate membrane was then washed consecutively with phosphate buffered saline and sterile distilled water.

Verification of antibody immobilization was performed by flowing 100 ml *Salmonella* suspension (7 log CFU/ml) over the antibody modified cellulose acetate using flow rates of 0.58, 0.69 or 0.89 ml/min. The membrane was then rinsed with sterile water to remove loosely attached cells. The captured cells were fixed onto the membrane by using 2 % glutaraldehyde and 1 % osmium oxide followed by dehydration in ethanol solutions as described by Gamliel (*14*). The membrane was then sputter coated with gold and viewed under a Hitachi S-3700N Scanning Electron Microscope. (Hitachi, Pleasanton, CA).

Capture efficiency of the flow-through immune sensor was determined using 100 ml *Salmonella* suspensions (100 ml) diluted from 7 log CFU/ml to 2 log CFU/ml. The suspension was circulated around the flow system at a rate of 0.89 ml/min (total time 2 h). Samples (1 ml) were removed at the start and end of the flow cell process and plated onto LB_{KAN} or XLD prior to

incubation at 37°C for 24 h. The capture efficiency was calculated by comparing *Salmonella* in the original suspension to levels retained within the suspension following the completion of the circulation through the immune-sensor. The degree of non-specific binding was determined by using gold spluttered cellulose acetate membranes containing no anti-*Salmonella* antibodies.

Electrochemical detection of *Salmonella*

Salmonella captured on the antibody modified cellulose acetate membranes were reacted for 20 min at room temperature with 25 µl of a secondary antibody (rabbit polyclonal with affinity for O and H antigens of *Salmonella*, 1 mg/ml; ab35156). The reaction cell was washed with 5 x 1 ml volumes of saline to remove unbound antibody prior to addition of 100 µl of biotin labelled goat polyclonal anti- rabbit (0.02 mg/ml; Abcam, ab6720). The antibody was allowed to attach for 20 min at room temperature prior to rinsing the cell with saline. Finally, 20 -120 µg glucose oxidase avidin conjugated glucose oxidase (Vector Laboratories, Burlington, Ontario, Canada), was applied to the membrane and allowed to bind to the biotin moiety of the anti-rabbit polyclonal for 40 min. The reaction cell was rinsed with sterile water to remove unattached glucose oxidase and 1mlof electrolyte (0.1M KCl) added. The background current was allowed to attain a steady state value prior to the addition of 1 ml glucose (1 M). The increase in current was recorded and subtracted from the background to give the sensor response.

The concentration of active units of glucose oxidase antibody conjugate was determined using dianisidine-peroxidase assay. The assay consisted of 1.25 ml dianisidine-buffer mixture (0.1 % w/v Sigma-Aldrich), 100 µl 20 Purpurgalin units/ml peroxidase (Sigma-Aldrich) and 1 µl of the conjugated glucose oxidase. The reaction was started by the addition of 150 µl 18 % w/v glucose

and the increase in A_{460nm} recorded over 5 minutes using a Shimadzu spectrophotometer (Thermo-Fisher) . The units/mg enzyme was calculated as follows:

Units/mg = ΔA_{460nm}/min / (11.3 x mg enzyme / ml reaction mixture)

Statistical analysis and experimental design

Except where otherwise stated all tests were repeated three times. Means generated were analyzed by analysis of variance and Tukey test. In all cases, the level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Concentration of *Salmonella* suspensions using Tangential Flow Filtration

For optimization studies, 10 l of 0.8% w/v saline inoculated with 2 log CFU/ml (6 log CFU/10 l) *Salmonella* was circulated through the TFF operating at different flow rates and transmembrane pressure (TMP). The suspension was concentrated within the TFF system until the dead volume (100 ml) had been attained which took 21 min to 3 h depending on the applied flow rate and pressure.

The average concentration factor achieved using the various flow rate and TMP combinations was 126-fold (60% recovery) which is comparable to values previously reported by other workers for concentrating bacterial suspensions (*18*, *26*). Both flow rate and TMP did not have a significant ($P > 0.05$) effect on the levels of *Salmonella* recovered (Figure 2). This may have been unexpected given that flow rate and TMP collectively defined the shear forces imposed on the cells which, if excessive, results in membrane disruption (*26*). It has been previously reported

that high shear forces significantly decrease the recovery of yeasts cells by cross-flow filtration (*26*). However, in the current study it was evident that *Salmonella* was less sensitive to the generated shear forces thereby retaining viability during the filtration process. In practical terms it is desirable to have high flow rates to facilitate rapid filtration and low TMP to reduce the stresses imposed on the TFF thereby extending the working life-time of the filter (*39*). Spent irrigation water contains solids derived from bean exudates, in addition to microbial biomass and constituents that could impact on the filtration efficacy of TFF. It was found that the solids content of spent irrigation water collected at different time periods during the sprouting period varied significantly ($P < 0.05$; Figure 3). Spent irrigation water collected shortly after the beans had been soaked in water for 24 h contained the highest solids content compared to samples collected towards the end of the sprouting period (Figure 3). The changes in solids content can be attributed to the release of exudates from the germinating beans that are maximal at the early stages of sprout development (*46*). Relevant to the current study, the high solids content of the spent irrigation water negatively affected the flux rate of TFF (Figure 3). The effect of solids content on flux rates can be attributed to the accumulation of organic matter within the pores of the TFF hollow fibre that impedes the flow of water through the membrane (*2*, *9*, *29*).

The solids content of spent irrigation water collected from mung bean beds 48-96 h into the sprouting period was not significantly ($P > 0.05$) different. However, the flux rate observed for irrigation water collected at either 48 or 72 h was significantly ($P < 0.05$) lower compared to samples withdrawn at 96 h (Figure 3). Such differences may be attributed to the composition of solids content. For example, polysaccharide materials results in agglomeration of particulates which would result in increased biofouling compared to low molecular weight constituents such

as organic acids (*8*, *19*). Regardless of this fact the results indicated that spent irrigation water collected at the end of the sprouting period resulted in less biofouling of the membrane and facilitated high filtration rates.

The reduced flux with increasing solids content could be compensated to a degree by increasing the TMP (Figure 3). By using a higher TMP (3.5 lb in^2) an increase in shear force is achieved that minimizes the accumulation of organic matter within the pores of the TFF hollow fibre thereby maintaining high flux rates (*8*).

For optimal recovery of *Salmonella* it is desirable to collect samples when levels of the enteric pathogen within the sprouting mung bean bed are maximal. Therefore, trials were performed to determine the levels of *Salmonella* associated with spent irrigation water collected at different times during the sprouting period. To mimic the worst-case-scenario, *Salmonella* was introduced at low levels (1.3 log CFU/g) into 500 g mung bean batches. The levels of *Salmonella* remained relatively constant during the initial 48 h into the sprouting period then increased thereafter attaining levels of 2.5 ± 0.4 log CFU/ml at 96 h. The results were in agreement with Hora et al (15) who also reported low *Salmonella* levels in spent irrigation water when the enteric pathogens were introduced at comparable cell densities as used in the current study. The low level of *Salmonella* was unexpected given that other reports have demonstrated rapid growth of the pathogen on sprouting seeds (*17*, *42*). However, it should be noted that in the majority of studies to date seeds were inoculated with a relatively high inoculum level (>4 log CFU/g) and also levels of the enteric pathogen were enumerated on sprouts as opposed to spent irrigation water.

Concentration and detection of *Salmonella*

The low *Salmonella* levels within spent irrigation water observed in the current study can be explained by the strong attachment of the human pathogen on sprouts (*7*). Consequently, *Salmonella* is more likely to be retained within the sprout bed than washed out during irrigation (*15*). A further possibility is the suppression of *Salmonella* growth by the antagonistic action of endogenous microflora (*10*). In the current study it was noted that the Total Aerobic Count (TAC) associated with spent irrigation water progressively increased during the sprouting period (Figure 4). Therefore, it is likely that *Salmonella* introduced at low levels could not effectively compete in the early stages of the sprouting period.

It has been recommended that spent irrigation water should be sampled 48 h into the sprouting period (*1*). Based on the results obtained in this study, sampling early in the sprouting production would reduce the possibility of detecting *Salmonella* if present. However, by performing spent irrigation water screening at the end of the sprouting period, the levels of *Salmonella* are maximal and consequently more likely to be detected (*15*).

The recovery of *Salmonella* inoculated into spent irrigation water was evaluated. Here spent irrigation water was collected from sprouting mung bean beds 96 h into the sprout production and inoculated with defined levels of *Salmonella* using the 7 log CFU/ml suspension (Table 1). The inoculated spent irrigation water was then concentrated using the optimized TFF. It was found that the cell density of pathogens could be increased by 10 – 16 fold by using TFF (Table 1). The percent recovery of *Salmonella* in spent irrigation water was lower than that observed in saline (Figure 2). In addition, it was noted that the % recovery of *Salmonella* was significantly ($P < 0.05$) decreased when 2 log CFU/ml suspensions were processed compared to 4.5 log CFU/ml (Table 1). The lower recovery yields with dilute *Salmonella* suspensions could have

been due to cells binding to the membrane surface which occurs during the filtration process (*26*).

Clean-in-Place of Tangential Flow Filtration Unit

Although the TTF cartridges used in the current study were only intended for single-use, it can be envisaged that in commercial practice the filter would perform multiple runs. This necessitates developing clean-in-place protocols that can ensure inactivation and removal of residual microbial cells without compromising the integrity of the filter membrane. From the sanitizers tested it was evident that sodium hydroxide proved least effective with *Salmonella* being recovered following the CIP cycle. In contrast, both hypochlorite and acidified sodium chlorite were both effective at sanitizing the TFF unit. However, applying acidified sodium chlorite caused accumulation of white crystals within the filter and hence negatively impacted on performance. Consequently, sodium hypochlorite was used in subsequent studies for sanitizing the TFF between pressure runs. In general the filters could be used to process 8 times before failing (i.e. recovery of *Salmonella* from the permeate). No progressive decrease in filtration performance (i.e. capture yield, flux rate) was observed between TFF runs. However, when membrane failure occurred there was an abrupt loss of TMP which coincided with *Salmonella* being recovered in the filtrate.

Flow-through amperometric immuno-sensor design and optimization

A flow-through immuno-assay was designed based on passing the retentate derived from the TFF step over a membrane modified with anti-*Salmonella* antibodies. In addition to acting as a solid support, the membrane also functioned as a perm-selective membrane to prevent biofouling

of the electrode which was used to detect the hydrogen peroxide enzymatic product of the glucose oxidase antibody conjugate. The three different base membrane types evaluated were cellulose acetate, plasticized PVC and poly phenyl ether sulphone (PES). All three polymers are widely used in biosensor devices due to biocompatibility or perm-selectivity characteristics (*3*, *37*). The PES membrane was too brittle and could not be overlaid over the electrode without breaking and PVC was readily passivated by proteins. However, cellulose acetate membranes were both biocompatible and permeable to hydrogen peroxide. Biocompatibility was evaluated by determining the response to hydrogen peroxide in the presence and absence of BSA or heat killed cells. Although the hydrogen peroxide amperometric response was lower in the presence of BSA or heat killed cells this was insignificantly ($P>0.05$) different compared to when dose response curves were performed in KCl alone (Figure 5).

Immobilization of antibodies onto cellulose acetate membranes

Anti-*Salmonella* antibody was immobilized onto the surface of gold sputtered cellulose acetate membranes via thiol coupling. Verification of antibody immobilization was performed by reacting the modified cellulose acetate membrane with diluted *Salmonella* suspensions (6 log CFU/ml). After incubating at room temperature for 40 min the membrane was rinsed to remove non-attached *Salmonella* prior to viewing under SEM. *Salmonella* cells were observed on antibody modified films although negligible numbers were encountered on non-modified membranes (Figure 6). The results confirm that the antibody modified cellulose acetate resulted in specific binding of *Salmonella* with minimal non-specific binding.

Concentration and detection of *Salmonella*

The anti-*Salmonella* modified cellulose acetate membrane was integrated into a flow cell and capture efficiency determined. Here, different *Salmonella* serovar suspensions (100ml) were flowed (0.89 ml/min) over the antibody layer and changes in levels of the enteric pathogen remaining being used to calculate the capture efficacy (Table 2). The capture efficiency of anti-*Salmonella* modified cellulose acetate membranes ranged from 64 – 95% (Table 2). The similar capture efficiencies with the different serovars confirm that the sensor could be used to capture different *Salmonella* types tested.

The level of *Salmonella* captured is comparable to immune-columns (*5*) and paramagnetic beads (*40*) both of which report efficiencies of 95%. In the current study the high capture efficiencies could be attributed of the high affinity of the anti-*Salmonella* antibodies. However, it should be noted that the non-specific binding (i.e. films containing no antibodies) was relatively high (12-36%; Table 2). The non-specific binding was likely caused by the attachment of cells to the tubing and reaction cell. However, the capture of *Salmonella* was consistently higher with antibody modified films indicating the majority of binding was specific and not due to passive attachment.

The captured cells were detected by reacting *Salmonella* with a secondary rabbit anti-*Salmonella* biotin-labelled antibody followed by avidin-tagged glucose oxidase. Glucose enzyme substrate was then added and the hydrogen peroxide product detected amperometrically at the underlying platinum electrode. The assay was optimized in terms of assay time, temperature, concentration of antibody and glucose oxidase. The optimal sensor response was found by reacting 6 µl (2 mg/ml) biotin-labelled antibody with120 µg glucose oxidase at 23°C for 40 min (results not shown).

Evaluation of the integrated tangential flow filtration and amperometric immunosensor-to detect *Salmonella* in spent irrigation water

Spent irrigation water (2 l) was collected from mung bean beds initially inoculated with different levels of *Salmonella* (1.3 – 3.3 log CFU/g; Table 3). The level of *Salmonella* recovered from the spent irrigation water at the end of the 96 h sprouting period was dependent on the initial inoculation introduced onto the mung beans (Table 3). The same finding has been reported for the growth of *Salmonella* introduced at different levels within sprouting alfalfa sprouts (24). The concentration factor when the spent irrigation water was passed through the TFF was found to be dependent on the *Salmonella* concentration in the sample (Table 3). Low levels of *Salmonella* in the spent irrigation water resulted in the lowest concentration factor which was again possibly due to binding of cells to the filtration unit. It was noted that the concentration factors were higher compared to when *Salmonella* was directly inoculated into spent irrigation water (Table 1). This may have been attributed to the physiological state of the cells which enhanced tolerance to shear effects and/or reduced attachment to the filter membrane.

The response of the amperometric immunosensor correlated ($r^2 = 0.91$) with the *Salmonella* levels within the retentate. However, it is unlikely that the sensor could be used to quantify *Salmonella* present within the sprouting mung bean bed given the variability in recovery yield using TFF and capture efficiency of the antibody modified membranes. The lack of quantitative measurement should not be considered a weakness of the sensor given that any *Salmonella* detected would be considered a hazard. In this respect the lower detection limit is of greater significance and as observed, the sensor could detect *Salmonella* concentrations on the order of 2 log CFU/ml following TFF. The lower detection limit if the flow-through immune-sensor in

isolation is comparable to other reported flow-through immune-sensors which typically can detect levels on the order of 3-4 log CFU/ml (*6*, *31*).

To test the reliability of the integrated TFF-sensor system 20 trials were performed where mung bean beds were inoculated with 1.3 log CFU/g *Salmonella* and sprouted for 96 h (Figure 7). The spent irrigation water (2 l) was passed through the TFF system and retentate transferred to the flow through immune-sensor. Each TFF filter cartridge was re-used seven times with the CIP hypochlorite decontamination step as previously described. A fresh antibody modified cellulose acetate membrane was used on each occasion.

The sensor response was found to be variable although consistently higher than the baseline (spent irrigation water derived from non-inoculated mung bean beds). *Salmonella* was not detected in spent irrigation water taken from the control (non-inoculated) mung bean batches confirming that no false-positives were generated by the sensor. Inoculated mung beans in Trial 2 gave a low response (Figure 7) which was not significantly different to the baseline value and hence can be regarded as a false-negative given that the retentate sample tested positive for *Salmonella* upon enrichment. Several samples resulted in high sensor responses which may be related to variation in the fabricated antibody modified membrane or levels of *Salmonella* within the retentate. However, regardless of this fact the results demonstrated the consistency of the integrated system to detect *Salmonella* within spent irrigation water. Furthermore, *Salmonella* captured on the membrane could be isolated by enrichment and subsequent plating onto selective media thereby enabling conformational tests to be performed in addition to epidemiology investigations.

In conclusion, the study has provided proof-of-principle for an integrated device to screen spent irrigation water derived from sprouting mung bean beds. The method is highly sensitive and can detect *Salmonella* in spent irrigation water within 4 h. Due to the rapid analysis time it is possible to take samples at the end of the sprouting process when *Salmonella* numbers are high. The system can be potentially automated and TFF cartridges re-used both thereby making the system a commercially viable alternative to current testing procedures. However, further studies are needed to verify the device performance in capturing and detecting a wider range of *Salmonella* serovars that may be encountered in sprouted seed production.

