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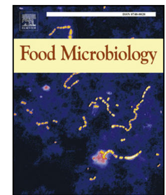
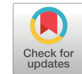


Sequential treatment of hydrogen peroxide, vacuum packaging, and dry heat for inactivating *Salmonella* Typhimurium on alfalfa seeds without detrimental effect on seeds viability



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ABSTRACT

The aim of this study was to inactivate *Salmonella* Typhimurium loaded onto alfalfa seeds with sequential treatment of hydrogen peroxide, drying, vacuum packaging, and dry heat. Also, we verified the effect of vacuum packaging in dry heat treatment. Populations of *Salmonella* on alfalfa seeds after sequential treatment were not detected after 8 or 3 h of dry heat treatment at 70 or 73 °C. *Salmonella* populations including injured cells were also reduced to below the detection limit after 8 or 4 h of dry heat treatment. The germination rate of alfalfa seeds subjected to the harshest treatment was not significantly different ($P > 0.05$) from that of untreated seeds. Vacuum packaging is conducive to preserving the moisture content of alfalfa seed and improving the efficiency of dry heat treatment. This study suggests that sequential treatment is recommended and applicable for decontaminating alfalfa seeds for sprout production.

1. Introduction

Since demand for healthful dietary foods has been increasing worldwide, sprouts have been attracting much consumer attention since they are highly nutritious. Among the various kinds of sprouts, alfalfa sprouts are one of the most common and popular, and their consumption around the world has also been increasing. Regardless of their nutritive value, paradoxically, alfalfa sprouts often pose a serious human health concern since they can carry foodborne pathogens when eaten raw or lightly cooked. A number of outbreaks associated with consumption of raw alfalfa sprouts support this view, and *Salmonella* spp. has been involved in many of these outbreaks. Since 1996, more than 30 cases of outbreaks linked to sprouts have been reported (CDC, 2011). An outbreak of *Salmonella* Saintpaul associated with alfalfa sprouts, which was the largest recorded case, occurred simultaneously in 14 states of the USA in 2009, resulting in 256 infections and 8 hospitalizations (CDC, 2009). In 2010, *Salmonella* Newport was the cause of a multistate outbreak which engendered 44 infections (CDC, 2010). In 2016, 36 people infected with the outbreak strains of *Salmonella* Reading or *Salmonella* Abony were reported from 9 states (CDC, 2016a,b). Also, 26 people infected with the outbreak strains of *Salmonella* Muenchen (25 people) or *Salmonella* Kentucky (1 person) were reported from 12 states (CDC, 2016a,b).

It is known that seeds are the most likely source of introducing pathogenic bacteria in sprout-associated outbreaks (NACMCF, 1999a,b). Various potential routes that can contaminate seeds during sprouting exist, including contaminated irrigation water, unsanitary production equipment, misuse of animal fertilizers, and inadequate worker hygiene (NACMCF, 1999a,b). Since the sprout-producing environment provides perfect conditions for pathogenic bacteria to proliferate and gives pathogens opportunity to internalize into sprouts, adequate interventions are needed for safe consumption of sprouts.

The U.S. Food and Drug Administration (FDA) and the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recommends a 5 log reduction of pathogen load on seeds and the FDA's guidance suggest seeds for sprouting should be sanitized with such as 20,000 ppm of calcium hypochlorite (US FDA, 1999). However, this method cannot guarantee elimination of pathogens on seeds. In addition, no study to date has reported both complete inactivation of pathogens while simultaneously preserving seed germination with a single treatment. Numerous studies have been conducted using various treatments, such as organic acids, hypochlorite, ozonated water, electrolyzed water, high hydrostatic pressure and gamma irradiation, to eliminate pathogens completely without affecting seed germination, but most were insufficient to achieve this goal (Lang et al., 2000;

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Rajkowski and Thayer, 2000; Sharma et al., 2002; Sharma and Demirci, 2003; Wuytack et al., 2003).

Thermal treatment has been evaluated and it was proved effective to reduce pathogen load on seeds to some degree (Jaquette et al., 1996; Neetoo and Chen, 2011; Weiss and Hammes, 2005). Especially, dry heat has been effectively applied alone or combined with other treatments to decontaminate seeds while simultaneously maintaining the germination rate under a suitable temperature range.

Hydrogen peroxide (H_2O_2) has a germicidal effect and it is designated as GRAS (Generally recognized as safe). It forms hydroxyl free radicals (OH^-) which act as an oxidant, and attacks bacteria by destroying essential components such as lipids, proteins, and DNA (Mcdonnell and Russell, 1999). Hydrogen peroxide also has been studied extensively, but individual treatment did not reduce pathogen level sufficiently. Weissinger and Beuchat (2000) reported that immersing inoculated alfalfa seeds in solutions containing 2% hydrogen peroxide for 10 min reduced populations of *Salmonella* by 0.67 log CFU/g. Also, treatment of alfalfa seeds for 10 min with 8% hydrogen peroxide reduced populations of *Salmonella* by 3.27 log CFU/g (Holliday et al., 2001).

