Survival of *Salmonella* Typhimurium on soybean sprouts following treatments with gaseous chlorine dioxide and biocontrol *Pseudomonas* bacteria

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Abstract Control of *Salmonella* Typhimurium on sprouts is crucial for food and consumer safety. In this study, natural microflora on soybean seed was assessed and effects of gaseous chlorine dioxide (ClO₂) and biocontrol *Pseudomonas* on the survival of *S. Typhimurium* on soybean sprouts were evaluated. Sprouts were dip-inoculated with *S. Typhimurium* prior to the application of the biocontrol (*P. chlororaphis* and *P. fluorescens*). After inoculation with *S. Typhimurium*, the sprouts were treated with ClO₂ at 0.4 mg/L for 1 h (90% R.H., 13°C). *Pseudomonas* strains and *Salmonella* were recovered on *Pseudomonas* Agar F (PAF) and xylose lysine tergitol-4 (XLT-4) media, respectively. *Pseudomonas* strains reduced *Salmonella* by <1 log colony forming units (CFU)/g of sprouts, whereas *S. Typhimurium* on soybean sprouts was reduced from 2.55 to 5.35 logs CFU/g by ClO₂. Gaseous ClO₂ treatment reduced *S. Typhimurium* by 3.90 (0 h), 4.47 (24 h), and 3.61 log CFU/g (168 h). It was concluded that ClO₂ and biocontrol treatment can enhance sprout safety.

Keywords: soybean sprouts, chlorine dioxide, biocontrol, *Salmonella enterica*, *Pseudomonas*

Introduction

Contamination of sprouts, leafy greens, and other minimally processed foods by pathogenic bacteria is a food safety problem of perennial occurrence in the United States and various parts of the world. The consumption of sprouts, such as alfalfa (*Medicago sativa*), soybean (*Glycine max* L.), clover (*Trifolium repens*), and radish (*Raphanus sativus*), have recently increased due to increased intake of fresh and fresh-cut fruits and vegetables (1). The increased consumption of sprouts and leafy greens has been attributed to the health benefits as well as convenience of pre-packed fresh produce. However, reports of increase in foodborne outbreaks and illnesses have occurred alongside the consumption of fresh produce (2,3). In the United States, approximately 5% of foodborne outbreaks and illnesses is linked to contamination and consumption of sprouts and sprout products (4). Various pathogenic microorganisms, such as *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Cyclospora* spp., have been associated with contamination of sprouts and leafy greens and implicated in disease outbreaks as well as illnesses of consumers in various countries (5,6).

Outbreaks of foodborne illnesses in various countries have been

attributed to contamination of alfalfa sprouts by *S. Typhimurium* serovar Newport (7) and salmonellosis (8). In a related study, a multistate outbreak of *E. coli* O157:H7 in the United States was reported to be a result of alfalfa sprouts contamination (9). In addition, an outbreak of *E. coli* O104:H4 was associated with contamination of sprouts in Germany, resulting in over 4,000 illnesses (10), as well as *E. coli* on white radish among school children in Japan (11). With regard to beans/soybeans, there are several documented reports of foodborne illnesses such as in the case from the United Kingdom (12).

Intervention approaches utilized for inactivation of foodborne pathogens on sprouts and other minimally processed produce include chemical and physical control measures. Chemical sanitizers such as chlorine wash, oxychlorine wash, and ammonia-based sanitizers have shown various degrees of effectiveness on foodborne pathogens (13). In this research, the authors documented that 3–4 log colony forming units (CFU)/g of cocktail of *E. coli* O157:H7 and *Salmonella* could be inactivated by an oxychloro-based sanitizer at a concentration of 50–100 ppm. Gamma irradiation and other physical measures including cold plasma have been shown to be effective for control of foodborne pathogens on sprouts (14–16). In addition,

combinations of biological control (Enterocin AS-48) when used in a washing solution of organic acids (lactic acid, peracetic acid, polyphosphoric acid, and EDTA) and sodium hypochlorite were found to be effective against Gram-negative bacteria (17).

The biocontrol potential of competitive bacteria comprising various strains of *Pseudomonas fluorescens* or *P. chlororaphis* has been previously applied for control of plant and foodborne pathogens *in vitro*, on produce, and on agronomic crops. Fluorescent *Pseudomonads* have been shown to affect the survival or growth of *Salmonellae* on sprouts (18). *P. fluorescens* isolated from produce have been shown to inhibit spoilage and pathogenic bacteria (19,20). Similarly, strains of *Pseudomonas* species have been shown to inhibit the growth of *Salmonella* serovars on media and sprouts (21). The potential of biocontrol mechanism for *Pseudomonas* spp. may include the inhibition of microbial growth through diffusion of antibiotics and volatile compounds, competition for nutrients and colonization sites, as well as competition for iron (mineral) by microbial produced siderophores (19,22,23).