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REFERENCES

1. Anonymous 1999. Microbiological safety evaluations and recommendations on sprouted seeds. National Advisory Committee on Microbiological Criteria for Foods. *Int.J.Food Microbiol.*52: 123-153.

2. Baruah, G. L., A. Nayak, E. Winkelman and G. Belfort. 2006. Purification of monoclonal antibodies derived from transgenic goat milk by ultrafiltration. *Biotechnol.Bioeng.*93: 747-754.

3. Benmakroha Y, I. Christie I, M. Desai and P. Vadgama. 1996. Poly(vinyl chloride), polysulfone and sulfonated polyether-ether sulfone composite membranes for glucose and hydrogen peroxide perm-selectivity in amperometric biosensors. *Analyst*12: 521-526.

4. Bilitewski, U., M. Genrich, S. Kadow and G. Mersal. 2003. Biochemical analysis with microfluidic systems. *Anal.Bioanal Chem.*377: 556-569

5. Brewster, JD. 2003. Isolation and concentration of Salmonellae with an immunoaffinity column. *J.Microbiol.Methods* 55: 287-293

6. Brooks, J. L., B. Mirhabibollahi and R. G. Kroll. 1992. Experimental enzyme-linked amperometric immunosensors for the detection of salmonellas in foods. *J.Appl.Bacteriol.*73: 189-196

7. Charkowski, A. O., J. D. Barak, C. Z. Sarreal and R. E. Mandrell. 2002. Differences in growth of *Salmonella* enterica and *Escherichia coli* O157:H7 on alfalfa sprouts. *Appl.Environ.Microbiol.* 68: 3114-3120

8. Colton, R. H., I. Pahl, L. E. Ottaviano, T. Bodeutsch and F. Meyeroltmanns. 2002. Study of protein adsorption effects on crossflow filtration using BSA and milk protein PDA J.*Pharm.Sci. Technol.* 56: 20-30

9. Daubert, I., M. Mercier-Bonin, C. Maranges, G. Goma, C. Fonade and C. Lafforgue. 2003. Why and how membrane bioreactors with unsteady filtration conditions can improve the efficiency of biological processes. *Ann.N.Y.Acad.Sci.*984: 420-435.

10. Fett, W. E.. 2006. Inhibition of *Salmonella* enterica by plant-associated pseudomonads *in vitro* and on sprouting alfalfa seed. *J.Food Prot.*69: 719-728.

11. Fu, T. J., D. Stewart, K. Reineke, J. Ulaszek, J. Schlesser and M. Tortorello. 2001. Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J.Food Prot.* 64: 802-806

12. Fu, T.-J.; Reineke, K.; Krupinski, T.; VanPelt, O.; Maks-Warren, N. Parisi, B.; Slade, P. 2005. Integrating tangential flow filtration with rapid tests for detection of *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in large volumes of spent sprout irrigation water. IAFP Annual Meeting. August 13-17, Baltimore, MD.

13. Genovesi, C. S. 1983. Several uses for tangential-flow filtration in the pharmaceutical industry. *J. Parenter. Sci. Technol.* 37: 81-86

14. Gamliel, H. 1985. Optimum fixation conditions may allow air drying of soft biological specimens with minimum cell shrinkage and maximum preservation of surface features. *Scanning Electron Microscopy* 4: 1649-1664.

15. Hora, R., M. Kumar, L. Garcia, B. Schumacher, J. Odumeru and K. Warriner. 2005. Spatial distribution of *Salmonella*, *Escherichia coli* O157:H7, and other bacterial populations in commercial and laboratory-scale sprouting mung bean beds. *J. Food Prot.* 68: 2510-2518.

16. Hora, R., K. Warriner, B. J. Shelp and M. W. Griffiths. 2005. Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. *J. Food Prot.*68: 2506-2509.

17. Howard, M. B. and S. W. Hutcheson. 2003. Growth dynamics of *Salmonella* enterica strains on alfalfa sprouts and in waste seed irrigation water. *Appl.Environ.Microbiol.*69: 548-553.

18. Jiang, S. C. J. M. Thurmond, S. L. Pichard and J. H. Paul. 1992. Concentration of microbial-populations from aquatic environments by vortex flow filtration. *Marine Ecol. Prog.* 80: 101-107.

19. Johns, M. R. and E. Noor. 1991. Recovery and purification of polysaccharides from microbial broth. *Aust. J. Biotechnol.*5: 73-77.

20. Johnston, L. M., D. Elhanafi, M. Drake and L. A. Jaykus. 2005. A simple method for the direct detection of *Salmonella* and *Escherichia coli* O157:H7 from raw alfalfa sprouts and spent irrigation water using PCR. *J. Food Prot.* 68: 2256-2263.

21. Johnson, R. N. and J. R. Baker, 1998. Accuracy of devices used for self-monitoring of blood glucose *Annals Clin Biochem.* **35: 68-74.**

22. Kramer, M. F., and D. V. Lim. 2004. A rapid and automated fiber optic-based biosensor assay for the detection of *Salmonella* in spent irrigation water used in the sprouting of sprout seeds. *J. Food Prot.*67: 46-52

23. Liao, C. H. 2008. Growth of *Salmonella* on sprouting alfalfa seeds as affected by the inoculum size, native microbial load and *Pseudomonas fluorescens* 2-79. *Lett. Appl. Microbiol.*46: 232-236

24. Liu, B. and D. W. Schaffner. 2007. Quantitative analysis of the growth of *Salmonella* stanley during alfalfa sprouting and evaluation of *Enterobacter aerogenes* as its surrogate. *J. Food Prot.*70: 316-322

25. Marquette, C. A. and L. J. Blum. 2006. State of the art and recent advances in immunoanalytical systems. *Biosens. Bioelectron.*211: 424-433.

26. Mawson, A. J. 1993. Crossflow microfiltration of cell suspensions. *Australas. Biotechnol.* 3: 348-352

27. Mohle-Boetani, J. C., J. Farrar, and P. Bradleyl. 2008. *Salmonella* infections associated with mung bean sprouts: epidemiological and environmental investigations. *Epidemiol. Infect.* 1-10

28. Ordonez, S. S. and E. Fabregas. 2007. New antibodies immobilization system into a graphite-polysulfone membrane for amperometric immunosensors. *Biosens. Bioelectron.*22: 965-972

29. Phelps, T. J., A. V. Palumbo and B. L. Bischoff. 2007. Micron-pore-sized metallic filter tube membranes for filtration of particulates and water purification. *J. Microbiol. Methods* (*Available online 24 August 200)7*

30. Reddy, S. M., S. P. Higson, I. M. Christie and P. M. Vadgama. 1994. Selective membranes for the construction and optimization of an amperometric oxalate enzyme electrode. *Analyst*119: 949-952

31. Ricci, F., G. Volpe, L. Micheli and G. Palleschi. 2007. A review on novel developments and applications of immunosensors in food analysis. *Anal. Chim. Acta*. 605: 111-129

32. Stewart, D. S., K. F. Reineke and M. L. Tortorello. 2002. Comparison of assurance gold *Salmonella* EIA, BAX for screening/*Salmonella*, and GENE-TRAK Salmonella DLP rapid assays for detection of *Salmonella* in alfalfa sprouts and sprout irrigation water. *J.AOAC Int.*85: 395-403.

33. Stewart, J. R., R. D. Ellender, J. A. Gooch, S. Jiang, S. P. Myoda and S. B. Weisberg. 2003. Recommendations for microbial source tracking: lessons from a methods comparison study. *J.Water.Health.*1: 225-231

34. Taormina, P. J. and L. R. Beuchat. 1999. Behavior of enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. J. Food Prot. 62: 850-856.

35. Tiefenauer, L. X., S. Kossek, C. Padeste and P. Thiebaud. 1997. Towards an amperometric immunosensor devices. *Biosens. Bioelectron.*12: 213-223

36. Urraca, J. L., E. Benito-Pena, C. Perez-Conde, M. C. Moreno-Bondi and J. J. Pestka. 2005. Analysis of zearalenone in cereal and Swine feed samples using an automated flow-through immunosensor. *J. Agric. Food Chem.*53: 3338-3344.

37. Vadgama. P. 2007. Sensor biocompatibility: final frontier in bioanalytical measurement. *Analyst*13: 2495

38. van Reis R. and A. Zydney. 2001. Membrane separations in biotechnology. *Curr. Opin. Biotechnol.*12: 208-211

39. vanReis, R., S. Gadam S, L.N. Frautschy, S. Orlando , E. M. Goodrich , S, Saksena, R. Kuriyel, C.M Simpson C. M, S. Pearl and A. Zydney 1997. High performance tangential flow filtration. *Biotech. Bioeng.* 56: 71-82.

40. Varshney, M., L. Yang, X. L. Su and Y. Li. 2005. Magnetic nanoparticle-antibody conjugates for the separation of *Escherichia coli* O157:H7 in ground beef. *J. Food Prot.* 68: 1804-1811.

41. Wang, S., X. Ji and Z. Yuan. 1995. Study of cellulose acetate membrane-based glucose biosensors. *Chin. J. Biotechnol.*11: 199-205.

42. Warriner, K., S. Spaniolas, M. Dickinson, C. Wright and W. M. Waites. 2003. Internalization of bioluminescent *Escherichia coli* and *Salmonella* Montevideo in growing bean sprouts. *J. Appl. Microbiol.* 95: 719-727.

43. Winthrop, K.. L., M. S. Palumbo and J. A. Farrar, 2003. Alfalfa sprouts and *Salmonella* Kottbus infection: a multistate outbreak following inadequate seed disinfection with heat and chlorine. *J. Food Prot.* 66: 13-17.

44. Yamazaki, M., K. Mitamura and M. Ichikawal. 2004. Evaluation of flow-through immunoassay for rapid detection of influenza A and B viruses. *Kansenshogaku Zasshi* 78: 865-871.

45. Zeravik, J., T. Ruzgas and M. Franek. 2003. A highly sensitive flow-through amperometric immunosensor based on the Peroxidase chip and enzyme-channeling principle. *Biosens. Bioelectron.*18: 1321-1327.

46. Zheng, S. H. and M. Kawabata 2000. Exudation of amino acids and sugars from imbibing seeds of several leguminous crops. *J. Crop Sci* 69: 380-384.

Figure 1: Schematic diagram of the integrated Tangential Flow Filtration (TFF) and amperometric flow-through ELISA. The spent irrigation water sample is recirculated through a TFF unit to concentrate bacterial cells. The retentate is then flowed over capture antibodies immobilized on the surface of a cellulose acetate membrane. The captured Salmonella *are detected via the addition of secondary antibody glucose oxidase conjugate.*

Figure 2: Effect of TFF flow rate and transmembrane pressure on the concentration of Salmonella *from a suspensions originally containing 2 log CFU/ml.* Salmonella *suspensions were prepared in saline and passed through TFF unit at different transmembrane pressure and flow rate. The cell density of* Salmonella *within the retentate following filtration was then determined and used to calculate the concentration factor.*

Figure 3. Effect of solids content of spent mung bean irrigation water on the flux rate through tangential flow filtration unit. Mung bean batches (500g) were sprouted for 96h at 25°C and watered daily with 2 l irrigation water. The spent irrigation water was collected and 100ml samples withdrawn for dry weight analysis (♦). The remaining spent irrigation water was passed through TFF with an operating transmembrane pressure at either 3.5 lb in2 (■) or 0.5 lb in2 (▲) and flow rate of 0.51 l/min.

Figure 4: Total aerobic count and Salmonella *within spent irrigation water derived from sprouting of mung bean batches at different time periods during sprouting. Batches (500g) of mung beans were inoculated with* Salmonella*(1.3 log CFU/g). The beans were sprouted over 96h at 25°C and irrigated daily with 2 l of water. The spent water was collected and* Salmonella *levels determined.*

Figure 5: Response of a cellulose acetate modified platinum electrode to hydrogen peroxide in the presence of 0.1% BSA (A) or heat killed (4 log CFU/ml) Salmonella(B). Step calibration curves were performed by addition of 1mM hydrogen peroxide (♦) to a polarized (0.65V vs Ag/AgCl) platinum electrode modified with a cellulose acetate membrane. The dose response to hydrogen peroxide was repeated in the presence of BSA or heat killed cells (■) using the same cellulose acetate membrane. Finally the dose response of the cellulose acetate membrane to hydrogen peroxide alone was assessed to determine the degree of biofouling (▲). Data points represent the average of three different cellulose acetate membranes

Figure 6: Scanning electron micrographs of cellulose acetate membranes without (A) and with (B) immobilized capture antibody. A thin gold layer was deposited onto the surface of cellulose acetate membranes upon which capture antibody was immobilized. The membrane was reacted with a Salmonella suspension and subsequently was to remove unbound cells. The control was prepared in the same way except that no capture antibody was immobilized on the membrane surface.

Figure 7: Verification of integrated Tangential Flow Filtration and immune-sensor to detect Salmonella *in spent irrigation water collected from mung bean beds 96 h into the sprouting process.* Salmonella *was inoculated into mung bean batches (1.3 log CFU/g) and sprouted over 96 h with daily irrigation with 2 l water. At the end of the sprouting period, 2 l of spent irrigation water was collected and concentrated using TFF. The retentate was then applied to the amperometric ELISA as described in the legend to Table 4. For sensor response for individual samples was plotted (■) and compared to the average response obtained for samples derived from non-inoculated controls (-----).*

Table 1: Increased concentration and percent recovery after concentrating 2 l of spent irrigation water containing Salmonella. *Spent irrigation water was collected from mung bean batches sprouted over 96h at 25°C. The spent irrigation water was inoculated with Salmonella and passed through the TFF system.The levels of target bacteria were enumerated from the retentate and used to calculate the concentration factor and percent recovery.TMP 3.5 Ib in^2, flow rate 511 ml/min*

Initial (log CFU/ml)	**Retentate log CFU/ml (CF[a])**	**% Recovery[b]**
4.5	5.7±0.58 (15.85)	82 ± 8
2.0	3.0±0.13(10.00)	55 ± 8

[a]CF: Concentration factor = CFU retentate/CFU initial suspension

[b] % Recovery = (total CFU retentate/total CFU initial suspension)*100

Table 2. *Capture of different* Salmonella *serovars on anti-*Salmonella *modified and non-modified cellulose acetate membranes.* Salmonella *suspensions (3 log CFU/ml) were flowed over (0.89 ml/min) cellulose acetate membrane and levels of the enteric pathogen retained within the suspension enumerated.*

Salmonella **serovar**	**Log CFU/ml anti-*Salmonella* CA[a] (% Capture)[b]**	**Log CFU/ml CA[a] control (% Capture)[b]**
Heidelberg	2.17 ± 0.35 (90%)[A]	2.96 ± 0.17 (36%)[B]
Senftenberg	1.88 ± 0.39 (95%)[A]	3.10 ± 0.31 (12%)[B]
Montevideo	2.58 ± 0.23 (73%)[A]	3.03 ± 0.32 (25%)[B]
Newport	2.12 ± 0.20 (91%)[A]	3.06 ± 0.29 (20%)[B]
Meleagridis	2.71 ± 0.21 (64%)[A]	3.04 ± 0.31 (24%)[B]
Oranienburg	2.39 ± 0.32 (83%)[A]	2.99 ± 0.32 (32%)[B]

[a]CA: Cellulose acetate

[b] % Recovery = (CFU Remaining in suspension/total CFU Initial)*100

Values within rows followed by the same letter are not significantly different

Table 3: *Concentration and detection of* Salmonella *from spent irrigation water derived from sprouting mung bean beds.* Salmonella *was inoculated into mung bean batches (1.3 -3.3 log CFU/g) and sprouted over 96 h. Spent irrigation samples (2 l) were collected at the end of the sprouting period and circulated through tangential flow filtration unit (511 ml/min, TMP 3.5 lb in^2). The retentate from the TFF was then passed through immune-flow cell (0.98 ml/min) and captured cells reacted with secondary glucose oxidase conjugate. The amperometric sensor response upon addition of glucose substrate was then recorded.*

Initial Loading **log CFU/g**	**Spent Irrigation Water log CFU/ml**	**Retentate log CFU/ml (CF)[a]**	**Sensor Response (nA)**
0	0	0	32.6±19.82^A
1.3	2.43±0.39	3.0±0.13 (4)	342.1±135.04^B
1.9	3.74±0.36	5.7±0.58 (91)	364.9±137.10^B
3.3	4.17±0.46	7.47±0.46 (3715)	668.4±71.91^C

