An Effective Treatment to Reduce *Escherichia coli* O157:H7 and *Salmonella* on Alfalfa Seeds

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Abstract

Since 1994, raw sprouts have been implicated as vehicles of outbreaks of *E. coli* O157:H7 and *Salmonella* both nationally and internationally. Most outbreaks were associated with alfalfa sprouts, but cress, mung bean, and clover sprouts have been implicated. Many treatments, including heat and chemicals [NaOCl, $Ca(OCl)_2$, acidified $NaClO_2$, acidified ClO_2, Na_3PO4, Vegi-Clean, Tsunami, Vortexx, and H_2O_2] have been evaluated for their ability to reduce *E. coli* O157:H7 contamination on alfalfa seeds, but none can definitely eliminate the pathogen and render seeds with acceptable germination rates. The purpose of our study was to determine the best concentrations and exposure time of a levulinic acid plus sodium dodecyl sulfate (SDS) treatment to reduce *E. coli* O157:H7 and *Salmonella* populations on alfalfa seeds and yield viable seeds with acceptable germination rates. A 5-strain mixture of *E. coli* O157:H7 or *S*. Typhimurium at 10^8 CFU/g was inoculated on alfalfa seeds. The seeds were dried at 21°C for up to 72 h. A 0.5% levulinic acid and 0.05% SDS treatment for 5 min at 21°C reduced *E. coli* O157:H7 and *S*. Typhimurium populations to undetectable levels (<5 CFU/g), however, some treated seeds were pathogen-positive by selective enrichment culture.

Introduction

A large multicountry outbreak of salmonellosis associated with alfalfa sprout occurred in Sweden and Finland in 1994, with almost 500 cases reported. *S*. Bovismorbificans was the causative agent and was traced to Australian alfalfa seeds that were treated with a solution of 0.5% NaOCl for 45 min prior to sprouting. A year later, three alfalfa sprout-associated outbreaks caused by *Salmonella* of different serotypes occurred in the US, with some also involving Finland and Canada. Since then there have been many more outbreaks of salmonellosis and *E. coli* O157:H7 infections associated with alfalfa sprout consumption. We have developed an antimicrobial treatment composed of levulinic acid plus SDS that effectively kills *Salmonella* and *E. coli* O157:H7 on foods and in treatment solutions, and does not adversely affect the sensory characteristics of the treated food. The purpose of our study was to determine the best concentration and exposure time of this chemical treatment to kill *Salmonella* and *E. coli* O157:H7 on alfalfa seeds and yield seeds with acceptable germination rates.

Materials and Methods

Bacterial strains. To facilitate enumeration of *E. coli* O157:H7, nalidixic acid-resistant (50 µg/ml) strains were used. Five isolates of *Escherichia coli* O157:H7, including 932 (human isolate), E009 (beef isolate), E0018 (cattle isolate), E0122 (cattle isolate), E0139 (deer jerky isolate) or five isolates of *Salmonella* Typhmurium DT104, including H2662 (cattle isolate), 11942A (cattle isolate), 13068A (cattle isolate), 152N17-1 (dairy isolate) and H3279 (human isolate) were used as 5-strain composite mixtures.

Chemicals and chemical treatments. Levulinic acid at 0.5% and 0.05% sodium dodecyl sulfate (SDS) were tested in combination at 21 ± 2°C as a wash treatment for their killing effect on *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seeds. Calcium hypochlorite [20,000 µg/ml (ppm)] was used as a positive control and deionized water was used as a negative control.

Water. Deionized, unchlorinated water (filter sterilized through a 0.2-µm regenerated cellulose filter), tap water and autoclaved tap water were used.

Inoculation of alfalfa seeds. Alfalfa seeds were obtained from Caudill Seeds Co., Louisville, Ky., and had a germination rate of approximately 91%. Dry seeds (50 g) were placed in a sterilized glass beaker (1 L) and 5 ml of a 5-strain mixture of *E. coli* O157:H7 or *S.* Typhimurium DT 104 (10^8-10^9 CFU/ml or 10^3-10^4 CFU/ml) was inoculated on the surface of the seeds then dried in a laminar flow hood for 1, 4, 24, 48, and 72 h.

Determination of *Salmonella* and *E. coli* O157:H7 inactivation on alfalfa seeds. Inoculated and dried alfalfa seeds (50-g samples) were placed in a 1000-ml glass beakers containing 200 ml of levulinic acid plus SDS or controls and agitated at 150 rpm with a magnetic stir bar at 21°C for 0, 1, 2, 5, 10, 20, 30 and 60 min. Following treatment, the sample (1 or 25/g or ml) was placed in a stomacher bag containing 9 ml or 25 ml of 0.1 M phosphate buffer, pH 7.2 (PBS), or neutralizing buffer and pummeled for 1 minute at 150 rpm in a stomacher blender. The suspension was serially (1:10) diluted in 0.1% peptone water and 0.1 ml of each dilution was surface-plated in duplicate onto plates of TSA and Sorbitol MacConkey agar each containing 50 µg nalidixic acid/ml (TSA-NA and SMA-NA) for *E. coli* O157:H7; and TSA and XLD containing ampicillin (32 µg/ml), tetracycline (16 µg/ml) and streptomycin (64 µg/ml) (TSA+ and XLD+) for *S.* Typhimurium DT 104. All plates were incubated at 37°C for 48 h.

Determination of seed germination percentage. To determine the germination percentage, treated and control seeds (5 gram per replicate) were placed on the surface of a plastic tray. A second tray containing 200 ml of sterile deionized water was placed with tray with seeds and water dropped into lower tray to maintain uniform moisture. The seeds were incubated at approximately 22°C for 72 h.