Due to the increasing outbreaks and food safety concerns associated with sprouts and pathogen internalization on produce, innovative ways to control foodborne contamination on sprout are paramount. In this research, soybean sprouts were utilized due to limited published data that document the efficacy of intervention measures for control of pathogenic bacteria on soybean sprouts, despite high level of consumer utilization. Combinations of biological control measures and gaseous chlorine dioxide (ClO_2) have been previously investigated against *S. Typhimurium* on tomatoes (24–26). Gaseous ClO_2 has also been previously shown to be effective against *Salmonella* on alfalfa and mung bean sprouts (26). Therefore, the objective of this research is to evaluate the efficacy of gaseous ClO_2 and biocontrol *Pseudomonas* to control *Salmonella* Typhimurium on soybean sprouts.

Materials and Methods

Bacteria strains The bacterial strains comprised *P. fluorescens* (2–79), *P. chlororaphis* (30–84), *S. Typhimurium* (ATCC 2380). The isolates of *P. fluorescens* and *P. chlororaphis* (non-human and non-plant pathogens) were obtained from USDA-ARS, Biocontrol Laboratory at Pullman, WA, USA. *S. Typhimurium* was obtained from the culture collection of USDA-ARS, ERRC at Wyndmoor, PA, USA. Stock cultures of the bacteria were maintained in Tryptic Soy broth (TSB; BBL, Sparks, MD, USA) containing 20% glycerol at -80°C . Before use in the experiments, a loopful (~ 0.05 mL) of stock culture was transferred to 9 mL of TSB and incubated at 37°C for *Salmonella* and at 26°C for *Pseudomonas* species for 24 h. Bacterial cells were collected by centrifugation ($10,000\times g$, 5 min at 5°C). The bacterial cell pellet was washed twice with 0.1% buffered peptone water (BPW, BBL) and re-suspended in sterile distilled water to produce cultures of approximately $9 \log$ CFU/mL. The suspended culture was

reconstituted in phosphate buffer saline (PBS) to give final cell concentration of approximately $7\text{--}8 \log$ CFU/mL. This culture was used as the inoculum.

Microflora associated with soybean seeds Soybean (*Glycine max* L.) seeds were purchased from a produce supplier (Sprout House, Lake Katrine, NY, USA) and stored at room temperature (25°C) until used. To determine the microflora on soybean seed prior to generating the sprouts, 25 g of seed was weighed in sterile weighing boats in 4 replicates. Each replicate was assayed separately by adding neutralizing buffer to the soybean seed (1:1 ratio of seed to neutralizing buffer on w/v) in a stomacher bag (Dynatech Lab, Alexandria, VA, USA). Seeds were stomached for 10 min at 250 rpm (400 Circulator; Seward Ltd., West Sussex, UK). A ten-fold serial dilution of samples in buffer peptone water tubes were conducted and plated on different media. To quantify *Pseudomonas* species, serial dilutions were plated on *Pseudomonas* Agar F (PAF; BBL) and incubated at 26°C for 48 h. Aerobic mesophilic bacteria were quantified on plate count agar (PCA; BBL) by incubating plates at 30°C for two days. Lactic acid bacteria were enumerated on de Man, Rogosa and Sharpe (MRS; BBL) medium by incubation at 30°C for two days. Yeast and molds were enumerated on potato dextrose agar (PDA) at 25°C for two days. CFU on the plates at different dilutions were enumerated.

Production of soybean sprouts Soybean seeds (350 g) were weighed on 3 sterilized trays representing three replicates for each treatment. Seeds were sterilized in sodium hypochlorite/chlorine wash solution (300 ppm) for 3 min and rinsed in sterile distilled water for 2 min. Soybean was subsequently soaked in sterile distilled water for 24 h at 25°C to imbibe water. The seeds were placed on a moistened sterile blotter and incubated (26°C) for 3–4 days until germination and sprouts were produced.