[a]CF: Concentration factor = CFU retentate/CFU initial suspension

Means followed by the same letter are not significantly ($P<0.05$) different

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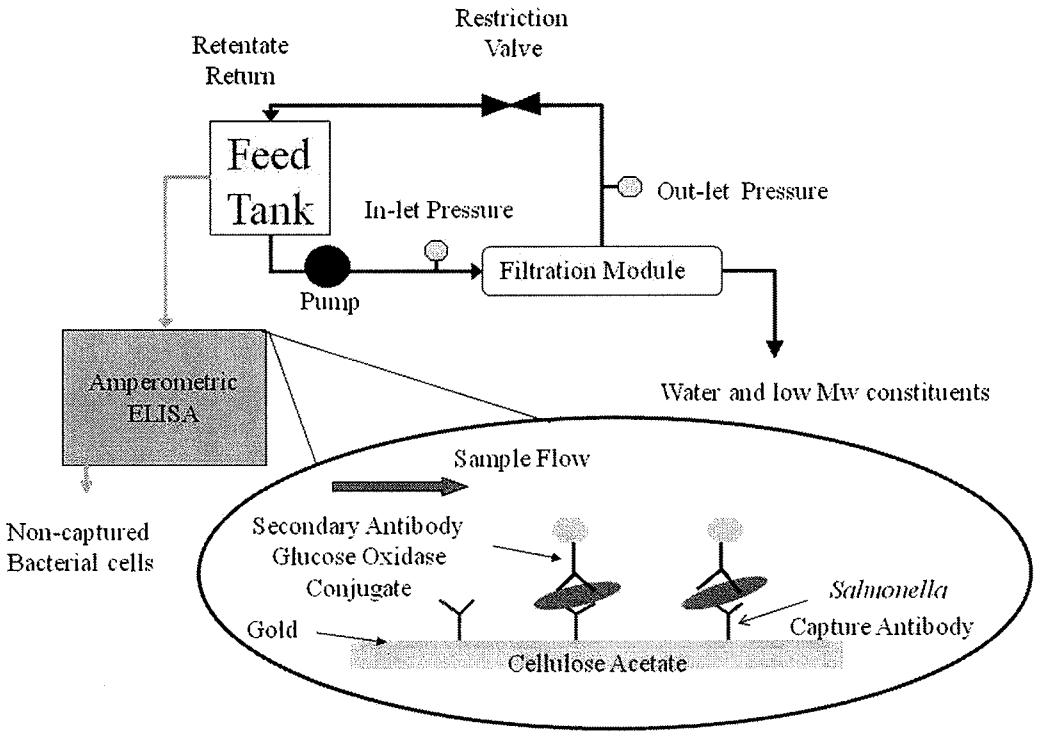
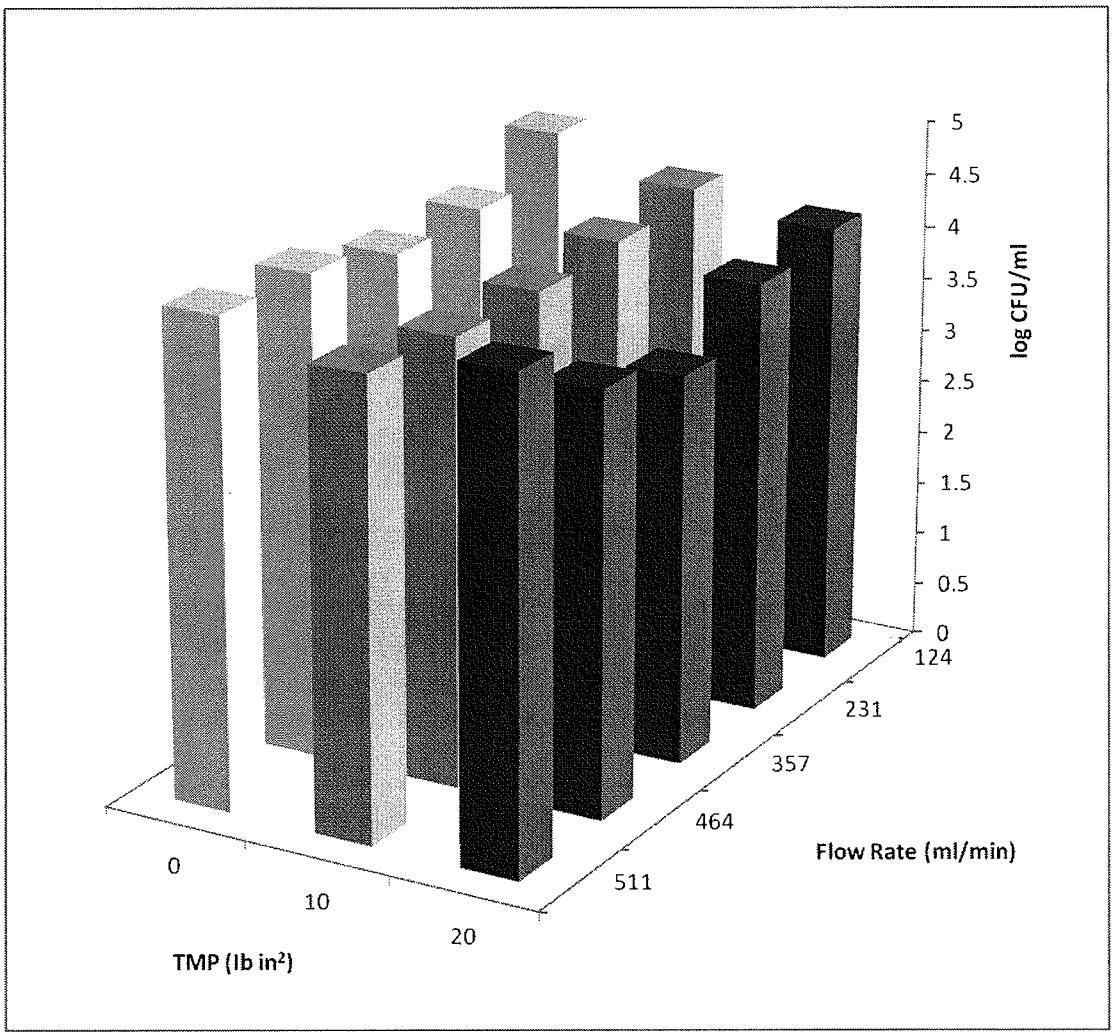


Figure 2: Effect of TFF flow rate and transmembrane pressure on the concentration of Salmonella *from a suspensions originally containing 2 log CFU/ml.* Salmonella *suspensions were prepared in saline and passed through TFF unit at different transmembrane pressure and flow rate. The cell density of* Salmonella *within the retentate following filtration was then determined and used to calculate the concentration factor.*



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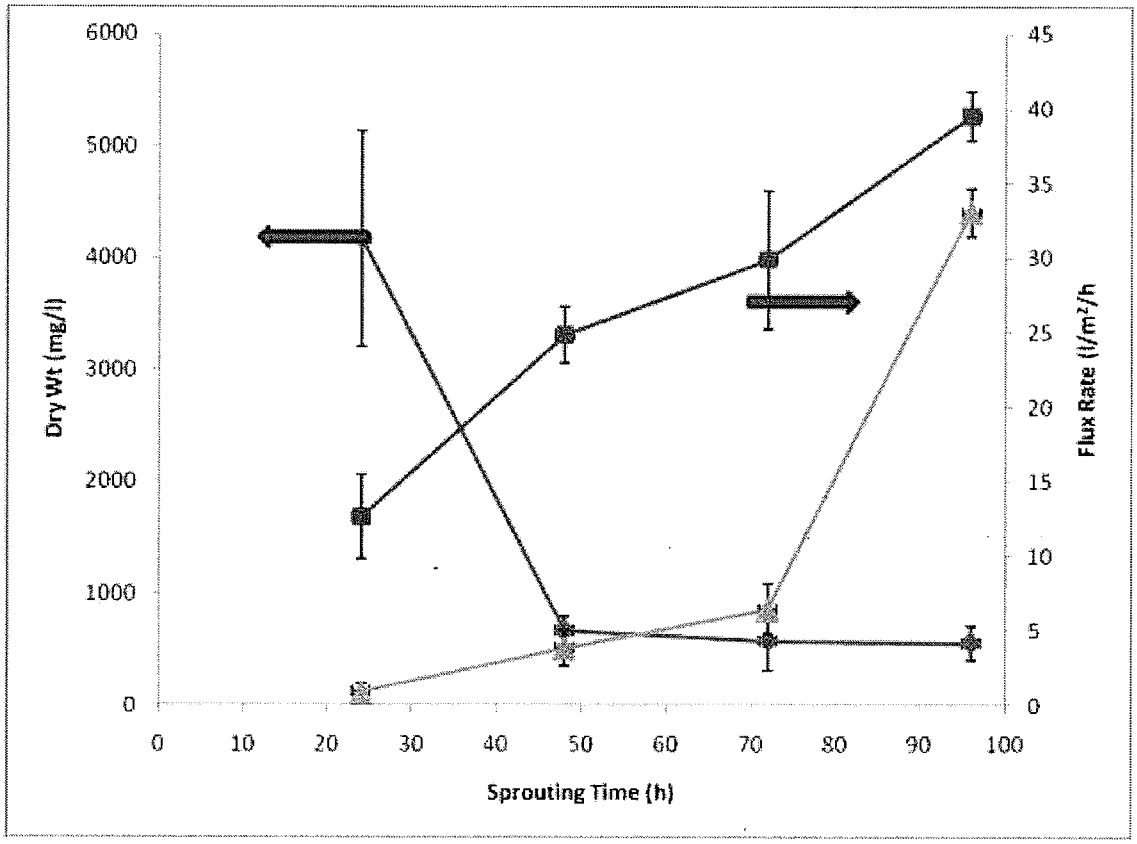

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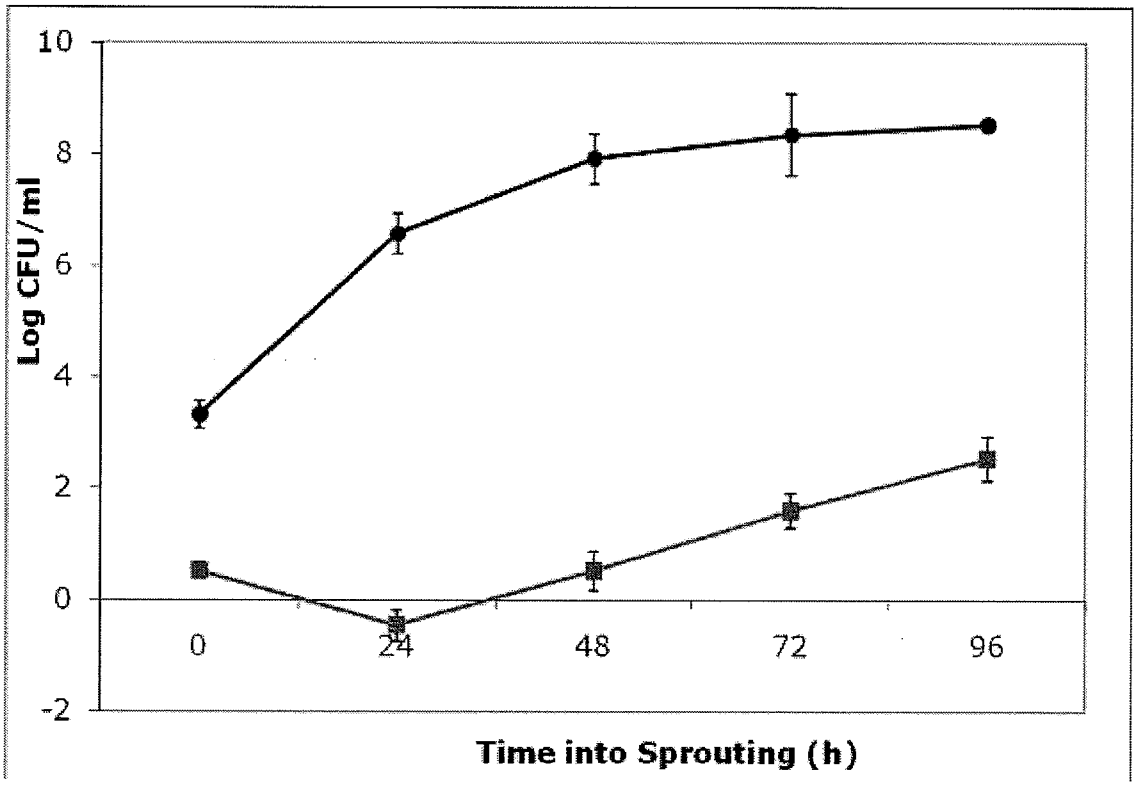

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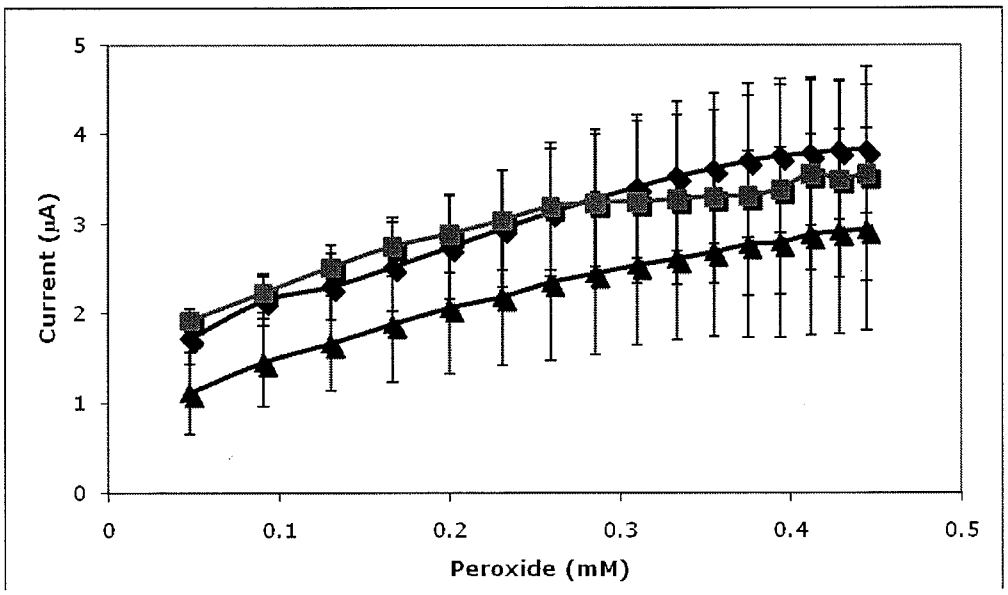

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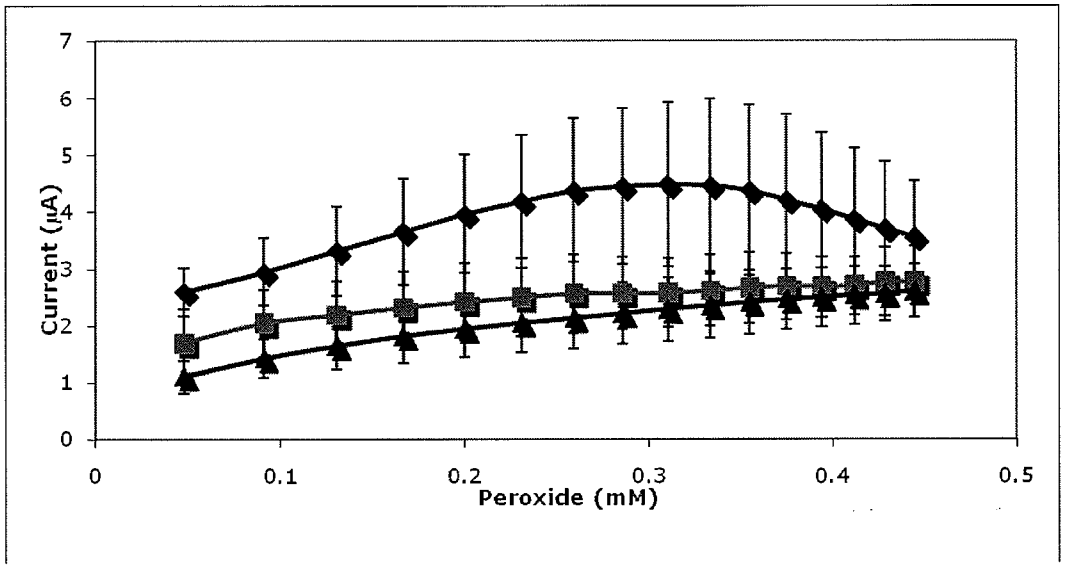

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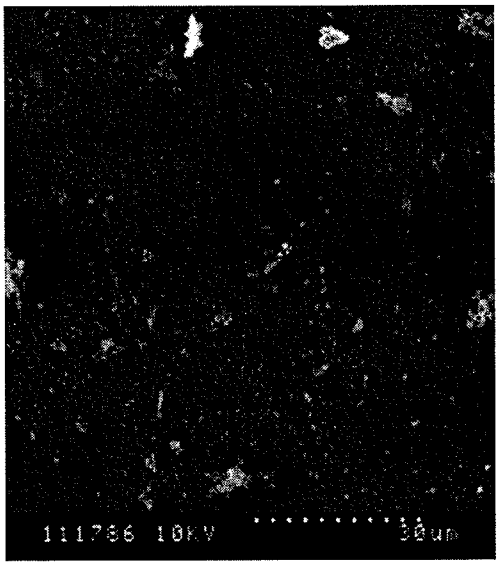
A