Normally, vacuum packaging has been used to extend shelf-life of fresh food or meat products by inhibiting growth of aerobic bacteria and preventing oxidative reactions (Church and Parsons, 1995). Accordingly, vacuum packaging has not been considered as an inactivation method, and no research has been done thus far combining heat treatment and vacuum packaging to inactivate pathogens.

The purpose of this study was to examine the efficacy of hydrogen peroxide, vacuum packaging, and dry heat when these treatments were applied alone or sequentially; and determine the impact of these treatments on seeds germinability. Furthermore, we aimed to demonstrate the impact of vacuum heating for improving dry heat efficiency.

2. Materials and methods

2.1. Bacterial strains and preparation of inoculum

Three strains of *Salmonella enterica* subsp. *enteric* serovar Typhimurium (ATCC, 19585, ATCC 43971, and DT 104) used for inoculation were obtained from the School of Food Science bacteria culture collection of Seoul National University (Seoul, Korea). Stock cultures were stored at −80 °C combining 0.7 ml of culture with 0.3 ml of sterile 50% glycerol. Active cultures were produced by streaking stock cultures onto tryptic soy agar (TSA; Difco, BD) and incubating at 37 °C for 24 h. A single colony of each strain was transferred to 5 ml of tryptic soy broth (TSB; Difco, BD) individually and incubated at 37 °C for 24 h. One ml of each strain's culture was transferred and spread onto TSA for producing a bacterial lawn. Each strain was spread-plated onto two plates for a total of six plates. The six plates were incubated at 37 °C for 24 h. To harvest the bacteria lawn, 9 ml of 0.2% peptone water was added to each plate, and cell suspensions were collected by dislodging cells off the agar surface with a sterile swab (3 M pipette swab, 3 M Korea Ltd.). Cell suspensions (ca. 10^{12} CFU/ml) were augmented by combining all strains with 450 ml of 0.2% peptone water to yield about 500 ml of culture cocktail in total, corresponding to approximately 10^{10} CFU/ml.

2.2. Inoculation of culture cocktail on alfalfa seeds

Commercially retailed alfalfa seeds were purchased from a local sprouting seed company (Danong, Gyeonggi province, Korea Rep.). Approximately 500 g of alfalfa seeds were soaked in 500 ml of prepared cell suspension, and the mixture was intermittently stirred for 5 min. Inoculated alfalfa seeds were drained from the cell suspension completely, placed onto aluminum foil and dried in a laminar flow hood at 21 °C for 20 h. Drying time was determined from our previous study's result which showed optimal time for reinstating original conditions such as moisture content and water activity (Hong and Kang, 2016). The dried seeds were packed in resealable bag and stored in a 4 °C refrigerator for no more than one week.

2.3. Hydrogen peroxide solution preparation and treatment

Two percent (v/v) aqueous hydrogen peroxide (H_2O_2, 30%, Junsei Chemical Co. Ltd., Tokyo, Japan) solution was prepared by adding sterile distilled water (DW). This diluted solution was used immediately after preparation. DW was used as a control for H_2O_2 solution. One hundred gram of inoculated seeds were immersed in 400 ml of 2% H_2O_2 solution or DW for 10 min. Drained seeds were transferred to aluminum foil and dried in laminar flow hood at 21 °C for 14 h.

2.4. Evaluation of drying time for hydrogen peroxide treated seeds

After H_2O_2 treatment, drained seeds were moved to aluminum foil and dried in laminar flow hood at 21 °C for up to 18 h. Moisture content (dry basis) was measured every two hours using a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH).

2.5. Vacuum packaging of alfalfa seeds and dry heat treatment

Aseptic nylon-polyethylene bags (150 × 200 mm) with 0.06 mm film thickness (YH Korea, Seoul, Korea Rep.) were used in this study. Water transmission rate of the packaging film is 62.97 g/m^2·day at 38 °C and 100% relative humidity. Bags were tailored so that final products had a size of 100 × 100 mm. Each 10 g portion of untreated, DW treated, and H_2O_2 treated seeds was transferred to a nylon-polyethylene bag and vacuum packaged using a vacuum packaging machine (Airzero, Ansan, Korea Rep.). The samples were moved to an oven (Thermostable™ ON-32, Wisd laboratory instruments) with a temperature of 70 or 73 °C for up to 8 h with 2 h time increments or up to 4 h with 1 h time increments, respectively. Unpackaged samples were also dry-heated to serve as a control.