Results and Discussions

Results revealed that a viable population of 10^8 CFU *E. coli* O157:H7/g of alfalfa seeds was present after drying for 4 h (Table 1). Treatments with 20,000 ppm calcium hypochlorite or 0.5% levulinic acid plus 0.05% SDS for up to 60 min reduced the *E. coli* O157:H7 population by greater than 6 and 5 log CFU/g, respectively.

The population of *E. coli* O157:H7 was reduced byt 3 log CFU/g after drying for 24 h. Treatment with calcium hypochlorite and 0.5% levulinic acid plus 0.05% SDS for 5 min reduced *E. coli* O157:H7 populations to levels only detectable by enrichment culture. Similar results were observed with seeds dried for 48 and 72 h (Table 1).

Results revealed that a viable population of 10^6 to 10^7 CFU *S.* Typhimurium DT 104/g of alfalfa seeds was present after drying for 4 h. Treatments with 20,000 ppm calcium hypochlorite or 0.5% levulinic acid plus 0.05% SDS provided similar results, inactivating all *Salmonella, including by enrichment culture,* within 5 min (Table 2).

Drying seeds for 24, 48, or 72 h reduced the population of *Salmonella* by ca. 4 log CFU/g. Treatment with 20,000 ppm calcium hypochlorite or 0.5% levulinic acid plus 0.05% SDS for 5 min reduced *Salmonella* to levels undetectable by direct plating, but still detectable by enrichment culture (Table 2).

Both chemical treatment solutions were negative for *E. coli* O157:H7 or *Salmonella* following treatment of contaminated seeds. Seeds treated for 10 min were transferred to a stomacher bag and pummeled for another 10 min at 200 rpm. Results revealed that all five samples treated with 20,000 ppm calcium hypochlorite or 0.5% levulinic acid and 0.05% SDS were *E. coli* O157:H7- and *Salmonella*-negative by direct plating, only (two of ten samples) treated with 0.5% levulinic acid and 0.05% SDS were negative by enrichment culture.

The germination rate of alfalfa seed treated with 0.5% levulinic acid plus 0.05% SDS for 1 h at 21°C was 80%, with tap water was 71%, and for 20,000 ppm caicium hypochlorite was 47.3%.

Conclusion

Similar results of *E. coli* O157:H7 and *Salmonella* inactivation on alfalfa seeds were obtained with treatments of 20,000 ppm calcium hypochlorite, pH 11.4, or 0.5% levulinic acid plus 0.05% SDS, pH 3.2. Alfalfa seed germination percentages were substantially greater when treated with levulinic acid plus SDS than calcium hypochlorite.

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Table 1. *E. coli* O157:H7 counts on alfalfa seeds with an initial inoculum of 10^8 CFU/g and dried at 21°C in a laminar hood for different times

Treatment Method	*E. coli* O157:H7 counts (CFU/g) on seeds dried for 4 h and treated for minutes:							
	0	1	2	5	10	20	30	60
A	8.1	8.2	8.2	8.1	8.2	8.3	8.3	8.1
B	+	+	-	+	1.7	2.0	1.7	+
C	1.7	2.7	3.0	2.5	2.8	2.0	2.6	2.2
	E. coli O157:H7 counts (CFU/g) on seeds dried for 24 h							
A	4.7	4.8	4.9	5.0	4.7	4.9	4.8	4.9
B	+	-	-	-	+	-	-	+
C	1.7	1.4	0.7	+	+	+	-	+
	E. coli O157:H7 counts (CFU/g) on seeds dried for 48 h							
A	4.0	4.1	4.0	4.1	4.1	4.0	4.0	3.9
B	+	-	+	-	-	-	-	-
C	2.7	2.1	+	+	+	+	+	+
	E. coli O157:H7 counts (CFU/g) on seeds dried for 72 h							
A	3.8	3.9	3.9	4.0	4.0	4.1	4.0	4.1
B	+	+	+	-	+	+	-	-
C	1.9	1.4	1.1	+	+	-	-	+

A: 0.1 M PBS, pH 7.2; B: 20,000 ppm $Ca(OCl)_2$, pH 11.4; C: 0.5% levulinic acid + 0.05% SDS, pH 3.2

Table 2. *S*. Typhimurium DT 104 counts on alfalfa seeds with an initial inoculum of 10^8 CFU/g and dried at 21°C in a laminar hood for different times

Treatment Method	*S*. Typhimurium DT 104 counts (CFU/g) on seeds dried for 4 h and treated for minutes:							
	0	1	2	5	10	20	30	60
A	6.4	6.8	6.3	6.4	6.6	6.3	6.3	6.0
B	+	-	-	-	-	-	-	-
C	3.1	+	+	-	-	-	-	-
	S. Typhimurium DT 104 counts (CFU/g) on seeds dried for 24 h							
A	4.4	4.2	4.3	4.4	4.5	4.6	4.6	4.3
B	+	+	-	-	-	-	-	-
C	1.6	2.4	1.2	+	+	-	-	-
	S. Typhimurium DT 104 counts (CFU/g) on seeds dried for 48 h							
A	4.0	4.1	4.2	4.3	4.2	4.4	4.4	4.3
B	+	+	-	-	+	-	-	-
C	3.0	+	-	-	+	-	+	-
	S. Typhimurium DT 104 counts (CFU/g) on seeds dried for 72 h							
A	4.0	4.0	3.9	4.1	4.1	4.5	4.1	4.2
B	-	-	-	-	-	+	+	-
C	2.3	+	+	+	-	-	+	+

A: 0.1 M PBS, pH 7.2; B: 20,000 ppm $Ca(OCl)_2$, pH 11.4; C: 0.5% levulinic acid + 0.05% SDS, pH 3.2