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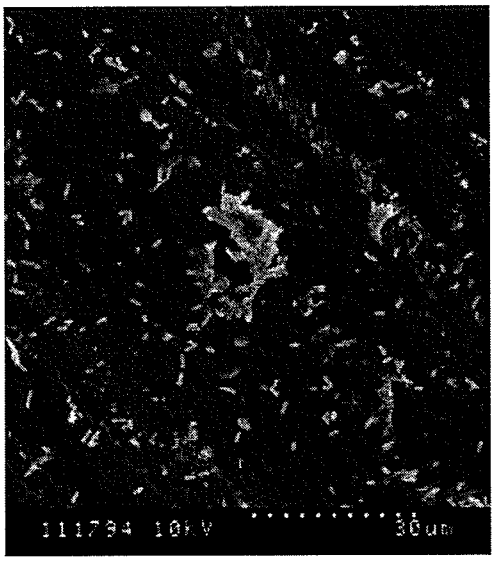

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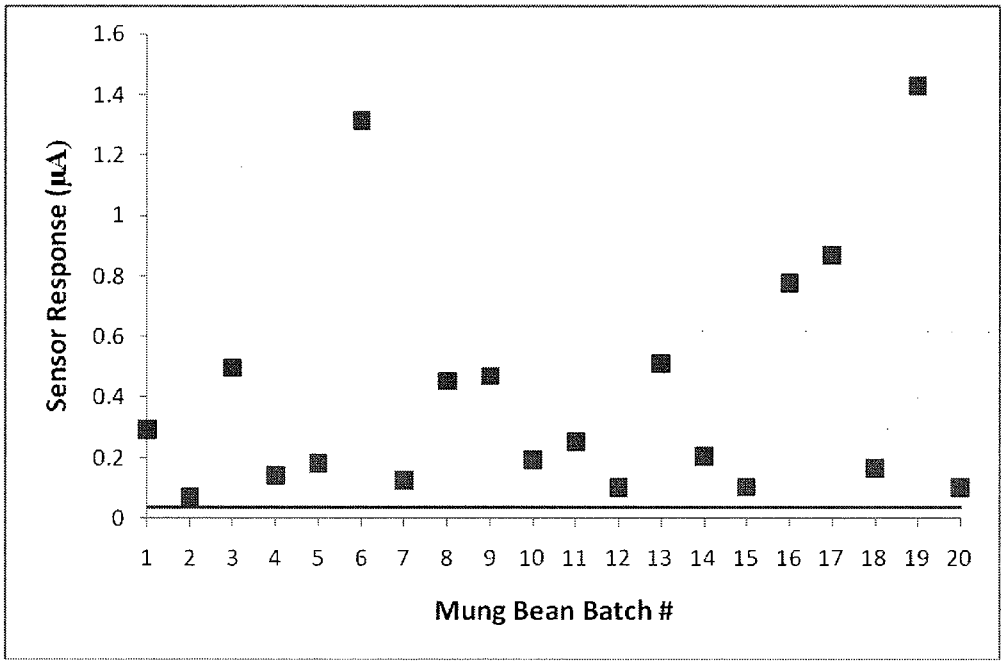

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Spatial Distribution of *Salmonella, Escherichia coli* O157:H7, and Other Bacterial Populations in Commercial and Laboratory-Scale Sprouting Mung Bean Beds

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ABSTRACT

The reliability of testing spent irrigation water to assess the microbiological status of sprouting mung bean beds has been investigated. In commercial trials, the distribution of opportunistic contaminants within 32 bean sprout beds (25 kg of mung beans per bin) was assessed 48 h after germination. The prevalence of generic *Escherichia coli,* thermotolerant coliforms, and *Aeromonas* in sprouts (n = 288) was 5, 11, and 39%, respectively, and 57, 70, and 79% in the corresponding spent irrigation water samples (n = 96). Contamination was heterogeneously distributed within the seedbed. In laboratory trials, beans inoculated with a five-strain cocktail of either *Salmonella* or *E. coli* O157:H7 (10^3 to 10^4 CFU/g) were introduced (1 g/500 g of noninoculated seeds) at defined locations (top, middle, or base), and the beans were then sprouted for 48 h. When seeds inoculated with pathogens were introduced at the base or top of the seedbed, the pathogens were typically restricted to these sites and resulted in 44% of the spent irrigation water samples returning false-negative results. Introducing inoculated beans into the middle or at the presoak stage enhanced the distribution of both pathogens within the subsequent sprout bed and resulted in comparable levels recovered in spent irrigation water. The study demonstrated that even though screening a single sample of spent irrigation water is more reliable than testing sprouts directly, it does not provide an accurate assessment of the microbiological status of sprouting mung bean beds. Such limitations may be addressed by ensuring that bean batches are mixed prior to use and by taking spent irrigation water samples from multiple sites at the latter stages of the sprouting process.

Sprouted seeds have been implicated in numerous outbreaks of foodborne illness *(18). Salmonella* remains the most common human pathogen associated with sprouts, although outbreaks linked to *Escherichia coli* O157:H7 have also been reported *(4).* Between 1973 and 2001, 34 major outbreaks of foodborne illness were directly linked to sprouted seeds, the majority of which have involved alfalfa and bean sprouts *(25).* To address the increasing number of foodborne illness outbreaks associated with sprouts, the FDA (U.S. Food and Drug Administration) issued guidelines to enhance food safety standards *(18).* However, despite the increased focus on food safety, outbreaks of foodborne illness and product recalls continue to occur *(23).* Among other measures, the guidelines recommend decontaminating seeds with 20,000 ppm of calcium hypochlorite prior to sprouting *(18).* However, studies have demonstrated that, although seed decontamination can reduce populations of human pathogens present, it cannot ensure the production of pathogen-free sprouts *(23).* Therefore, there has been an increased reliance on detecting pathogens (if present) by screening spent irrigation water *(23).* The screening of spent irrigation water is preferred over testing sprouts directly because of uniformity issues, ease of collection, and analysis *(18).* More significantly, because irrigation water runs over the sprouts, this method is thought to provide a more representative assessment of the microbiological status of the sprouting seedbed than the screening of individual sprout samples *(18).*

Current recommendations state that 1 liter of spent irrigation water samples should be taken from individual sprouted seed batches 48 h into the sprouting process. Subsamples of the spent irrigation water are then screened for the presence of *Salmonella* and *E. coli* O157:H7 *(18).* The protocols for sampling spent irrigation water were validated through studies that used inoculated and noninoculated alfalfa seeds *(9, 12, 21).* It is assumed that the sampling protocols developed for alfalfa can be extrapolated to other seed types. However, methods applied to sprout alfalfa seeds differ significantly from those used to sprout beans. Specifically, alfalfa is commonly sprouted in rotating drums or as a monolayer of seeds on trays, with irrigation water delivered for 10 s every 10 min. In contrast, mung beans are sprouted in large quantities (25 kg) within deep containers *(18).* The depth of the seedbed is approximately 15 cm but can increase threefold within 48 h and 10-fold upon completion of the sprouting process (unpublished data). During the sprouting of mung beans, irrigation water is delivered via a moving or static overhead sprinkler system for 1 min every 3 to 6 h. The water drains through the bed and exits via slats at the base of the bin.

The contrasting sprouting methods used for alfalfa and bean sprouts could lead to a difference in the distribution

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of human pathogens within the seedbed. Therefore, it is possible that screening a single spent irrigation water sample (as recommended for alfalfa) is inadequate in assessing the microbiological status of an entire batch of sprouting seeds. However, no definitive studies have been performed to determine the homogeneity of contamination within commercial bean sprout beds.

Determining the uniformity of contamination within large batches of sprouting mung beans is problematic, because screening for *Salmonella* or *E. coli* O157:H7 directly is unrealistic, given the rarity in which each pathogen is encountered. On the assumption that human pathogens would occur sporadically on seeds and not in entire batches, there is a need to screen for bacteria that are also occasionally encountered on bean sprouts. Clearly, total aerobic counts and coliforms would be unsuitable, as both are found at consistently high levels in sprouted seeds *(15)*. Potential alternatives are generic *E. coli,* thermotolerant coliforms (TCs), and mesophilic *Aeromonas.* All are opportunistic contaminants on sprouts, although specific prevalence rates have yet to be fully determined. A survey performed in the United Kingdom estimated that the carriage rate of generic *E. coli* in bean sprouts is 4% *(14)*. The prevalence of TCs in bean sprouts has been estimated to be 13% *(20)*. There are no data on the carriage rate of mesophilic *Aeromonas* associated with bean sprouts, although the bacterium is frequently associated with salad vegetables *(5, 16, 19)*. Therefore, generic *E. coli,* TCs, and *Aeromonas* represent opportunistic contaminants that are not ubiquitous within sprouted seeds but that do occur at a greater frequency than pathogens such as *Salmonella* or *E. coli* O157:H7.

The present study consists of two parts. Specifically, sampling trials performed within a commercial sprout facility were undertaken to evaluate the distribution of microflora within large-scale sprouting mung bean beds and how this was reflected in screening spent irrigation water. The second part of the study determined how low levels of *E. coli* O157:H7 and *Salmonella* become distributed within sprouting mung bean beds. Collectively, the goal of this study was to determine if the currently recommended single-sampling protocols provided an adequate method for detecting contamination within sprouting mung bean beds.

MATERIALS AND METHODS

Description of the commercial sprout house and sprouting process. The commercial sprout production facility consisted of several separate growth rooms within which six to eight fixed, slatted floor, sprouting bins (approx. 2.7 by 1.2 m) were housed. Mung beans (25 kg) were rinsed with potable water prior to being soaked for 3 h in 200 ppm of calcium hypochlorite solution (ca. 1:1 seed:sanitizer ratio). The beans were then transferred to a sprouting bin (initial depth of seeds was ca. 15 cm) and watered every 6 h via a moving overhead irrigation water system. The temperature of the growth room varied from 24 to 30°C. Between batches of sprouts, the growth rooms and bins were sanitized in accordance with Good Manufacturing Practice.

Sample collection. Approximately 48 h after the initiation of the sprouting process, samples of sprouts and spent irrigation water were collected from four randomly selected bins within a single growth room. To determine the distribution of bacteria within the sprouting seedbeds, individual bins were arbitrarily separated into three sections (Fig. 1). Spent irrigation water (3 × 3 liters) was collected from under the sprout bed at designated locations (right, center, and left). Sprouts (100 g per sample) were then collected at differing depths of ca. 5 cm (top), 15 cm (middle), and 25 cm (base) within individual columns. The samples were taken above the site from which the spent irrigation water was collected. For each bin, columns of sprouts were collected 15 cm from the left edge, center, and right edge (Fig. 1). Therefore, for each bin, three spent irrigation water samples and nine sprout samples were collected in total. Incoming municipal irrigation water (2 × 3 liters) was also collected. All samples were transferred to a cooler and screened for the target bacteria within 24 h.

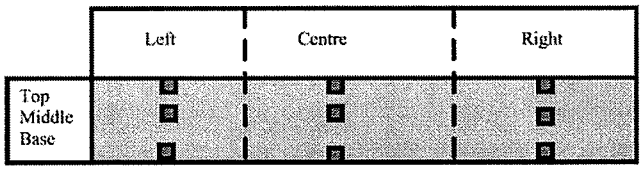

FIGURE 1. *Sampling regime performed in a commercial sprout house. Each bin was subdivided into columns (left, center, and right) within which sprout samples were withdrawn from the top, middle, or base. Prior to extracting the sprout samples, spent irrigation water was collected from under each column.*

Mesophilic *Aeromonas* detection. The detection of *Aeromonas* in sprout samples was performed as described by Neyts et al. *(19)*. Sprouts (25 g) were suspended in 225 ml of tryptic soya broth containing 10 μg/ml of ampicillin (TSBA) and homogenized in a stomacher for 2 min. The homogenate was incubated at 28°C for 18 to 24 h, from which a dilution series was prepared in Butterfield phosphate diluent. Aliquots (0.1 ml) of the 10^{-1} to 10^{-6} dilutions were spread plated onto the surface of starch ampicillin (Oxoid, Basingstoke, UK) agar plates prior to their incubation at 28°C for 18 to 24 h.

Spent irrigation water (100 ml) was filtered through a 45-mm diameter, 0.45-μm-pore-size filter membrane (Fisher, Ottawa, Ontario, Canada). The membrane was then suspended in 10 ml of TSBA and homogenized by stomaching for 2 min. The homogenates were incubated at 28°C for 24 h, and a dilution series was prepared in sterile Butterfield phosphate diluent. Aliquots (0.1 ml) were plated onto starch ampicillin agar plates prior to being incubated at 28°C for 24 h.

Confirmation of *Aeromonas* isolates was made with triple sugar iron agar slants and oxidase-positive reactions *(19)*. The detection limit for mesophilic *Aeromonas* on sprouts was 1 CFU/25 g of sprouts or 100 ml of spent irrigation water.

***E. coli* and TC detection.** Generic *E. coli* was detected by plating sprout homogenates (25 g of sprouts suspended in 225 ml of Butterfield phosphate diluent and stomached for 2 min) onto *E. coli*/coliform petri films (3M, London, Ontario, Canada) that were subsequently incubated at 37°C for 24 h. The lower detection limit for generic *E. coli* (when plating was performed in duplicate) was 5 CFU/g. TCs were screened by the use of m-FC (Difco, Becton Dickinson, Sparks, Md.) agar incubated at 44°C for 24 h (lower detection limit was 50 CFU/g). Spent irrigation water (100 ml) was passed through a 0.45-μm sterile filter that was subsequently suspended in 10 ml of Butterfield phosphate diluent and stomached for 2 min prior to plating onto the appropriate media. The lower detection limit for generic *E. coli* was 0.1 CFU/ml, and

TABLE 1. Salmonella *serovars and* Escherichia coli *O157:H7 strains used during the study*

E. coli O157 strain[a]	Original source	*Salmonella* serovars[a]	Original source
E. coli O157:H7-C1033	Water sediments	Meleagridis E1	Alfalfa sprouts
E. coli O157:H7-C1032	Soil	Oranienburg C1	Alfalfa sprouts
E. coli O157-C652	Clinical samples	Newport C2	Alfalfa sprouts
E. coli O157-C476	Clinical samples	Senftenberg	Alfalfa sprouts
E. coli O157-C477	Clinical samples	Montevideo	Tomatoes

[a] All *Escherichia coli* O157:H7 strains and *Salmonella* Montevideo serovars were donated by the Canadian Research Institute of Food Safety. The remaining *Salmonella* strains were provided by Professor Poppe of Health Canada (Guelph, Ontario, Canada).

for TCs, the lower detection limit for generic *E. coli* was 1 CFU/ml.

Laboratory trials: bacteria and preparation of cell suspensions. *E. coli* O157:H7 and *Salmonella* strains used in the study were selected from environmental, clinical, or sprout isolates (Table 1). Each of the strains was cultivated individually in 50 ml of TSB for 24 h at 37°C. Bacterial cells were harvested by centrifugation (5,500 × *g* for 10 min at 4°C) and washed once in 0.8% (wt/vol) sterile saline. The cell pellet was lastly resuspended in saline to give a final cell density of 10^6 CFU/ml (optical density at 600 nm = 0.02). Equal volumes of the *E. coli* O157:H7 or *Salmonella* suspensions were combined to form a five-strain cocktail that was subsequently used to inoculate seeds.

Inoculation of mung beans and sprouting. Seeds (250 g) were soaked in 250 ml of the appropriate five-strain cocktail of *E. coli* O157:H7 or *Salmonella* for 20 min. The seeds were allowed to dry for 48 h at room temperature and either used immediately or stored at 4°C until required (maximum of 5 days).

Sprouting of inoculated mung beans. Noninoculated mung beans were soaked for 3 h at 25°C in distilled water (1:1 ratio). The soaked seeds were then introduced into cylindrical containers (60 by 6.5 cm) with 5-mm holes drilled into the base to permit the drainage of irrigation water. The depth of the bean column was ca. 15 cm to reflect depths encountered in commercial practice. Inoculated seeds (20 g) were soaked in separate containers (3 h at 25°C), and 1-g lots were introduced into the batch of noninoculated seeds (500 g) at the base, middle, or top of the cylindrical seedbed. The cylinders were then transferred to an environmental growth chamber (Percival Scientific, Inc., San Antonio, Tex.) maintained at 25°C and were watered periodically (ca. every 8 h) with 2-liter volumes of municipal water. Control batches of noninoculated beans were sprouted in parallel.

In a further set of experiments, the inoculated seeds (1 g) were manually mixed with 500 g of noninoculated beans prior to soaking for 3 h in 200 ppm of calcium hypochlorite or sterile distilled water. The seeds were then transferred to cylindrical containers and sprouted as described.