Inoculation of soybean sprouts with *S. Typhimurium* and biocontrol *Pseudomonads* (*P. fluorescens* and *P. chlororaphis*) Sprouts (350 g) were placed in a sterilized beaker and bacterial inocula diluted in PBS were used for inoculating the sprouts. The inocula were prepared depending on the treatments used and consisted of monocultures of 1) *P. fluorescens* (Pf), 2) *P. chlororaphis* (Pc), 3) *S. Typhimurium* 2380, 4) Pf+S. Typhimurium 2380, and 5) Pc+S. Typhimurium 2380. For monocultures of bacteria, sprouts were dip-inoculated in 160 mL of inocula for 5 min in laminar hood, removed with sterile forceps, and placed on a weighing boat to air dry for 30 min. In treatments in which combinations of the pathogen and biocontrol were used, soybean sprouts were dip-inoculated in *S. Typhimurium* for 5 min, air dried in the hood for 30 min, and then subsequently dip-inoculated with the biocontrol bacterium (*P. fluorescens*). The same procedure described above was used for inoculation of the pathogen followed by *P. chlororaphis*. Samples were stored for 24 h at 5°C prior to processing. Treatments were

replicated three times, and the experiment was repeated.

Media for microbiological assays The bacteriological media comprised PAF and xylose–lysine–tergitol-4 (XLT-4) media for assaying populations of *P. fluorescens*, *P. chlororaphis*, and *S. Typhimurium*, respectively (Difco, Detroit, MI, USA). The XLT-4 medium supplemented with sodium tetradecyl sulfate has specificity for *Salmonella* (27). The media for isolation and differentiation of bacteria were prepared based on standard procedure.

Recovery of bacteria populations (*S. Typhimurium* and *Pseudomonas* species) from soybean sprouts Inoculated sprouts (25 g) were placed in a stomacher bag, and 50 mL of BPW was added to the samples at the ratios of 1:2. Treatments of soybean sprouts were stomached for 10 min and ten-fold serial dilutions were performed. Samples were plated on XLT-4 and PAF selective media to enumerate *Salmonella* and *Pseudomonas* species, respectively. The PAF and XLT-4 plates were incubated at 26 and 37°C for 48 and 24 h, respectively. The populations of *P. fluorescens*, *P. chlororaphis*, and *S. Typhimurium* were enumerated.

Survival of *S. Typhimurium* on soybean sprouts following treatment with gaseous chlorine dioxide (ClO₂) and *Pseudomonas* biocontrol microbe The survival of *P. fluorescens* and *S. Typhimurium* individually and in combinations of *P. fluorescens*+*S. Typhimurium* was evaluated. A 2×3 factorial treatment comprising gaseous ClO₂ (2 treatments) by bacterial strain inoculations (3 treatments) was established. Soybean sprouts were either treated with gaseous ClO₂ or not treated (untreated). Soybean sprouts were inoculated with *S. Typhimurium*, *S. Typhimurium*+*P. fluorescens*, and *P. fluorescens* were air dried in laminar hood for 30 min, and stored at 5°C for 24 h prior to the application of gaseous ClO₂ treatment. Furthermore, soybean sprouts were subjected to gaseous ClO₂ treatment of 0.4 mg L⁻¹ (90% R.H., and 13°C) for 1 h. ClO₂ was generated on a ClorDiSys Minidox-L gas generator (ClorDiSys Solutions Inc., Lebanon, PA, USA). This is a self-contained unit with capacity to monitor concentrations and treatment time using a panel controller. Soybean sprouts were placed in rectangular trays in which each treatment had three replicates. There were three treatments for each run of the experiment. In the control treatment, soybean sprouts were inoculated with the biocontrol microbe and the bacterial pathogen, which were as follows: 1) *S. Typhimurium*, 2) *S. Typhimurium*+*P. fluorescens*, and 3) *P. fluorescens*. In the control treatment, the inoculated sprouts were not exposed to gaseous ClO₂ treatment. The sprouts treated with gaseous ClO₂ and untreated samples were stomached in a neutralizing buffer (BBL), serially diluted, and plated on XLT-4 and PAF selective media for *Salmonella* and *Pseudomonas*, respectively (16). Samples were plated in triplicate, and the plates were incubated at 37 and 26°C, respectively, for 24 and 48 h. Bacterial CFUs were enumerated.

Effects of *Pseudomonas* bacteria (biocontrol) on the survival of *S.*

Typhimurium on soybean sprouts To assess the efficacy of *P. fluorescens* and *P. chlororaphis* for biocontrol of *S. Typhimurium* on soybean sprouts, soybean sprouts were inoculated (treated) with 1) *S. Typhimurium*, 2) *S. Typhimurium*+*P. chlororaphis*, 3) *S. Typhimurium*+*P. fluorescens*, 4) *P. fluorescens*, and 5) *P. chlororaphis* in three replicates. The treatments were stored (5°C) for 0 day (control), 1 day, and 5 days and processed by stomaching as described above to assess bacterial populations. The percentage reduction of the *S. Typhimurium* populations by *Pseudomonas* species was computed relative to the untreated control (populations of *S. Typhimurium* minus *S. Typhimurium*+*P. fluorescens*/untreated control×100%). Similar computations were made for treatments of *S. Typhimurium*+*P. chlororaphis*.