2.6. Microbiological analysis

For enumeration of *Salmonella* Typhimurium, each treated sample was taken from the oven and immediately transferred to a sterile stomacher strainer bag (Labplas Inc., Sainte-Julie. Quebec, Canada) containing 90 ml of 0.2% peptone water homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes, France). One ml aliquots of homogenized mixture were 10-fold serially diluted with 9 ml of 0.2% peptone water, and 0.1 ml of appropriate diluents were spread-plated onto selective medium, xylose lysine desoxycholate agar (XLD; Difco, BD). In the case of low levels of surviving cells, 1 ml of undiluted stomacher bag contents was equally divided into 0.25 ml portions and distributed between four XLD plates and spread-plated. All plates were incubated at 37 °C for 24 h and black colonie representative of *Salmonella* Typhimurium were counted. To confirm the identity, randomly selected 5 colonies were subjected to the *Salmonella* latex agglutination assay (Oxoid, Ogdensberg, NY).

To detect presence of sub-lethally injured cells which can resuscitate under favorable conditions, the agar overlay method was used (Lee and Kang, 2001). One tenth ml of serial dilutions were spread onto TSA and incubated at 37 °C for 2 h to allow recovery of injured cells. After incubation, approximately 7 ml of XLD tempered to 48 °C was poured over the plates and allowed to solidify at ambient temperature. After solidification, plates were incubated at 37 °C for another 22 h and counted with the same method mentioned above.

2.7. *Effect of sequential treatments on viability of alfalfa seeds*

Only the germination rate of alfalfa seed samples treated with the harshest protocol (H_2O_2, drying, and dry heating with vacuum packaging) and the untreated control were investigated. Two hundred randomly selected seeds of each sample were placed on aseptic cheesecloth in 90 mm diameter petri dishes, and regularly provided with sufficient distilled water. The seeds were nurtured at ambient temperature (23 ± 2 °C) for 5 days. Only seeds with a protruding hypocotyl were counted and ruptured or swollen seeds were excluded from counting. This was replicated three times.

2.8. *Effect of maintaining moisture content on inactivating Salmonella Typhimurium*

Four plates containing 10 g of alfalfa seeds were placed in the oven and subjected to dry heat for up to 4 h. Duplicate sets of alfalfa seeds were prepared and dry-heated at 70 °C for up to 4 h in an oven with relative humidity set for maintaining seeds' original moisture content. To maintain moisture content of seeds, 300 ml of saturated aqueous potassium sulfate (K_2SO_4) solution was used. At 1 h intervals, 10 g portions of seeds were taken out from the two ovens. After treatment, populations of *Salmonella* were enumerated using the selective medium XLD following the method described previously.

2.9. *Effect of moisture content level on inactivating Salmonella Typhimurium*

The moisture content of inoculated alfalfa seeds (100 g), which had an initial level of about 6% moisture, was modulated to about 9% and 12% by adding distilled water. Moisture-adjusted and non-adjusted seeds were divided into 10 g portions and each portion was vacuum packaged, transferred to the oven and subjected to dry heat at 70 °C for up to 4 h. Each package of every moisture level was removed at hourly intervals and microbiologically analyzed and its moisture content was measured as well.

2.10. *Statistical analysis*

All experiments were replicated three times. Data were analyzed by ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and separation of means by the least significant difference (LSD) test. A value of $P < 0.05$ was used to indicate significant differences.

3. Results

3.1. *Trend of moisture content of alfalfa seeds after hydrogen peroxide treatment*

To compare hydrogen peroxide treated sample with untreated samples exposed to the same conditions and to prevent seeds from damage by wet heat, moisture content was measured at regular intervals following hydrogen peroxide treatment (data not shown). The moisture content of alfalfa seeds declined dramatically and reached stability after 12 h (6.56 ± 0.44%) which was similar to its original condition (6.47 ± 0.28%). The moisture content after 14 h of drying was 6.56 ± 0.32%, and this drying time was determined to be an optimal for the next treatment, dry heat.

3.2. *The effect of treatments on inactivation of Salmonella on alfalfa seeds*

Survival of *Salmonella* Typhimurium on alfalfa seeds after sequential treatment according to dry heat exposure temperature and time is shown in Tables 1 and 2. As shown in Table 1, the initial population of *S*. Typhimurium was about 7 log CFU/g and when alfalfa seeds were subjected to DW or 2% H_2O_2 for 10 min and dried, populations on seeds

Table 1
Effect of sequential treatment on populations of Salmonella Typhimurium on alfalfa seeds (70 °C).