Approximately 48 h into the sprouting process, 2-liter volumes of water were applied to the top of the cylinder, and the spent irrigation water was collected in a sterile container (spent irrigation water). Two 500-ml samples of spent irrigation water (taken from the first and last 500 ml) were collected and pooled for microbiological analysis. Batches of sprouts (200 g) were withdrawn from the top, center, and base of the cylinder. Care was taken to minimize the mixing of sprouts taken from specific locations within the sprouting seedbed.

Detection and enumeration of *Salmonella*. Sprout samples were tested for the presence of *Salmonella* as described in the Health Canada *Compendium of Analytical Methods,* MFHPB-20 *(10). Salmonella* levels in positive sprout samples were enumerated by a five-dilution three-tube most-probable-number (MPN) technique. Batches (25 g) of sprouts were suspended in 225 ml of buffered peptone water (BPW) and stomached for 2 min. A dilution series was prepared in BPW; the tubes were subsequently incubated at 36°C for 18 to 24 h. Aliquots (1 ml) from each tube were then transferred to tetrathionate brilliant green broth and incubated at 42°C for 18 to 24 h. The selective enriched culture was plated onto brilliant green sulfa agar and bismuth sulfite agar and incubated at 35°C for 24 to 48 h. The MPN was obtained by means of MPN tables.

The spent irrigation water sample (100 ml) was filtered through a 0.45-μm membrane filter that was subsequently resuspended in 10 ml of BPW. The bacteria on the filter were then released by vortexing, and a dilution series (three-tube MPN method) was prepared in BPW. The tubes were incubated at 36°C for 24 h. Confirmation of *Salmonella* was made in accordance with the Health Canada MFHPB-20 method *(10). Salmonella* numbers were then derived from MPN tables.

Detection and enumeration of *E. coli* O157:H7. Sprout samples were tested for the presence of *E. coli* O157:H7 by a combination of the immunomagnetic separation technique (Health Canada, HPB MFLP-90) *(10)* and a lateral flow immunoassay method (Reveal O157:H7 kit, Neogen, Lansing, Mich.). *E. coli* O157:H7 was enumerated in positive sprout samples by means of MPN combined with the immunomagnetic separation method as described by Fegan et al. *(8)*. In this procedure, 25 g of sprouts was suspended in 225 ml of BPW and stomached for 2 min. A five-dilution three-tube MPN in 9-ml BPW tubes was prepared, and all tubes were incubated at 42°C for 20 h. Aliquots (1 ml) of each enriched broth were removed and tested by AIMS (antibody immunomagnetic separation) with anti–*E. coli* O157 beads (Dynal, Oslo, Norway) and the BeadRetriever (Dynal) according to the manufacturer's instructions. Volumes (100 μl) of the collected beads were plated onto CT-SMAC and CHROMagar O157 selective agars (Oxoid), which were subsequently incubated at 36°C for 24 to 48 h. Suspect colonies were confirmed as *E. coli* O157:H7 by serology (*E. coli* O157 test kit, Oxoid). Levels of *E. coli* O157:H7 in the original sprout sample were then derived from MPN tables.

Spent irrigation water (100-ml samples) was passed through a 0.45-μm filter, and bacteria were released by submerging the membrane in 10 ml of BPW and stomaching for 2 min. *E. coli* O157:H7 was detected and then enumerated as described.

Statistical analysis. Sampling trials at a commercial sprout house were performed weekly for 8 weeks. On each visit, sprouts and spent irrigation water samples were taken from four randomly selected seedbeds contained within the same growth room. Sprout and spent irrigation water samples were collected from designated areas (across and within the seedbed). Microbiological data were

reported as the presence and absence either in duplicate 25-g sprout samples or in 100 ml of spent irrigation water samples. The qualitative data derived from the commercial sprout sampling trial were analyzed by contingency table analysis (S-Plus software). The data were analyzed on the basis of sprout columns (i.e., three columns per bin) or individual bins ($n = 4$ per trial). Rank (Spearman) regression analysis was performed to assess the correlation between the prevalence of target bacteria in the sprout and spent irrigation water samples. The laboratory trials were performed with quadruplicate sprouting seed batches in parallel with duplicate (noninoculated) controls. The means generated from quantitative data were analyzed by the Student's t test and analysis of variance. In all cases, the level of significance was set at $P < 0.05$.

RESULTS

Prevalence of generic *E. coli*, TCs, and mesophilic *Aeromonas* associated with sprouting mung beans and spent irrigation water produced in a commercial sprout house. The occurrence of generic *E. coli* was not sporadic, but rather, changed during the 8-week trial (Fig. 2A). The prevalence of *E. coli* in spent irrigation water was significantly higher ($P < 0.01$) than that associated with sprouts (Fig. 2A). Nevertheless, there was a significant correlation between *E. coli* recovered from sprouts and spent irrigation water on an individual bin ($r_s = 0.47$ $P = 0.04$) basis.

The prevalence of TCs in spent irrigation water was significantly higher ($P < 0.01$) than in sprouts (Fig. 2B). No significant ($P > 0.05$) correlation existed between the frequency with which TCs were recovered from spent irrigation water when compared to sprouts ($r_s = 0.24$). In trials 4 to 6 (weeks 4 to 6), no TCs were recovered from sprouts, despite the high prevalence within spent irrigation water (Fig. 2B).

The proportion of sprout samples that tested positive for mesophilic *Aeromonas* was comparable to spent irrigation water during the initial three trials performed (Fig. 2C). However, the frequency with which *Aeromonas* was recovered from sprouts decreased during the subsequent trials, although the number of positive spent irrigation water samples remained high (Fig. 2C). Overall, there was a significant correlation ($r_s = 0.58$; $P < 0.01$) between the prevalence of *Aeromonas* in sprouts and spent irrigation water on the basis of the individual bins sampled.

As to the interaction between the different bacterial types, the prevalence of generic *E. coli* in spent irrigation water correlated significantly with TCs ($r_s = 0.88$; $P < 0.01$). However, *Aeromonas* did not show any significant ($P > 0.05$) correlation with respect to either generic *E. coli* or TCs.

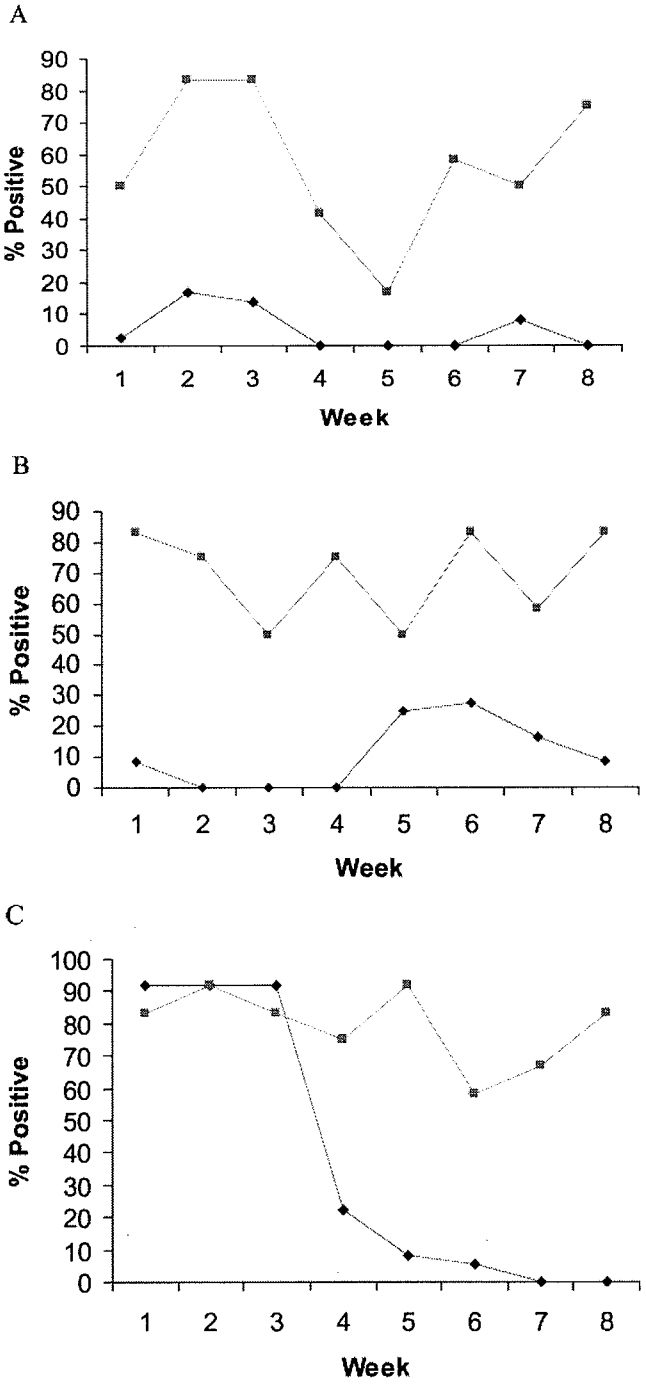

FIGURE 2. *Percent sprouts (♦) and spent irrigation water (■) testing positive for (A) generic* Escherichia coli, *(B) thermotolerant coliforms, and (C) mesophilic* Aeromonas *in commercially sprouted mung beans. During each visit (one per week for 8 weeks), sprouts and spent irrigation water samples were collected from four bins (12 columns). Data points represent percentage of positive samples collected per visit.*

Distribution of generic *E. coli*, TCs, and mesophilic *Aeromonas* within germinating mung bean beds. Generic *E. coli* was recovered from at least one sample (sprouts or spent irrigation water) in 28 of the 32 bins sampled. In total, 6% of the spent irrigation water samples returned false-negative results. In contrast, 49 (of 96) spent irrigation water samples tested positive for the bacterium, but the corresponding sprouts were negative. Generic *E. coli* was heterogeneously distributed throughout the sprouting seedbed, and no specific site was more likely to be contaminated with the bacterium (Table 2). *E. coli* was recovered throughout 11 of the 96 sprout columns sampled but never across the sprouting seedbed. The recovery of *E. coli* in spent irrigation water also showed a random distribution and was independent on the sampling site (Table 2). On 11 occasions, the spent irrigation water collected from all three

TABLE 2. *Number of positive samples from sprouts and spent irrigation water taken from different locations within the sprouting bin*[a]

Sampling site	No. of columns tested	No. of positive samples								
		Escherichia coli			Thermotolerant coliforms			Mesophilic *Aeromonas*		
		Left	Center	Right	Left	Center	Right	Left	Center	Right
Top	96	0	1	1	3	3	4	15	10	13
Middle	96	1	3	3	2	4	5	13	12	13
Base	96	2	2	2	2	3	5	12	11	13
Water	96	18 A	16 A	21 A	22 B	19 B	26 B	24 CD	22 D	30 C

[a] No significant difference ($P > 0.05$) was found between the number of positive sprout samples (for individual bacterial types) at different sampling locations. The number of spent irrigation water samples (for individual bacterial types) with the same letter are not significantly different ($P > 0.05$).

sites within a bin tested positive for *E. coli*. In the remaining bins, the bacterium was recovered from two (9 bins), one (6 bins), or none (6 bins) of the three spent irrigation water samples collected.

At least one sprout or spent irrigation water sample from the 32 sprouting seedbeds tested positive for TCs. Collectively, spent irrigation water samples taken from individual bins showed the presence of TCs in 97% of the seedbeds screened. TCs were recovered throughout the entire column of sprouts on 7 of the 96 tested. However, only a single bin contained TCs across the entire seedbed. This would suggest that contamination within the sprouting seedbed is localized and heterogeneously distributed (Table 2). With regard to spent irrigation water, TCs were recovered in all three samples taken from 15 of 32 bins. In the remaining 17 bins, TCs were present in only one (5 bins) or two (12 bins) of the three spent irrigation water samples taken.

Aeromonas was isolated in at least one spent irrigation water sample taken from each bin. *Aeromonas* was recovered throughout 8 of the 32 bins tested; however, in the remaining 24 containers, the bacterium was either absent in sprouts or localized at specific sites. Spent irrigation water tested positive in all three samples taken in 23 of the 32 bins. In the remaining 9 bins, irrigation water tested positive in one (4 bins) or two (5 bins) of the three samples taken. The frequency with which *Aeromonas* was present in spent irrigation water taken from the center of the seedbed was significantly lower ($P < 0.05$) than from the right edge (Table 2). However, there was no significant difference between the prevalence of the bacterium at specific locations within the sprouting mung bean bed.

Laboratory trials. The loading of *Salmonella* or *E. coli* O157:H7 on inoculated mung beans prior to use varied between 10^3 and 10^4 CFU/g. Significant variation in the distribution of *E. coli* O157:H7 was observed between trials when inoculated seeds were introduced (1 g/500 g of non-inoculated seeds) at the top of the seedbed. *E. coli* O157:H7 either failed to grow or was typically restricted to the site of introduction (Table 3). In all cases, the levels of *E. coli* O157:H7 on sprouts and spent irrigation water were low (Table 3). In one seedbed sampled, the sprouts tested positive (350 ± 157 MPN/g), but spent irrigation water tested negative.

Significant variations in *E. coli* O157:H7 levels were also observed when inoculated seeds were introduced into the middle of the bed. On two occasions (trials I and II), *E. coli* was distributed throughout the column of sprouting seeds at high levels (Table 3). However, only low numbers of *E. coli* O157:H7 were encountered in trials III and IV. In general, high *E. coli* O157:H7 levels within the sprout bed were reflective in the counts recovered in spent irrigation water (Table 3).

E. coli O157:H7 introduced to the base of the seedbed typically became localized at this site, with negligible contamination spreading to other parts of the sprouting seed column (Table 3). Significantly, in three of the four trials performed, sprouts tested positive for *E. coli* O157:H7, but spent irrigation water tested negative. Low levels of *E. coli* O157:H7 (17 MPN/ml) were recovered in one spent irrigation water sample, despite the sprouts at the base of the seedbed containing $>1.1 \times 10^3$ MPN/g (Table 3).

There was significant variation in *Salmonella* counts in sprouts when inoculated seeds were introduced at the top of the seedbed. Indeed, there was significant ($P < 0.01$) variation in the *Salmonella* levels in sprouts taken from the same location in several instances (Table 3). The highest *Salmonella* counts were recovered at the top of the seedbed but decreased toward the base. Spent irrigation water had levels of *Salmonella* comparable to those associated with sprouts. However, in one trial, *Salmonella* levels in the spent irrigation water were $>1.1 \times 10^3$ MPN/ml when the sprout counts ranged from 0.3 to 4.27 MPN/g. In trial IV, no *Salmonella* was recovered from either sprouts or spent irrigation water.

Similar to *E. coli* O157:H7, *Salmonella* introduced in the middle of the seedbed spread to the top and base of the column (Table 3). Spent irrigation water tested positive for *Salmonella* at levels comparable to those recovered in sprouts. However, in one spent irrigation water sample (trial III), the level recovered was $>1.1 \times 10^3$ MPN/ml, and sprout *Salmonella* counts were low, <23 MPN/g.

Sprouts derived from beds in which the inoculated seeds were introduced at the base either became localized within this area or spread throughout the column. Again, significant variations were observed in counts derived from the same sampling point. For example, in one sprout sample taken from the center of the seedbed (trial IV), counts var-

TABLE 3. *Distribution of* Escherichia coli *O157:H7 and* Salmonella *introduced at different points within a sprouting mung bean bed*[a]

Location from which samples were extracted	MPN/g or ml				
	<0.3	MDL[b]	MDL–10^2	10^2–10^3	>1.1 × 10^3
			***Escherichia coli* O157:H7**		
Top inoculated					
Top	1^{II}	1^{III}	1^{IV}	1^{I}	0
Middle	$3^{I,II,IV}$	1^{III}	0	0	0
Base	$3^{I,II,IV}$	0	1^{III}	0	0
Spent irrigation water	$2^{I,II}$	1^{IV}	1^{III}	0	0
Middle inoculated					
Top	1^{III}	1^{IV}	0	0	$2^{I,II}$
Middle	1^{III}	1^{IV}	0	0	$2^{I,II}$
Base	$2^{III,IV}$	0	0	0	$1^{I,II}$
Spent irrigation water	1^{III}	0	1^{IV}	1^{II}	1^{I}
Base inoculated					
Top	$4^{I–IV}$	0	0	0	0
Middle	$3^{I,III,IV}$	0	1^{II}	0	0
Base	1^{III}	0	1^{IV}	0	$2^{I,II}$
Spent irrigation water	$3^{I,III,IV}$	0	1^{II}	0	0
Inoculated seeds introduced during soaking stage					
Top	0	0	0	0	1
Middle	0	0	0	0	1
Base	0	0	0	0	1
Spent irrigation water	0	0	0	0	1
			Salmonella		
Top inoculated					
Top	1^{IV}	0.5^{III}	1^{II}, 0.5^{I}		$0.5^{I,III}$[c]
Middle	1^{IV}	1^{I}	1^{II}, 0.5^{III}[c]		0.5^{III}
Base	1^{IV}	1^{I}	$2^{II,III}$	0	0
Spent irrigation water	1^{IV}	0	$2^{I,II}$	0	1^{II}
Middle inoculated					
Top	1^{II}	1^{I}	$2^{III,IV}$	0	0
Middle	0	$2^{III,IV}$	0	1^{I}	1^{II}
Base	0	1^{IV}	1^{III}	1^{II}	1^{I}
Spent irrigation water	0	0	$2^{II,IV}$	1^{I}	1^{II}
Base inoculated					
Top	$2^{I,III}$	1^{II}	0	0	1^{IV}
Middle	1^{III}	1^{I}	0	0.5^{IV}[c]	1^{II}, 0.5^{IV}[c]
Base	0	0	0	0	$3^{I,III,IV}$[d]
Spent irrigation water	1^{III}	0	0	0	$3^{I,II,IV}$
Inoculated seeds introduced during soaking stage[e]					
Top	0	0	3	0	0
Middle	0	0	2.5	0	0.5[c]
Base	0	0	1	2	0
Spent irrigation water	0	0	0	3	0

[a] I–IV indicate the trial number from which sprouts and irrigation water samples were derived.
[b] MDL (method detection limit) = 0.3 MPN/g or ml.
[c] 0.5 denotes counts from duplicate sprout samples taken from the same location within the sprouting seedbeds but having pathogen levels in different categories.
[d] One sample (trial II) lost because of laboratory error.
[e] Values represent *Salmonella* counts from three separate trials.