Statistical analysis Populations of natural microflora recovered from soybean seed were converted in log CFU/g and the means and associated standard errors were analyzed by Proc Means of the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Populations of *S. Typhimurium*, *P. fluorescens*, and *P. chlororaphis* colony counts recovered from soybean seed and sprouts were converted to log CFU/g of produce. Similarly, data on the effects of gaseous ClO₂ and biocontrol *Pseudomonas* on the survival of *S. Typhimurium* on soybean sprouts were computed by Proc GLM of the Statistical Analysis System (Version 9.1, SAS Institute Inc.). Comparison of means and its significance was computed by Fishers' LSD statistics.

Results and Discussion

Microflora associated with soybean seeds The populations of natural microflora determined by microbial plating from the surface of soybean seed were low and ranged from 0.93 to 2.91 log CFU/g of seed (Table 1). The populations of aerobic mesophilic bacteria and *Pseudomonas* species differed significantly ($p<0.05$) from lactic acid bacteria as well as yeasts and molds (Table 1). The populations of aerobic mesophilic bacteria and *Pseudomonas* species were less than 2 log CFU/g of seed, whereas that of yeasts, molds and lactic acid bacteria was less than 1.2 log CFU/g of seed.

Low levels of microflora were detected on soybean seeds with aerobic mesophilic bacteria as well as yeasts and molds averaged at 2.04 and 1.1 log CFU/g of seed, respectively (Table 1). Our data on microflora populations are lower than that previously reported on pod and shelled vegetable soybean (28), in which aerobic mesophilic bacteria averaged at 3.4 and 3.1 log CFU/g of seed, whereas yeast and molds averaged at 2.3 and 2.1 log CFU/g on pods and beans, respectively. The microflora populations may not be expected to be similar since the soybean seeds used in the two experiments were from different sources and therefore variation in seed treatment conditions prior to shipment may account for the differences in microflora populations. In contrast to the above, when assessing the

Table 1. Populations of lactic acid bacteria, *Pseudomonas* species, aerobic mesophilic bacteria, and yeasts and molds recovered from surfaces of soybean seeds subsequent to plating on different media

Media ¹⁾	Microflora	Bacterial populations (log CFU/g) ²⁾
de Man, Rogosa and Sharpe (MRS)	Lactic acid bacteria	0.93±0.22a
Pseudomonas Agar F (PAF)	<i>Pseudomonas</i> sp.	2.91±0.09b
Plate Count Agar (PCA)	Aerobic mesophilic bacteria	2.04±0.38b
Potato Dextrose Agar (PDA)	Yeasts and molds	1.06±0.20a

¹⁾The populations of lactic acid bacteria, *Pseudomonas* spp., aerobic mesophilic bacteria, and yeasts and molds were enumerated after plating in MRS medium (30°C for three days), PAF incubated at 26°C for 48 h, PCA at 30°C for 2–3 days, and PDA at 25°C for 48 h, respectively.

²⁾Soybean seeds were stomached at 250 rpm for 10 min and plated immediately following serial dilutions. Data represent a means plus associated standard errors from 3 replications and two soybean seed lots. Means within each column with the same letters are not significantly different ($p>0.05$).

microbiological quality of fresh fruits, vegetables, and sprouts, sprouts (soybean and alfalfa) were found to be contaminated with mesophilic bacteria (7.9 log CFU/g), psychrotrophic microbes (7.3 log CFU/g), and *Enterobacteriaceae* (7.2 log CFU/g) and had high incidence of *E. coli* (40% of samples) although none was positive for *E. coli* O157:H7 (29). In comparison to quantification of aerobic mesophilic bacteria, yeast and molds, and *Pseudomonas* species on minimally processed produce, the populations of microflora are within range of the microbial load of that reported on minimally processed produce (3,30).

Recovery of bacteria populations from inoculated soybean sprouts

When soybean sprouts were inoculated with monocultures of *P. chlororaphis*, *P. fluorescens*, and *S. Typhimurium* at inocula levels of 7.8, 8.3, and 8.2 log CFU/g, populations of *P. chlororaphis* and *P. fluorescens* recovered were similar but differed significantly ($p<0.05$) from *S. Typhimurium* (Fig. 1). The bacterial populations were similar to the levels of initial inocula. In co-cultures of *S. Typhimurium*+*P. fluorescens* inoculated on soybean sprouts, the populations of *P. fluorescens* and *S. Typhimurium* differed significantly ($p<0.05$). Similarly, *S. Typhimurium* and *P. chlororaphis* recovered from the mixtures also differed significantly ($p<0.05$) from each other (Fig. 1).

Populations of *P. chlororaphis* and *Salmonella* were readily recovered from the inoculated soybean seed following monoculture and co-inoculations (data not shown). This demonstrates that the assay procedure can be effectively utilized to recover the biocontrol microbe and the pathogen from soybean seed. The recovery of *Salmonella* and other pathogenic bacteria and biocontrol microbes has been previously documented on mung beans, tomatoes, and other *in vitro* assays (25,26,31). The recovery of *P. chlororaphis* (7.8

Fig. 1. Recovery of *Pseudomonas chlororaphis*, *P. fluorescens*, and *Salmonella* Typhimurium from soybean sprouts. (A) control (un-inoculated) inocula prior to application on sprouts and monocultures of each bacterial species recovered from soybean sprouts, and (B) *S. Typhimurium* and *P. fluorescens* (Pf) recovered from soybean sprouts inoculated with mixture of *S. Typhimurium*+Pf, as well as *S. Typhimurium*, and *P. chlororaphis* (Pc) recovered from soybean sprouts inoculated sequentially with mixtures of *S. Typhimurium*+Pc. Means of populations recovered with the same letters on bars for each pathogen species are not significantly different ($p>0.05$).

log CFU/g), *P. fluorescens* (8.2 log CFU/g), and *S. Typhimurium* (8.2 log CFU/g) populations from soybean sprouts were similar to inoculum levels except for *S. Typhimurium* inoculum, implying that the assay procedure can be readily used for pathogen recovery from sprouts as well as the recovery of *Pseudomonas* and *Salmonella* from co-cultures of *S. Typhimurium*+Pf and *S. Typhimurium*+Pc from sequential inoculations.

Survival of *S. Typhimurium* on soybean sprouts following treatment with gaseous ClO_2 and *P. fluorescens* biocontrol microbe Gaseous ClO_2 application significantly ($p<0.05$) affected the survival of bacterial populations on soybean sprouts. The reductions of bacterial populations on inoculated soybean sprouts were greater for *S. Typhimurium* (CDC 2380) than on *P. fluorescens* (Table 2). At 1 h of treatment of soybean sprouts with ClO_2 (0.4 mg L⁻¹, 90% R.H, 13°C) followed by immediate processing of samples (0 h storage), *S. Typhimurium* populations were reduced by 3.99 log CFU/g of sprouts

Table 2. Effects of gaseous chlorine dioxide on the survival of *Pseudomonas fluorescens* (2-79) and *Salmonella* Typhimurium (2380) on soybean sprouts (log CFU/g)

Microbial populations ¹⁾	Storage time (h)	Untreated (control)	ClO ₂ (0.4 mg L ⁻¹) ³⁾	Bacteria reduction (log CFU/g) ⁴⁾
<i>S. Typhimurium</i> (2380)	0	5.94c ²⁾	1.95e	3.99d
<i>S. Typhimurium</i> (with <i>P. fluorescens</i>)	0	5.82c	3.27d	2.50e
<i>P. fluorescens</i> (2-79)	0	8.18a	6.78b	1.40f
<i>P. fluorescens</i> (with <i>S. Typhimurium</i>)	0	8.05a	6.74b	1.31f
<i>S. Typhimurium</i> (2380)	24	5.46c	0.99f	4.47c
<i>S. Typhimurium</i> (with <i>P. fluorescens</i>)	24	5.52c	<0.30g	5.22c
<i>P. fluorescens</i> (2-79)	24	8.26a	6.78b	1.48f
<i>P. fluorescens</i> (with <i>S. Typhimurium</i>)	24	8.16a	6.30b	1.78f
<i>S. Typhimurium</i> (2380)	168	5.39c	1.78e	3.61d
<i>S. Typhimurium</i> (with <i>P. fluorescens</i>)	168	5.65c	<0.30g	5.35c
<i>P. fluorescens</i> (2-79)	168	8.21a	6.68b	1.53f
<i>P. fluorescens</i> (with <i>S. Typhimurium</i>)	168	8.20a	5.47c	2.73e

¹⁾Soybeans were inoculated with monocultures of each bacteria suspension at approximately 7.96 log CFU/g for *P. fluorescens* and 8.03 log CFU/g of sprouts for *S. Typhimurium*, followed by the application of ClO₂ at 1 h. ClO₂ was applied at the rate of 0.4 mg L⁻¹, 90% R.H, and at 13°C using the ClorDiSys chlorine dioxide system.