TABLE 4. *Distribution of* Escherichia coli *O157:H7 and* Salmonella *within sprouting mung bean beds derived from inoculated seeds treated with calcium hypochlorite (200 ppm for 20 min)*

Pathogen/sprouting period/location sprouts were extracted from	MPN/g or ml				
	<0.3	MDL	MDL–10^2	10^2–10^3	>1.1 × 10^3
E. coli O157					
48 h					
Top	1	0	0	0	0
Middle	0	0	1	0	0
Base	0	0	0	0	1
Spent irrigation water	0	0	1	0	0
96 h					
Top	0	0	1	0	1
Middle	0	0	0	1	1
Base	0	0	0	0	2
Spent irrigation water	0	0	0	2	0
Salmonella					
48 h					
Top	2	0	1	0	0
Middle	2	0	0	0	1
Base	2	0	0	0	1
Spent irrigation water	2	0	0	0	1

ied from 149 to 1.1 × 10^3 MPN/g. High levels of *Salmonella* were associated with both spent irrigation water and sprouts (Table 3). However, in trial III, spent irrigation water tested negative, despite the sprouts at the base of the container containing levels >1.1 × 10^3 MPN/g.

When *Salmonella*- or *E. coli* O157:H7–inoculated seeds were introduced at the bean-soaking stage, the distribution of contamination was homogeneously spread throughout the subsequent column of mung bean sprouts. Pathogen counts in spent irrigation water were comparable to levels recovered in sprouts (Table 4).

Effect of seed decontamination on the distribution of *E. coli* O157:H7 and *Salmonella* in mung bean sprout beds. Calcium hypochlorite treatment of seeds inoculated with *E. coli* O157:H7 resulted in low pathogen levels in the subsequent sprouts (Table 4). However, when sprouts were sampled following a 96-h sprouting period, *E. coli* O157:H7 levels had increased to >1.1 × 10^3 MPN/g throughout the sprouted mung bean column.

Calcium hypochlorite (200 ppm) treatment appeared successful in decontaminating two of the three batches of seeds inoculated with *Salmonella* (Table 4). However, in a third seed batch, the human pathogen grew to high levels. Spent irrigation water samples contained counts similar to those associated with the corresponding sprout beds.

DISCUSSION

From a combination of commercial and laboratory studies, the distribution of bacterial contamination within sprouting mung bean beds has been determined. In the commercial sampling trial, the prevalence of generic *E. coli* and TCs was significantly higher than that reported in previous microbiological surveys of sprouted seeds *(14, 20)*. The source (environment versus seedborne) and reasons for the temporal variability of contamination within the commercial sprouting mung bean beds remain unclear. Nevertheless, the results clearly illustrate that generic *E. coli*, TCs, and mesophilic *Aeromonas* are heterogeneously distributed within sprouting mung bean beds. Moreover, although a correlation did exist between sprouts and spent irrigation water, the prevalence of the target bacteria was greater in the latter. This is in contrast to previous studies that used alfalfa, in which bacterial counts in sprouts were 1 log higher than those encountered in spent irrigation water *(9, 12, 21)*. The sprouts must have harbored the target bacteria for them to have been recovered in spent irrigation water. On this basis, the apparently higher prevalence of the opportunistic contaminants in spent irrigation water was likely because of the heterogeneous distribution of the target bacteria within the sprout bed.

Why contamination did not disseminate throughout the sprout bed is unclear. It has been reported that *E. coli* or *Salmonella* introduced onto inoculated seeds rapidly spreads throughout the sprout bed, even when introduced at low levels *(9, 12, 21, 24)*. However, in such studies, small batches of seeds were typically sprouted as a monolayer. In the present study, it is likely that the relatively deep mung bean bed physically restricted the movement of bacteria. Although the flow of irrigation water would have facilitated the spread of bacteria, it appears to have been limited to vertical as opposed to horizontal transmission. This was likely a consequence of the deposition of columns of water by the moving overhead irrigation system as it passed over the sprouting seedbed.

A heterogeneous distribution of contamination was also observed when seeds inoculated with either *Salmonella* or *E. coli* O157:H7 were introduced at specific points within a sprouting mung bean bed. Although both pathogens

were typically concentrated at the site of inoculation, it was evident that *Salmonella* was disseminated throughout the bed to a greater extent than was *E. coli* O157:H7 (Table 3). It has previously been reported that the attachment of *Salmonella* to sprouting alfalfa seeds is stronger than that of *E. coli* O157:H7 *(1, 6)*. Moreover, it has been established that the frequent watering of sprouting alfalfa seeds inoculated with *E. coli* O157:H7 results in lower counts of the pathogen on the subsequent sprouts *(6)*. Therefore, it is possible that the stronger attachment of *Salmonella* to sprouts resulted in a greater retention of the pathogen within the seedbed. In contrast, the low attachment of *E. coli* O157:H7 would have caused the bacterium to be washed out of the sprouting seed column. Nevertheless, *E. coli* O157:H7 was still retained within the sprouting seedbed at localized sites. This may have been due to the internalization of *E. coli* O157:H7 within the sprouting mung beans and thereby the lesser likelihood of being removed during the passage of irrigation water *(24)*. Significantly, unlike the commercial trial, screening spent irrigation water frequently resulted in false-negative results. This could have been due to the localization of pathogens within the site of inoculation and the removal of loosely attached cells by the successive watering cycles.

When inoculated seeds were introduced at the presoak stage, both *Salmonella* and *E. coli* O157:H7 became distributed throughout the sprouting mung bean bed. This result may have been unexpected, considering that the cell density of *E. coli* O157:H7 and *Salmonella* would have been more diluted than when introduced at specific points within the seedbed. However, during the seed-soaking step, both *E. coli* O157:H7 and *Salmonella* released from the seeds could freely move within the steeping solution, thereby contaminating a larger quantity of mung beans. Although present at low levels on steeped seeds, the nutrient-rich exudates released from the seeds *(12)* could have supported the growth of pathogens, thereby achieving the relatively high levels observed 48 h into the sprouting process. In practical ferms, the results illustrate that the mixing of mung bean batches during the soaking stage can have a significant effect on the distribution of contamination within the subsequent sprouting seedbed.

The actual levels of human pathogens encountered on naturally contaminated seeds remain open to speculation *(9, 17)*. However, pathogen numbers are assumed to be low and further reduced during seed decontamination *(3, 7, 11, 21)*. Therefore, the model in which inoculated seeds were introduced at specific locations within the seedbed may be more reflective of what is encountered in reality. This was the case when beans inoculated with *E. coli* O157:H7 were introduced prior to the soaking stage and were subsequently treated with 200 ppm of calcium hypochlorite. Here, the pathogen became localized in the middle or base of the sprout bed at varying levels 48 h into the sprouting process. Significantly, *E. coli* O157:H7 counts in the spent irrigation water at this time were close to the limit of detection. However, by performing sampling 96 h into the sprouting process, higher counts were obtained in both the sprouts and spent irrigation water.

Salmonella either was not detected or proliferated to high levels when the mung beans were treated with calcium hypochlorite prior to germination. In this respect, it is evident that *Salmonella* follows the distribution pattern similar to the pattern that occurs when inoculated seeds are introduced at the presoak stage. This further highlights the differences that exist between the distribution of the *Salmonella* and *E. coli* O157:H7 encountered within sprouting mung bean beds.

The apparent inactivation of *Salmonella* on beans by the use of relatively low concentrations of calcium hypochlorite was unexpected, considering that the treatment has previously been shown to have limited efficacy *(2, 22)*. However, given that both testing sprouts and spent irrigation water are not completely reliable, it may have been that *Salmonella* was present but not detected.

In conclusion, this study has illustrated the limitation of screening spent irrigation water to report on the microbiological status of sprouting mung bean beds. Although screening multiple spent irrigation water samples can partially compensate for the heterogeneous distribution of contamination, it cannot provide complete assurance that contamination can be detected (if present). This is especially true for *Salmonella* and *E. coli* O157:H7, because both showed a wide variation in distribution patterns within bean sprout beds. Without an effective seed decontamination method, the success of spent irrigation water testing can possibly be enhanced by ensuring that mung bean batches are well mixed during the soak stage and that spent irrigation water is sampled at multiple sites during the latter stages of the sprouting period. It is acknowledged that this will increase the cost of sampling and require an extended holding period before sprouts are released to market. However, rapid pathogen detection methods *(13)* are currently under development that should address such issues.

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REFERENCES

1. Barak, J. D., L. C. Whitehand, and A. O. Charkowski. 2002. Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. *Appl. Environ. Microbiol.* 68: 4758–4763.
2. Beuchat, L. R. 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. *Int. J. Food Microbiol.* 34:329–333.
3. Beuchat, L. R., T. E. Ward, and C. A. Pettigrew. 2001. Comparison of chlorine and a prototype produce wash product for effectiveness in killing *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *J. Food Prot.* 64:152–158.
4. Breuer, T., D. H. Benkel, R. L. Shapiro, W. N. Hall, M. M. Winnett, M. J. Linn, J. Neimann, T. J. Barrett, S. Dietrich, F. P. Downes, D. M. Toney, J. L. Pearson, H. Rolka, L. Slutsker, and P. M. Griffin. 2001. A multistate outbreak of *Escherichia coli* O157:H7 infections linked to alfalfa sprouts grown from contaminated seeds. *Emerg. Infect. Dis.* 7:977–982.
5. Callister, S. M., and W. A. Agger. 1987. Enumeration and characterization of *Aeromonas hydrophila* and *Aeromonas caviae* isolated from grocery store produce. *Appl. Environ. Microbiol.* 53:249–253.
6. Charkowski, A. O., J. D. Barak, C. Z. Sarreal, and R. E. Mandrell.

2002. Differences in growth of *Salmonella enterica* and *Escherichia coli* O157:H7 on alfalfa sprouts. *Appl. Environ. Microbiol.* 68:3114–3120.
7. Charkowski, A. O., C. Z. Sarreal, and R. E. Mandrell. 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. *J. Food Prot.* 64:1292–1298.
8. Fegan, N., G. Higgs, P. Vanderlinde, and P. Desmarchelier. 2004. Enumeration of *Escherichia coli* O157 in cattle faeces using most probable number technique and automated immunomagnetic separation. *Lett. Appl. Microbiol.* 38:56–59.
9. Fu, T., D. Stewart, K. Reineke, J. Ulaszek, J. Schlesser, and M. Tortorello. 2001. Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J. Food Prot.* 64:802–806.
10. Health Canada, Ottawa. 2003. Canadian compendium of analytical methods. Available at: http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/e_index.html. Accessed 29 May 2004.
11. Holliday, S. L., A. J. Scouten, and L. R. Beuchat. 2001. Efficacy of chemical treatments in eliminating *Salmonella* and *Escherichia coli* O157:H7 on scarified and polished alfalfa seeds. *J. Food Prot.* 64:1489–1495.
12. Howard, M. B., and S. W. Hutcheson. 2003. Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Appl. Environ. Microbiol.* 69:548–553.
13. Kramer, M. F., and D. V. Lim. 2004. A rapid and automated fiber optic-based biosensor assay for the detection of *Salmonella* in spent irrigation water used in the sprouting of sprout seeds. *J. Food Prot.* 67:46–52.
14. Little, C. L., and R. T. Mitchell. 2004. Microbiological quality of pre-cut fruit, sprouted seeds, and unpasteurised fruit and vegetable juices from retail and production premises in the UK, and the application of HAACP. *Commun. Dis. Public Health* 7:184–190.
15. Matos, A., J. L. Garland, and W. F. Fett. 2002. Composition and physiological profiling of sprout-associated microbial communities. *J. Food Prot.* 65:1903–1908.
16. McMahon, M. A., and I. G. Wilson. 2001. The occurrence of enteric pathogens and *Aeromonas* species in organic vegetables. *Int. J. Food Microbiol.* 70:155–162.
17. Montville, R., and D. Schaffner. 2005. Monte Carlo simulation of pathogen behavior during the sprout production process. *Appl. Environ. Microbiol.* 71:746–753.
18. National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on sprouted seeds. *Int. J. Food Microbiol.* 52:123–153.
19. Neyts, K., G. Huys, M. Uyttendaele, J. Swings, and J. Debevere. 2000. Incidence and identification of mesophilic *Aeromonas* spp. from retail foods. *Lett. Appl. Microbiol.* 31:359–363.
20. Robertson, L. J., G. S. Johannessen, B. K. Gjerde, and S. Loncarevic. 2002. Microbiological analysis of seed sprouts in Norway. *Int. J. Food Microbiol.* 75:119–126.
21. Stewart, D. S., K. F. Reineke, J. M. Ulaszek, and M. L. Tortorello. 2001. Growth of *Salmonella* during sprouting of alfalfa seeds associated with salmonellosis outbreaks. *J. Food Prot.* 64:618–622.
22. Taormina, P. J., and L. R. Beuchat. 1999. Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. *J. Food Prot.* 62:318–324.
23. Thomas, J. L., M. S. Palumbo, J. A. Farrar, T. B. Farver, and D. O. Cliver. 2003. Industry practices and compliance with U.S. Food and Drug Administration guidelines among California sprout firms. *J. Food Prot.* 66:1253–1259.
24. Warriner, K., S. Spaniolas, M. Dickinson, C. Wright, and W. M. Waites. 2003. Internalization of bioluminescent *Escherichia coli* and *Salmonella* Montevideo in growing bean sprouts. *J. Appl. Microbiol.* 95:719–727.
25. Winthrop, K. L., M. S. Palumbo, J. A. Farrar, J. C. Mohle-Boetani, S. Abbott, M. E. Beatty, G. Inami, and S. B. Werner. 2003. Alfalfa sprouts and *Salmonella* Kottbus infection: a multistate outbreak following inadequate seed disinfection with heat and chlorine. *J. Food Prot.* 66:13–17.

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Composite *versus* single sampling of spent irrigation water to access the microbiological status of sprouting mung bean beds

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Abstract

BACKGROUND: Spent irrigation water testing has been recommended in sprouted seed production to detect the presence of pathogens. However, because of the heterogeneous distribution of contamination within batches of sprouted seed, taking single samples of spent irrigation water may return false-negative results. The following evaluated whether spent irrigation water collected from multiple points provided a more representative assessment of the microbiological status of the sprouting mung bean bed compared to when single samples were taken.

RESULTS: Generic *Escherichia coli* or *Aeromonas* was recovered in one and 10 of the 160 sprout samples taken from 32 sprouting mung bean batches, respectively. Composite spent irrigation water samples tested positive for generic *E. coli* on 19 occasions compared to 12 when single samples were taken. Mesophilic *Aeromonas* was detected in 13 composite spent irrigation water samples which compared to eight single samples. The prevalence of either target bacterium in composite spent irrigation water samples was not significantly ($P > 0.05$) different compared to when a single sample was collected.

CONCLUSIONS: Sampling spent irrigation water from multiple points under sprouting mung bean beds does not significantly increase the probability of detecting contamination, if present. The findings of the study should be considered when devising sampling plans for spent irrigation water testing in bean sprout production.