²⁾Bacteria populations in untreated control. Means within each column with the same letters are not significantly different ($p < 0.05$) and data represent means for three replicates.

³⁾Bacteria treated with gaseous chlorine dioxide. Treated soybean sprouts were stored at 13°C for 24 h, prior to blending in buffer and plating for enumeration of *Salmonella* on XLT-4 medium (37°C) and for *Pseudomonas* on PAF after incubation for 24 and 48 h, respectively. Means within each column with the same letters are not significantly different ($p > 0.05$).

⁴⁾Reduction of bacterial populations were computed relative to the control. The mean log reductions within each column with the same letters are not significantly different ($p > 0.05$).

(Table 2). The larger bacteria reduction at 24 h than that at 168 h in *S. Typhimurium* (2380) may be explained by temperature effects (13°C) that was less than optimum for bacterial colonization and growth during storage. The population of *S. Typhimurium* that was co-inoculated with *P. fluorescens* (2-79) was reduced by 1.93 log CFU/g of sprouts. Low inactivation of *P. fluorescens* and *P. fluorescens* co-inoculated with *S. Typhimurium* were recorded (1.3–1.4 log CFU/g of sprout) when samples were processed immediately (0 storage time) after gaseous ClO₂ treatment.

When soybean sprouts were processed after 24 h of storage following gaseous ClO₂ treatment, *S. Typhimurium* and *S. Typhimurium* + *P. fluorescens* (co-inoculated with *P. fluorescens*) were reduced by 4.47 and 5.22 log CFU/g of sprouts, respectively (Table 2). In the two cases mentioned above, the surviving populations of *S. Typhimurium* were less than 0.99 and <0.30 log CFU/g of sprouts (un-detectable). After 24 h of storage, *P. fluorescens* and *P. fluorescens* co-inoculated with *S. Typhimurium* were reduced by 1.48 and 1.78 log CFU/g of sprouts, respectively (Table 2). The surviving populations of *P. fluorescens* and *P. fluorescens* co-inoculated with *S. Typhimurium* were 6.74 and 6.38 log CFU/g of sprouts, respectively. After ClO₂ treatment and storage of soybean sprouts for 168 h (7 days), the populations of *S. Typhimurium* and *S. Typhimurium* co-inoculated with *P. fluorescens* were reduced by 3.61 and 5.35 log CFU/g of produce. Similarly, reductions of *P. fluorescens* and *P. fluorescens* co-inoculated with *S. Typhimurium* were 1.53 and 2.73 log CFU/g of sprouts (Table 2). The application of biocontrol *Pseudomonas* after gaseous ClO₂ treatment did not result in further reductions of *S.*

Typhimurium when the samples were processed immediately at 0 h of storage but resulted in greater reductions at 24 h (1 day) and 168 h (7 days) of storage following gaseous ClO₂ treatment (Table 2).

Application of gaseous ClO₂ inactivated and reduced *S. Typhimurium* populations on soybean sprouts when inoculated with monoculture of *S. Typhimurium*, *S. Typhimurium* co-inoculated with *P. fluorescens* sequentially, and *P. fluorescens* co-inoculated with *S. Typhimurium* in sequence. Variations in reductions of *Salmonella* populations on soybean sprouts were recorded at 0 h (1.31–3.99 log CFU/g), 24 h (1.78–5.22 log CFU/g), and 168 h (2.73–5.35 log CFU/g) following treatment, implying that the effectiveness of inactivation increases with produce storage. This may be due to refrigeration conditions for storage (26,31). The reductions of *S. Typhimurium* on soybean sprouts suggest that 0.4 mg L⁻¹ of ClO₂ treatment for 1 h is sufficient to inactivate the pathogen on soybean sprouts. Our results are similar to that previously reported on other produce such as tomatoes, in which inactivation of *S. Typhimurium* with gaseous ClO₂ on tomatoes applied at 0.4 mg L⁻¹ resulted in bacterial reductions of 3–5 log CFU/g although the application duration for gaseous ClO₂ were at 2 and 4 h (24). The application of biocontrol *Pseudomonas* after gaseous ClO₂ treatment resulted in additional reductions of *S. Typhimurium* when the samples were processed at 24 and 168 h of storage following ClO₂ treatment. This suggests that addition of the biocontrol following gas treatment may enhance inactivation of *Salmonella* on soybean sprouts. In another study on ClO₂ application on tomatoes at 5, 10, and 20 ppm, *S. Typhimurium* reductions in excess of 5 logs were reported for treatment durations of 6, 4, and