Keywords: mung bean sprouts; spent irrigation water; testing; *Salmonella*; *Escherichia coli*; *Aeromonas*

INTRODUCTION

Sprouted seeds, such as bean sprouts, have been implicated in numerous outbreaks of foodborne illness.[1,2] The largest outbreak of foodborne illness linked to bean sprouts occurred within Ontario in 2005 and resulted in over 600 clinical cases of salmonellosis (Canadian Food Inspection Agency, http://www.inspection.gc.ca, 7 March 2007). To address the increasing number of foodborne illness outbreaks associated with sprouts, the US Food and Drugs Administration issued guidelines to enhance food safety standards.[3] Amongst other measures, the guidelines recommend screening spent irrigation water collected 48 h into the sprouting process to screen for the presence of pathogens such as *Salmonella* and *Escherichia coli* O157:H7.[4] The screening of spent irrigation water is preferred over testing sprouts directly due to uniformity, ease of collection and analysis.[3] More significantly, because irrigation water runs over the sprouts it is considered to provide a more representative assessment of the microbiological status of the sprouting seedbed compared to when individual sprout samples are screened.[3]

Current recommendations state that a single spent irrigation water sample should be taken from individual sprouted seed batches.[3] Screening single spent irrigation water samples has been considered satisfactory for sprouts (e.g., alfalfa) produced within rotating drums where the seeds are distributed as a monolayer and irrigation water intimately mixed with the sprouting seeds.[4,5] However, a study performed by Liu and Schaffner[6] illustrated that spent irrigation water sampling for alfalfa sprouted on trays is more problematic owing to the heterogeneous distribution of contamination. Here, contamination introduced via seed at a single point within a batch of sprouting alfalfa seed fails to disseminate through the entire bed and hence is restricted to localized sites. Consequently, when spent irrigation water is taken from a distance (>20 cm in the reported study)[6] from the original contamination site

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the probability of detecting the target bacteria is significantly reduced. The problems associated with the heterogeneous distribution of contamination are more significant when attempting to devise sampling plans for screening spent irrigation water derived from mung bean sprout production.[7] Unlike alfalfa, mung beans are sprouted in large quantities (25–75 kg) within deep bins and hence the distribution of microbial populations is more heterogeneous. This was confirmed by the findings of Hora *et al.*,[7] who reported on the distribution of opportunistic contaminants (generic *Escherichia coli* and mesophilic *Aeromonas*) within 25 kg batches of sprouting mung bean sprouts. The selection of generic *E. coli* and *Aeromonas* to assess the distribution of contamination within sprouting seed batches was based on the fact that both bacterial types are occasionally recovered from sprout batches.[7] Therefore, similar to human pathogens the bacteria would be present on a proportion of the seed and spread though the bed during the course of the sprouting process. Screening for pathogens, such as *Salmonella*, directly would have been problematic owing to the sporadic nature in which they are encountered and also the ethical issues associated with detecting a positive sample. In the study by Hora *et al.*,[7] three spent irrigation water samples were collected (left, center and right side of the bed) in addition to corresponding sprout samples at different depths over the sampling point. It was found that contamination was localized within the sprouting mung bean bed and consequently screening a single sample of irrigation water for pathogens would be unreliable in terms of assessing the microbiological status of the bed.[7] Laboratory-based trials using beans inoculated with either *Salmonella* or *E. coli* O157:H7 confirmed that contamination introduced at a specific point in the bed remains localized and does not spread throughout the sprout batch within 48 h into the sprouting process.[7]

A potential approach to address the heterogeneous distribution of contamination within sprouting mung bean batches is to take multiple spent irrigation water samples. However, increasing the number of samples will significantly add to the cost of testing, which would be commercially unfeasible in the majority of sprout operations. A more practical approach is to form a composite from multiple samples, thereby enabling a single test to be performed. Although composite sampling in microbiological analysis can reduce the cost of testing, there are disadvantages in terms of diluting contamination to below the level of detection.

The objective of the following study was to compare composite *versus* single sampling of spent irrigation water derived from sprouting mung bean beds. To address the issues relating to dilution of contamination by composite sampling the individual samples were enriched prior to preparing composites. Although this approach increased the number of steps in sample analysis the enrichment step is relatively less costly than selective detection and hence would not significantly add to the expense of testing.

MATERIALS AND METHODS

Description of the commercial sprout house and sprouting process

The commercial sprout facility sprouted 75 kg lots of mung beans within stainless steel bins (1.5 m^3) with narrow slits on the base of the bed to permit drainage of excess water. The mung beans were prepared for sprouting by an initial rinse in municipal water prior to loading into the sprouting bins. No seed sanitation step was included in the preparation of beans prior to sprouting. Irrigation water was delivered from a moving overhead shower every 3 h. The temperature of the growth room was maintained at 23 °C with filtered fresh air being constantly introduced. Between batches of sprouts, the growth rooms and bins were sanitized in accordance with Good Manufacturing Practice.

Spent irrigation water and sprout sample collection

Spent irrigation water for preparing composite samples was collected 48 h into the sprouting period using 10 × 100 mL sterile bottles (Pharm-Fisher, Ottawa, ON, Canada) positioned at random locations under the bed. In parallel, a 500 mL sample was collected from under the bed at a randomly selected location. In all cases the sampling bottles were temporally fixed in place using denture adhesive purchased from a local supermarket. Samples (500 mL) of incoming municipal water were also collected directly from the overhead shower. Five 100 g sprout samples were collected at random locations on and within the sprout bed and placed in sterile bags. All the samples were transferred to a portable cooler and processed within 24 h.

Microbiological analysis

Composite samples were prepared by subdividing the 100 mL water samples into two 45 mL volumes. Peptone water (Oxoid, Basingstoke, UK; 5mL, 10× concentration) was added to one set of samples and incubated overnight at 42 °C to enrich for generic *E. coli*. Alkaline peptone water (5 mL, pH 8, 10× concentration containing 100 $\mu g\,mL^{-1}$ ampicillin) was added to the second set of subsamples and incubated overnight at 37 °C to enrich for mesophilic *Aeromonas*. Upon completion of the incubation period the respective enriched cultures were combined to form a 500 mL composite sample. The single spent irrigation water sample was subdivided into two 225 mL samples and supplemented with either 25 mL of 10× concentration peptone water or alkaline peptone as described above.

Sprout samples (25 g) were suspended in •either 225 mL peptone water prior to incubating at 42 °C to enrich for generic *E. coli*. For *Aeromonas*, 225 mL alkaline peptone water containing 10 $\mu g\,mL^{-1}$ ampicillin was added to 25 g sprouts and incubated at 37 °C for 24 h.

Aeromonas in the enriched sprout or spent irrigation water samples was isolated by preparing a dilution

series of the enriched samples in Butterfield's phosphate diluent (BPD; Oxoid). Aliquots (0.1 mL) of the 10^{-4} to 10^{-6} dilutions were spread plated onto the surface of starch ampicillin agar (Oxoid) plates prior to incubating at 37 °C for 18–24 h. Confirmation of *Aeromonas* isolates was performed using triple sugar iron agar slants and amino acids, and oxidase-positive reaction.•[9]

Generic *E. coli* was detected in the enriched samples by preparing a dilution series in BPD that were subsequently dispensed on *E. coli*/coliform Petri films (3M, London, ON, Canada). The Petri films were subsequently incubated at 37 °C for 24 h and examined for typical colonies (purple/black with gas).

Statistical analysis

Sampling trials at a commercial sprout grower were performed two or three times a week over an 11-week period (32 sprout beds sampled in total). On each visit, sprouts and spent irrigation water samples were taken from two randomly selected bins contained within the same growth room. Microbiological data were reported as presence or absence in duplicate enriched sprouts. The composite and single spent irrigation water samples were also reported as presence/absence for generic *E. coli* or mesophilic *Aeromonas*. The qualitative data derived from the commercial sprout sampling trial were analyzed using contingency table analysis (S-Plus, Insightful Corp., NY, USA).

RESULTS

Generic *E. coli* and mesophilic *Aeromonas* were consistently recovered from spent irrigation water over the 11-week sampling period. From composite sampling 19 of the 32 beds screened tested positive for generic *E. coli* (Table 1). In comparison, 12 single spent irrigation water samples tested positive for generic *E. coli*, which is not significantly ($P = 0.08$) different compared to when composite sampling was performed (Table 1). On six occasions the composite sample tested positive for generic *E. coli* and the single spent irrigation water negative despite being derived from the same sprout bin. However, in one sample set the single spent irrigation water tested positive but the composite negative (Table 1). Only one sprout sample from a total of 160 screened tested positive for generic *E. coli*, which was significantly ($P < 0.05$) lower compared to the prevalence of the indicator bacterium in spent irrigation water.

Mesophilic *Aeromonas* was recovered from 13 beds sampled when performing composite sampling, which is not significantly ($P = 0.183$) different compared to when single samples were screened (Table 1). By using composite sampling, the spent irrigation water from nine beds tested positive for *Aeromonas* but not when single samples were screened. In contrast, four single spent irrigation water samples tested positive for the bacterium with the composite sampling returning negative results.

Sprout samples taken from 10 sprouting mung bean beds tested positive for *Aeromonas*. In two beds, *Aeromonas* was recovered in all sprout samples tested but was only recovered in one to three samples in the other sprouting seed beds testing positive. This would confirm that the distribution of *Aeromonas* was heterogeneous. In two mung bean beds *Aeromonas* was detected in the sprout samples screened but not in either the single or composite spent irrigation water samples. However, overall there was no significant ($P > 0.05$) difference between the number of positive *Aeromonas* in sprout and spent irrigation water samples.

DISCUSSION

In total, generic *E. coli* was recovered from 20 (63%) of the spent irrigation water derived from 32 individual sprouting mung bean beds sampled, which compares with 57% found in a previous study from a commercial sprout producer.[7] The prevalence of generic *E. coli* associated with sprouts was 0.63%, which compares to 4% reported for sprouts sampled at retail[10] or 5% for sprouts sampled 48 h into the sprouting process.[7]

Mesophilic *Aeromonas* was recovered in 41% of spent irrigation water and, collectively, 15% of sprout samples taken from sprouting mung bean beds. In comparison, the prevalence of mesophilic *Aeromonas* in spent irrigation water and sprouts in a previous study was 79% and 39% respectively.[7]

The origins of *E. coli* and *Aeromonas* were not identified in the current study. The incoming water used to irrigate sprouts tested negative for both bacterial types, although it is possible that environmental sources contributed to the microflora of sprout beds. However, it is well established that the seed used for sprouting is a common source of contamination recovered from sprouts.[3] Regardless of the origins, the prevalence of generic *E. coli* and mesophilic *Aeromonas* it can be confirmed that spent irrigation water provides a more reliable index of the microbiological status of sprouting mung bean beds compared to sprouts.[7] Nevertheless, it was interesting to note that on several occasions sprouts tested positive for mesophilic *Aeromonas* but negative in spent irrigation water. Therefore, although spent irrigation water provides a more reliable assessment of the microbiological status of sprouting mung bean beds it should not be used as a sole intervention to prevent contaminated products reaching the market.

The main objective of the study was to evaluate whether composite spent irrigation water sampling provided a more representative assessment of the microbiological status of sprouting mung bean beds compared to when single samples of spent irrigation water were taken. Although numerically, composite sampling identified more *Aeromonas* and generic *E. coli* positive sprout beds compared to when single samples were screened, such differences were found to

Table 1. Prevalence of generic *Escherichia coli* and mesophilic *Aeromonas* in sprouting mung bean bins as assessed by screening sprouts, composite or single spent irrigation water samples

	Generic *E. coli*			Mesophilic *Aeromonas*		
	Spent irrigation water[a]			Spent irrigation water[a]		
Mung bean bed	Composite	Single	Sprouts[b]	Composite	Single	Sprouts[b]
1	–	–	–	–	–	1/5
2	+	–	–	+	–	–
3	–	–	–	+	+	–
4	–	–	–	–	–	–
5	–	–	–	–	–	–
6	+	–	–	–	–	–
7	+	+	–	–	–	–
8	+	+	–	+	–	2/5
9	–	–	–	+	+	1/5
10	+	–	–	–	–	–
11	+	–	–	+	–	–
12	+	+	–	+	–	–
13	–	+	–	+	–	3/5
14	–	–	–	+	–	3/5
15	+	+	–	–	–	–
16	+	+	–	–	–	–
17	+	+	–	+	+	–
18	–	–	–	–	–	–
19	+	–	–	–	+	–
20	–	–	–	+	–	–
21	–	–	–	+	–	–
22	+	+	–	–	–	–
23	+	–	–	+	+	5/5
24	+	–	–	+	–	5/5
25	–	–	–	–	–	–
26	+	+	1/5	–	–	–
27	+	+	–	–	–	–
28	–	–	–	–	–	–
29	+	+	–	–	+	1/5
30	+	+	–	–	+	–
31	–	–	–	–	+	2/5
32	+	–	–	–	–	1/5
Total positive beds	19A	12A	1B	13A	8A	10A

+, positive; –, negative.

[a] Spent irrigation water was collected from the same 32 sprouting mung bean bins.

[b] Five sprout samples per bin.

Values for total positive beds for individual bacterial types followed by the same letter are not significantly different ($P > 0.05$).

be insignificant. This may have been unexpected given that sampling at more sites would have increased the probability of capturing contamination, if present. It is possible that the target bacteria were homogeneously distributed within the sprouting mung bean beds, thereby negating the benefits of composite sampling. This is unlikely given that the *Aeromonas* and especially generic *E. coli* were sporadically recovered from sprout samples. A more probable explanation is that even though multiple samples were taken from under the sprouting mung bean bins, this only represented a relatively small area of the entire production bed. It is also possible that collecting a single 500 mL sample from a single site, compared to 10 × 100 mL from multiple locations, increased the probability of capturing contamination from a specific area under the bed. The increased probability of detected contamination in 500 mL single samples, compared to 10 × 100 mL, was supported by the finding that a number of single spent irrigation water samples tested positive for target bacteria, with the composite samples testing negative. The results of the study are in agreement with those of Liu and Schaffner,[6] who reported that testing single larger volumes (100 mL *versus* 0.1 mL) significantly increased the probability of detecting contamination in sprouting alfalfa seed batches.

In conclusion, the study has demonstrated that composite sampling does not provide a significantly greater probability in detecting contamination in sprouting mung bean beds compared to screening single spent irrigation water samples.

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REFERENCES

1 Fett WF and Cooke PH, Reduction of *Escherichia coli* O157:H7 and *Salmonella* on laboratory-inoculated alfalfa seed with commercial citrus-related products. *J Food Prot* **66**:1158–1165 (2003).

2 Fett WF, Interventions to ensure the microbiological safety of sprouts, in *Microbiology of Fruit and Vegetables*, ed. by Sapers GM, Gorny JR and Yousef AE. CRC Press, Boca Raton, FL, pp. 187–210 (2005).

3 Anonymous, Microbiological safety evaluations and recommendations on sprouted seeds. National Advisory Committee on Microbiological Criteria for Foods. *Int J Food Microbiol* **52**:123–153 (1999).

4 Fu T, Stewart D, Reineke K, Ulaszek J, Schlesser J and Tortorello M, Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J Food Prot* **64**:802–806 (2001).

5 Howard MD and Hutcheson SW, Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Appl Environ Microbiol* **69**:548–553 (2003).

6 Liu B and Schaffner DW, Mathematical modeling and assessment of microbial migration during the sprouting of alfalfa in trays in a nonuniformly contaminated seed batch using *Enterobacter aerogenes* as a surrogate for *Salmonella* Stanley. *J Food Prot* **70**:2602–2605 (2007).

7 Hora R, Kumar M, Garcia L, Schumacher B, Odumeru J and Warriner K, Spatial distribution of *Salmonella, Escherichia coli* O157:H7, and other bacterial populations in commercial and laboratory-scale sprouting mung bean beds. *J Food Prot* **68**:2510–2518 (2005).

8 Pearce RA and Bolton DJ, Excision vs sponge swabbing: a comparison of methods for the microbiological sampling of beef, pork and lamb carcasses. *J Appl Microbiol* **98**:896–900 (2005).

9 Neyts K, Huys G, Uyttendaele M, Swings J and Debevere J, Incidence and identification of mesophilic *Aeromonas* spp. from retail foods. *Lett Appl Microbiol* **31**:359–363 (2000).

10 Little CL and Mitchell RT, Microbiological quality of pre-cut fruit, sprouted seeds, and unpasteurised fruit and vegetable juices from retail and production premises in the UK, and the application of HAACP. *Commun Dis Public Health* **7**:184–190 (2004).

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Composite *versus* single sampling of spent irrigation water to access the microbiological status of sprouting mung bean beds

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Abstract

BACKGROUND: Spent irrigation water testing has been recommended in sprouted seed production to detect the presence of pathogens. However, because of the heterogeneous distribution of contamination within batches of sprouted seed, taking single samples of spent irrigation water may return false-negative results. The following evaluated whether spent irrigation water collected from multiple points provided a more representative assessment of the microbiological status of the sprouting mung bean bed compared to when single samples were taken.

RESULTS: Generic *Escherichia coli* or *Aeromonas* was recovered in one and 10 of the 160 sprout samples taken from 32 sprouting mung bean batches, respectively. Composite spent irrigation water samples tested positive for generic *E. coli* on 19 occasions compared to 12 when single samples were taken. Mesophilic *Aeromonas* was detected in 13 composite spent irrigation water samples which compared to eight single samples. The prevalence of either target bacterium in composite spent irrigation water samples was not significantly ($P > 0.05$) different compared to when a single sample was collected.

CONCLUSIONS: Sampling spent irrigation water from multiple points under sprouting mung bean beds does not significantly increase the probability of detecting contamination, if present. The findings of the study should be considered when devising sampling plans for spent irrigation water testing in bean sprout production.

Keywords: mung bean sprouts; spent irrigation water; testing; *Salmonella*; *Escherichia coli*; *Aeromonas*

INTRODUCTION

Sprouted seeds, such as bean sprouts, have been implicated in numerous outbreaks of foodborne illness.[1,2] The largest outbreak of foodborne illness linked to bean sprouts occurred within Ontario in 2005 and resulted in over 600 clinical cases of salmonellosis (Canadian Food Inspection Agency, http://www.inspection.gc.ca, 7 March 2007). To address the increasing number of foodborne illness outbreaks associated with sprouts, the US Food and Drug Administration issued guidelines to enhance food safety standards.[3] Amongst other measures, the guidelines recommend screening spent irrigation water collected 48 h into the sprouting process to screen for the presence of pathogens such as *Salmonella* and *Escherichia coli* O157:H7.[4] The screening of spent irrigation water is preferred over testing sprouts directly due to uniformity, ease of collection and analysis.[3] More significantly, because irrigation water runs over the sprouts it is considered to provide a more representative assessment of the microbiological status of the sprouting seedbed compared to when individual sprout samples are screened.[3]

Current recommendations state that a single spent irrigation water sample should be taken from individual sprouted seed batches.[3] Screening single spent irrigation water samples has been considered satisfactory for sprouts (e.g., alfalfa) produced within rotating drums where the seeds are distributed as a monolayer and irrigation water intimately mixed with the sprouting seeds.[4,5] However, a study performed by Liu and Schaffner[6] illustrated that spent irrigation water sampling for alfalfa sprouted on trays is more problematic owing to the heterogeneous distribution of contamination. Here, contamination introduced via seed at a single point within a batch of sprouting alfalfa seed fails to disseminate through the entire bed and hence is restricted to localized sites. Consequently, when spent irrigation water is taken from a distance (>20 cm in the reported study)[6] from the original contamination site

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the probability of detecting the target bacteria is significantly reduced. The problems associated with the heterogeneous distribution of contamination are more significant when attempting to devise sampling plans for screening spent irrigation water derived from mung bean sprout production.[7] Unlike alfalfa, mung beans are sprouted in large quantities (25–75 kg) within deep bins and hence the distribution of microbial populations is more heterogeneous. This was confirmed by the findings of Hora *et al.*,[7] who reported on the distribution of opportunistic contaminants (generic *Escherichia coli* and mesophilic *Aeromonas*) within 25 kg batches of sprouting mung bean sprouts. The selection of generic *E. coli* and *Aeromonas* to assess the distribution of contamination within sprouting seed batches was based on the fact that both bacterial types are occasionally recovered from sprout batches.[7] Therefore, similar to human pathogens the bacteria would be present on a proportion of the seed and spread though the bed during the course of the sprouting process. Screening for pathogens, such as *Salmonella*, directly would have been problematic owing to the sporadic nature in which they are encountered and also the ethical issues associated with detecting a positive sample. In the study by Hora *et al.*,[7] three spent irrigation water samples were collected (left, center and right side of the bed) in addition to corresponding sprout samples at different depths over the sampling point. It was found that contamination was localized within the sprouting mung bean bed and consequently screening a single sample of irrigation water for pathogens would be unreliable in terms of assessing the microbiological status of the bed.[7] Laboratory-based trials using beans inoculated with either *Salmonella* or *E. coli* O157:H7 confirmed that contamination introduced at a specific point in the bed remains localized and does not spread throughout the sprout batch within 48 h into the sprouting process.[7]

A potential approach to address the heterogeneous distribution of contamination within sprouting mung bean batches is to take multiple spent irrigation water samples. However, increasing the number of samples will significantly add to the cost of testing, which would be commercially unfeasible in the majority of sprout operations. A more practical approach is to form a composite from multiple samples, thereby enabling a single test to be performed. Although composite sampling in microbiological analysis can reduce the cost of testing, there are disadvantages in terms of diluting contamination to below the level of detection.

The objective of the following study was to compare composite *versus* single sampling of spent irrigation water derived from sprouting mung bean beds. To address the issues relating to dilution of contamination by composite sampling the individual samples were enriched prior to preparing composites. Although this approach increased the number of steps in sample analysis the enrichment step is relatively less costly than selective detection and hence would not significantly add to the expense of testing.

MATERIALS AND METHODS

Description of the commercial sprout house and sprouting process

The commercial sprout facility sprouted 75 kg lots of mung beans within stainless steel bins (1.5 m^3) with narrow slits on the base of the bed to permit drainage of excess water. The mung beans were prepared for sprouting by an initial rinse in municipal water prior to loading into the sprouting bins. No seed sanitation step was included in the preparation of beans prior to sprouting. Irrigation water was delivered from a moving overhead shower every 3 h. The temperature of the growth room was maintained at 23 °C with filtered fresh air being constantly introduced. Between batches of sprouts, the growth rooms and bins were sanitized in accordance with Good Manufacturing Practice.

Spent irrigation water and sprout sample collection

Spent irrigation water for preparing composite samples was collected 48 h into the sprouting period using 10 × 100 mL sterile bottles (Pharm-Fisher, Ottawa, ON, Canada) positioned at random locations under the bed. In parallel, a 500 mL sample was collected from under the bed at a randomly selected location. In all cases the sampling bottles were temporally fixed in place using denture adhesive purchased from a local supermarket. Samples (500 mL) of incoming municipal water were also collected directly from the overhead shower. Five 100 g sprout samples were collected at random locations on and within the sprout bed and placed in sterile bags. All the samples were transferred to a portable cooler and processed within 24 h.

Microbiological analysis

Composite samples were prepared by subdividing the 100 mL water samples into two 45 mL volumes. Peptone water (Oxoid, Basingstoke, UK; 5mL, 10× concentration) was added to one set of samples and incubated overnight at 42 °C to enrich for generic *E. coli*. Alkaline peptone water (5 mL, pH 8, 10× concentration containing 100 μg mL^{-1} ampicillin) was added to the second set of subsamples and incubated overnight at 37 °C to enrich for mesophilic *Aeromonas*. Upon completion of the incubation period the respective enriched cultures were combined to form a 500 mL composite sample. The single spent irrigation water sample was subdivided into two 225 mL samples and supplemented with either 25 mL of 10× concentration peptone water or alkaline peptone as described above.

Sprout samples (25 g) were suspended in ~~either~~ 225 mL peptone water prior to incubating at 42 °C to enrich for generic *E. coli* ~~or~~ *Aeromonas*, 225 mL alkaline peptone water containing ~~10~~ μg mL^{-1} ampicillin was added to 25 g sprouts and incubated at 37 °C for 24 h.

Aeromonas in the enriched sprout or spent irrigation water samples was isolated by preparing a dilution

series of the enriched samples in Butterfield's phosphate diluent (BPD; Oxoid). Aliquots (0.1 mL) of the 10^{-4} to 10^{-6} dilutions were spread plated onto the surface of starch ampicillin agar (Oxoid) plates prior to incubating at 37 °C for 18–24 h. Confirmation of *Aeromonas* isolates was performed using triple sugar iron agar slants and amino acids, and oxidase-positive reaction.[9]

Generic *E. coli* was detected in the enriched samples by preparing a dilution series in BPD that were subsequently dispensed on *E. coli*/coliform Petri films (3M, London, ON, Canada). The Petri films were subsequently incubated at 37 °C for 24 h and examined for typical colonies (purple/black with gas).

Statistical analysis

Sampling trials at a commercial sprout grower were performed two or three times a week over an 11-week period (32 sprout beds sampled in total). On each visit, sprouts and spent irrigation water samples were taken from two randomly selected bins contained within the same growth room. Microbiological data were reported as presence or absence in duplicate enriched sprouts. The composite and single spent irrigation water samples were also reported as presence/absence for generic *E. coli* or mesophilic *Aeromonas*. The qualitative data derived from the commercial sprout sampling trial were analyzed using contingency table analysis (S-Plus, Insightful Corp., NY, USA).

RESULTS

Generic *E. coli* and mesophilic *Aeromonas* were consistently recovered from spent irrigation water over the 11-week sampling period. From composite sampling 19 of the 32 beds screened tested positive for generic *E. coli* (Table 1). In comparison, 12 single spent irrigation water samples tested positive for generic *E. coli*, which is not significantly ($P = 0.08$) different compared to when composite sampling was performed (Table 1). On six occasions the composite sample tested positive for generic *E. coli* and the single spent irrigation water negative despite being derived from the same sprout bin. However, in one sample set the single spent irrigation water tested positive but the composite negative (Table 1). Only one sprout sample from a total of 160 screened tested positive for generic *E. coli*, which was significantly ($P < 0.05$) lower compared to the prevalence of the indicator bacterium in spent irrigation water.

Mesophilic *Aeromonas* was recovered from 13 beds sampled when performing composite sampling, which is not significantly ($P = 0.183$) different compared to when single samples were screened (Table 1). By using composite sampling, the spent irrigation water from nine beds tested positive for *Aeromonas* but not when single samples were screened. In contrast, four single spent irrigation water samples tested positive for the bacterium with the composite sampling returning negative results.

Sprout samples taken from 10 sprouting mung bean beds tested positive for *Aeromonas*. In two beds, *Aeromonas* was recovered in all sprout samples tested but was only recovered in one to three samples in the other sprouting seed beds testing positive. This would confirm that the distribution of *Aeromonas* was heterogeneous. In two mung bean beds *Aeromonas* was detected in the sprout samples screened but not in either the single or composite spent irrigation water samples. However, overall there was no significant ($P > 0.05$) difference between the number of positive *Aeromonas* in sprout and spent irrigation water samples.

DISCUSSION

In total, generic *E. coli* was recovered from 20 (63%) of the spent irrigation water derived from 32 individual sprouting mung bean beds sampled, which compares with 57% found in a previous study from a commercial sprout producer.[7] The prevalence of generic *E. coli* associated with sprouts was 0.63%, which compares to 4% reported for sprouts sampled at retail[10] or 5% for sprouts sampled 48 h into the sprouting process.[7]

Mesophilic *Aeromonas* was recovered in 41% of spent irrigation water and, collectively, 15% of sprout samples taken from sprouting mung bean beds. In comparison, the prevalence of mesophilic *Aeromonas* in spent irrigation water and sprouts in a previous study was 79% and 39% respectively.[7]

The origins of *E. coli* and *Aeromonas* were not identified in the current study. The incoming water used to irrigate sprouts tested negative for both bacterial types, although it is possible that environmental sources contributed to the microflora of sprout beds. However, it is well established that the seed used for sprouting is a common source of contamination recovered from sprouts.[3] Regardless of the origins, the prevalence of generic *E. coli* and mesophilic *Aeromonas* it can be confirmed that spent irrigation water provides a more reliable index of the microbiological status of sprouting mung bean beds compared to sprouts.[7] Nevertheless, it was interesting to note that on several occasions sprouts tested positive for mesophilic *Aeromonas* but negative in spent irrigation water. Therefore, although spent irrigation water provides a more reliable assessment of the microbiological status of sprouting mung bean beds it should not be used as a sole intervention to prevent contaminated products reaching the market.

The main objective of the study was to evaluate whether composite spent irrigation water sampling provided a more representative assessment of the microbiological status of sprouting mung bean beds compared to when single samples of spent irrigation water were taken. Although numerically, composite sampling identified more *Aeromonas* and generic *E. coli* positive sprout beds compared to when single samples were screened, such differences were found to

Table 1. Prevalence of generic *Escherichia coli* and mesophilic *Aeromonas* in sprouting mung bean bins as assessed by screening sprouts, composite or single spent irrigation water samples

	Generic *E. coli*			Mesophilic *Aeromonas*		
	Spent irrigation water[a]			Spent irrigation water[a]		
Mung bean bed	Composite	Single	Sprouts[b]	Composite	Single	Sprouts[b]
1	–	–	–	–	–	1/5
2	+	–	–	+	–	–
3	–	–	–	+	+	–
4	–	–	–	–	–	–
5	–	–	–	–	–	–
6	+	–	–	–	–	–
7	+	+	–	–	–	–
8	+	+	–	+	–	2/5
9	–	–	–	+	+	1/5
10	+	–	–	–	–	–
11	+	–	–	+	–	–
12	+	+	–	+	–	–
13	–	+	–	+	–	3/5
14	–	–	–	+	–	3/5
15	+	+	–	–	–	–
16	+	+	–	–	–	–
17	+	+	–	+	+	–
18	–	–	–	–	–	–
19	+	–	–	–	+	–
20	–	–	–	+	–	–
21	–	–	–	+	–	–
22	+	+	–	–	–	–
23	+	–	–	+	+	5/5
24	+	–	–	+	–	5/5
25	–	–	–	–	–	–
26	+	+	1/5	–	–	–
27	+	+	–	–	–	–
28	–	–	–	–	–	–
29	+	+	–	–	+	1/5
30	+	+	–	–	+	–
31	–	–	–	–	+	2/5
32	+	–	–	–	–	1/5
Total positive beds	19A	12A	1B	13A	8A	10A

+, positive; –, negative.

[a] Spent irrigation water was collected from the same 32 sprouting mung bean bins.

[b] Five sprout samples per bin.

Values for total positive beds for individual bacterial types followed by the same letter are not significantly different ($P > 0.05$).

be insignificant. This may have been unexpected given that sampling at more sites would have increased the probability of capturing contamination, if present. It is possible that the target bacteria were homogeneously distributed within the sprouting mung bean beds, thereby negating the benefits of composite sampling. This is unlikely given that the *Aeromonas* and especially generic *E. coli* were sporadically recovered from sprout samples. A more probable explanation is that even though multiple samples were taken from under the sprouting mung bean bins, this only represented a relatively small area of the entire production bed. It is also possible that collecting a single 500 mL sample from a single site, compared to 10 × 100 mL from multiple locations, increased the probability of capturing contamination from a specific area under the bed. The increased probability of detected contamination in 500 mL single samples, compared to 10 × 100 mL, was supported by the finding that a number of single spent irrigation water samples tested positive for target bacteria, with the composite samples testing negative. The results of the study are in agreement with those of Liu and Schaffner,[6] who reported that testing single larger volumes (100 mL *versus* 0.1 mL) significantly increased the probability of detecting contamination in sprouting alfalfa seed batches.

In conclusion, the study has demonstrated that composite sampling does not provide a significantly greater probability in detecting contamination in sprouting mung bean beds compared to screening single spent irrigation water samples.

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REFERENCES

1 Fett WF and Cooke PH, Reduction of *Escherichia coli* O157:H7 and *Salmonella* on laboratory-inoculated alfalfa seed with commercial citrus-related products. *J Food Prot* 66:1158–1165 (2003).

2 Fett WF, Interventions to ensure the microbiological safety of sprouts, in *Microbiology of Fruit and Vegetables*, ed. by Sapers GM, Gorny JR and Yousef AE. CRC Press, Boca Raton, FL, pp. 187–210 (2005).

3 Anonymous, Microbiological safety evaluations and recommendations on sprouted seeds. National Advisory Committee on Microbiological Criteria for Foods. *Int J Food Microbiol* 52:123–153 (1999).

4 Fu T, Stewart D, Reineke K, Ulaszek J, Schlesser J and Tortorello M, Use of spent irrigation water for microbiological analysis of alfalfa sprouts. *J Food Prot* 64:802–806 (2001).

5 Howard MD and Hutcheson SW, Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Appl Environ Microbiol* 69:548–553 (2003).

6 Liu B and Schaffner DW, Mathematical modeling and assessment of microbial migration during the sprouting of alfalfa in trays in a nonuniformly contaminated seed batch using *Enterobacter aerogenes* as a surrogate for *Salmonella* Stanley. *J Food Prot* 70:2602–2605 (2007).

7 Hora R, Kumar M, Garcia L, Schumacher B, Odumeru J and Warriner K, Spatial distribution of *Salmonella, Escherichia coli* O157:H7, and other bacterial populations in commercial and laboratory-scale sprouting mung bean beds. *J Food Prot* 68:2510–2518 (2005).

8 Pearce RA and Bolton DJ, Excision vs sponge swabbing: a comparison of methods for the microbiological sampling of beef, pork and lamb carcasses. *J Appl Microbiol* 98:896–900 (2005).

9 Neyts K, Huys G, Uyttendaele M, Swings J and Debevere J, Incidence and identification of mesophilic *Aeromonas* spp. from retail foods. *Lett Appl Microbiol* 31:359–363 (2000).

10 Little CL and Mitchell RT, Microbiological quality of pre-cut fruit, sprouted seeds, and unpasteurised fruit and vegetable juices from retail and production premises in the UK, and the application of HAACP. *Commun Dis Public Health* 7:184–190 (2004).

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