2 s, respectively (32). This treatment duration is lower than that in our study although the produce was different. In a study on growth and quality of soybean sprouts, at gamma irradiation (D_{10} values) of 3 kGy was noted to be useful for microbial safety of soybean sprouts (33). In a totally different research on inactivation of *S. Typhimurium* on broccoli and red radish sprouts using electron beam radiation, the D_{10} values were 0.30 and 0.23 kGy on the two produce, respectively (34). The radiation (D_{10} values) for gamma rays on *S. Typhimurium* on broccoli and red radish sprouts averaged at 0.13 and 0.14 kGy, respectively.

Effects of *Pseudomonas* bacteria (biocontrol) on the survival of *S. Typhimurium* on soybean sprouts The survival of *S. Typhimurium* on soybean sprouts when co-inoculated with *P. fluorescens* (2-79) and *P. chlororaphis* (30-84) when the samples were processed immediately after inoculation (without storage) did not differ significantly ($p>0.05$). The average reductions of *S. Typhimurium* bacterium in the above mentioned treatments were 0.37–0.79 log CFU/g of sprouts (Table 3). After 24 h of storage (5°C) subsequent to inoculations with the biocontrol microbes, *S. Typhimurium* co-inoculated with *P. fluorescens* and *P. chlororaphis* populations were reduced by 0.40 and 1.07 log CFU/g of produce, respectively (Table 3). The differences were also significant ($p<0.05$) between the two treatments. At 168 h (7 days) of storage, the reduction of *S. Typhimurium* when co-inoculated with *P. fluorescens* and *P. chlororaphis* was not statistically significant ($p>0.05$). The reductions of *S. Typhimurium* attributed to *P. fluorescens* and *P. chlororaphis* were 0.43 and 0.57 log CFU/g of sprout. In this experiment, quality evaluation of soybean sprouts after treatment with *Pseudomonas* sp. was not done, however; the images of sprouts before and after treatment (Fig. 2A and 2B) indicated that soybean sprouts had some discoloration but not extensively at day 7.

Fig. 2. Soybean sprouts prior to treatment with non-pathogenic biocontrol *Pseudomonas* spp. (A) and after inoculation with *Pseudomonas* spp. and storage for 7 days at 5°C (B).

In contrast to the gaseous ClO_2 treatment, suppression of *S. Typhimurium* on soybean sprouts by biocontrol *Pseudomonas* were comparatively lower, implying that bacterial reductions attributed to biocontrol microbe is not as efficacious as ClO_2 gas treatment. The reductions obtained as a result of biocontrol *Pseudomonas* bacteria ranged from 0.40 to 0.79 log CFU/g of sprouts at 24 and 168 h of storage at 5°C (Table 3). This is contrary to the expectations as greater reductions and biocontrol efficacy would be expected at longer storage durations to allow for biocontrol colonization.

Table 3. Reduction of *Salmonella* Typhimurium populations (log CFU/g) on soybean sprouts following artificial inoculation with *Pseudomonas fluorescens* and *P. chlororaphis* on and storage at 5°C for 7 days

Treatments ¹⁾	Storage time (h)	Bacterial populations (log CFU/g) ²⁾	Reduction of <i>Salmonella</i> (log CFU/g) ³⁾
<i>S. Typhimurium</i> 2380 (control)	0	5.55±0.07a	-
<i>S. Typhimurium</i> (with <i>P. fluorescens</i> 2-79)		5.18±0.12ab	0.37e
<i>S. Typhimurium</i> (with <i>P. chlororaphis</i>)		4.76±0.04bc	0.79d
<i>S. Typhimurium</i> 2380 (control)	24	5.63±0.06a	-
<i>S. Typhimurium</i> (with <i>P. fluorescens</i> 2-79)		5.11±0.05ab	0.52de
<i>S. Typhimurium</i> (with <i>P. chlororaphis</i>)		5.21±0.09ab	0.42de
<i>S. Typhimurium</i> 2380 (control)	168	5.11±0.18ab	-
<i>S. Typhimurium</i> (with <i>P. fluorescens</i> 2-79)		4.41±0.29c	0.70de
<i>S. Typhimurium</i> (with <i>P. chlororaphis</i>)		4.65±0.09bc	0.46de

¹⁾Soybean sprouts were inoculated with monocultures of *S. Typhimurium* and with combinations of *S. Typhimurium*+*P. fluorescens* and *S. Typhimurium*+*P. chlororaphis*. *P. fluorescens* and *P. chlororaphis* were used as biocontrol agents against *Salmonella*.

²⁾*Salmonella* Typhimurium (2380) populations were recovered and quantified on an XLT-4 medium (37°C for 24 h), whereas *Pseudomonas* strains were enumerated on PAF (26°C after incubation for 48 h). Means of bacterial populations within each column with the same letters are not significantly different ($p>0.05$).

³⁾Reduction of *Salmonella* populations were computed relative to the untreated control. The mean log reductions within each column with the same letters are not significantly different ($p>0.05$).

Table 4. Populations of *P. fluorescens* and *P. chlororaphis* (log CFU/g) on soybean sprouts following artificial inoculation as monocultures and co-inoculated with *S. Typhimurium* and storage at 5°C for 7 days

Treatments ¹⁾	Storage time (h)	Populations of <i>Pseudomonas</i> (log CFU/g) ²⁾
<i>P. fluorescens</i> (Pf) 2-79	0	7.68±0.06ab
<i>P. fluorescens</i> (Pf) 2-79	24	7.55±0.08ab
<i>P. fluorescens</i> (Pf) 2-79	168	7.81±0.14ab
<i>P. chlororaphis</i> (Pc) 30–84	0	7.34±0.16b
<i>P. chlororaphis</i> (Pc) 30–84	24	7.46±0.06ab
<i>P. chlororaphis</i> (Pc) 30–84	168	7.64±0.15ab
<i>Pf</i> ± <i>S. Typhimurium</i> (co-inoculated)	0	7.62±0.02ab
<i>Pf</i> ± <i>S. enteric</i> (co-inoculated)	24	7.69±0.04ab
<i>Pf</i> ± <i>S. Typhimurium</i> (Co-inoculated)	168	7.97±0.03a
<i>Pc</i> ± <i>S. Typhimurium</i> (Co-inoculated)	0	7.61±0.06ab
<i>Pc</i> ± <i>S. Typhimurium</i> (Co-inoculated)	24	7.59±0.05ab
<i>Pc</i> ± <i>S. Typhimurium</i> (Co-inoculated)	168	7.64±0.09ab

¹⁾Soybean sprouts were inoculated with monocultures of *P. fluorescens* and *P. chlororaphis* and co-inoculated with *P. fluorescens*+*S. Typhimurium* and *P. chlororaphis*+*S. Typhimurium* as biocontrol agents against *Salmonella*.

²⁾*Salmonella* Typhimurium (2380) populations were quantified on XLT-4 medium (37°C for 24 h), while *Pseudomonas* strains were enumerated on PAF agar (26°C after incubation for 48 h). The means of bacterial populations within each column with the same letters are not significantly different ($p>0.05$).

However, because soybean sprouts were stored at 5°C in accordance with previous storage temperatures for produce studies (31,35), the possible efficacy of biocontrol microbes could have been negated. Storage studies that would apply biocontrol agents on sprout inoculated produce and storing at 25°C would perhaps yield beneficial results. In a previous research, low to moderate reductions of *S. Typhimurium* populations by biocontrol *Pseudomonas* (*P. fluorescens* and *P. chlororaphis*) were recorded on tomatoes (25). The variation in suppressive effects of biocontrol *Pseudomonas* in that research was attributed to the similar cell density ratios of *Pseudomonas* to *Salmonella* used in produce inoculations, as well as the differential interaction of biocontrol strains and pathogenic *Salmonella* serovars. The populations of *Pseudomonas* bacteria in monocultures and co-inoculated with *Salmonella* at different storage times were similar (Table 4).

We conclude that inactivation of foodborne pathogens is essential for food safety. We evaluated the survival of *S. Typhimurium* on soybean sprouts subsequent to treatment with gaseous ClO₂ and biocontrol *Pseudomonas* bacteria. *S. Typhimurium* inactivated on soybean sprouts by ClO₂ varied with storage time. The bacterial populations on sprouts were reduced by 3.2 (0 h), 5.09 (24 h), and 3.99 log CFU/g (168 h). The reductions of *Salmonella* on sprouts co-inoculated with *Pseudomonas* strains+*S. Typhimurium* ranged from

0.40 to 1.1 log CFU/g of sprouts. Biocontrol *Pseudomonas* applied after gaseous ClO₂ treatment resulted in further reductions of *S. Typhimurium* on sprouts processed 24 and 168 h of storage. These results suggest that biocontrol microbes applied after gaseous ClO₂ treatment may enhance inactivation of *Salmonella* on sprouts and produce safety.

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