Temperature (°C)	Treatment	Dry heat time (Hour)									
		0[a]		2		4		6		8	
		XLD	OV XLD	XLD	OV XLD	XLD	OV XLD	XLD	OV XLD	XLD	OV XLD
		Population (log CFU/g)									
70	Dry heat	6.99 ± 0.06 Aa	7.20 ± 0.05 *Aa*	6.35 ± 0.15 Ba	6.77 ± 0.11 *Ba*	5.85 ± 0.10 Ca	6.38 ± 0.15 *Ca*	5.67 ± 0.23 Ca	6.24 ± 0.13 *Ca*	5.71 ± 0.20 Ca	6.40 ± 0.17 *Ca*
	Vacuum heat[b]	6.99 ± 0.06 Aa	7.20 ± 0.05 *Aa*	4.79 ± 0.25 Bbc	5.18 ± 0.44 *Bc*	3.52 ± 0.15 Cc	4.24 ± 0.73 *Cc*	2.77 ± 0.14 Dc	3.81 ± 0.28 *CDc*	2.66 ± 0.10 Dc	3.20 ± 0.42 *Dc*
	DW + Dry heat	6.14 ± 0.12 Ab	6.41 ± 0.09 Ab	5.58 ± 0.27 Bb	5.88 ± 0.22 Bb	4.72 ± 0.19 Cb	5.28 ± 0.10 Cb	4.41 ± 0.21 Cb	4.88 ± 0.22 *CDb*	4.37 ± 0.18 Cb	4.60 ± 0.41 *Db*
	DW + Vacuum heat	6.14 ± 0.12 Ab	6.41 ± 0.09 Ab	3.47 ± 0.34 Bd	4.04 ± 0.11 Bd	2.61 ± 0.56 Cd	3.25 ± 0.18 Cd	2.08 ± 0.48 Cd	2.51 ± 0.23 *Dd*	1.94 ± 0.31 Cd	1.84 ± 0.21 Ed
	H_2O_2 + Dry heat	5.49 ± 0.04 Ac	5.79 ± 0.09 *Ac*	4.72 ± 0.26 Bc	5.08 ± 0.09 *Bc*	4.42 ± 0.13 BCb	4.97 ± 0.08 BCb	4.12 ± 0.17 CDb	4.67 ± 0.13 *CDb*	3.86 ± 0.57 Db	4.50 ± 0.44 *Db*
	H_2O_2 + Vacuum heat	5.49 ± 0.04 Ac	5.79 ± 0.09 *Ac*	3.14 ± 0.13 Bd	3.76 ± 0.11 Bd	1.96 ± 0.39 Ce	2.82 ± 0.12 Cd	1.20 ± 0.35 De	2.25 ± 0.67 *Dd*	ND[c] De	ND *Ee*

Values represent means and standard deviations of three replications.
Within the same row, different uppercase letters indicate significant differences ($P < 0.05$) in populations relative to dry heat exposure time.
Within the same column, different lowercase letters indicate significant differences ($P < 0.05$) in populations relative to each treatment.
Italic font indicates significant differences between populations on overlaid XLD medium.

[a] Control *Salmonella* population is 6.99 log CFU/g and samples were treated with distilled water or 2% hydrogen peroxide for 10 min and followed by drying in a laminar flow hood for 14 h. After drying, levels of *Salmonella* on DW or H_2O_2 treated sample were 6.14 and 5.49 log CFU/g, respectively.

[b] Vacuum heat indicates that the sample was vacuum packaged and dry-heated.

[c] ND indicates 'not detected'.

Table 2
Effect of sequential treatment on populations of Salmonella Typhimurium on alfalfa seeds (73 °C).

Temperature (°C)	Treatment	Dry heat time (Hour)									
		0[a]		1		2		3		4	
		XLD	OV XLD	XLD	OV XLD	XLD	OV XLD	XLD	OV XLD	XLD	OV XLD
		Population (log CFU/g)									
73	Dry heat	7.08 ± 0.09 Aa	7.32 ± 0.10 *Aa*	6.39 ± 0.15 Ba	6.78 ± 0.20 *Ba*	6.06 ± 0.23 Bca	6.66 ± 0.30 *Bca*	5.77 ± 0.11 Cda	6.43 ± 0.17 *Ca*	5.57 ± 0.38 Da	6.31 ± 0.16 *Ca*
	Vacuum heat[b]	7.08 ± 0.09 Aa	7.32 ± 0.10 *Aa*	5.14 ± 0.53 Bbc	5.69 ± 0.27 *Bbc*	3.76 ± 0.37 Cc	4.62 ± 0.34 Cb	3.18 ± 0.05 CDd	4.10 ± 0.16 *Dc*	2.79 ± 0.62 Dd	3.58 ± 0.30 Ed
	DW + Dry heat	6.25 ± 0.18 Ab	6.90 ± 0.73 *Aa*	5.46 ± 0.12 Bb	5.85 ± 0.15 Bb	4.95 ± 0.07 Cb	5.28 ± 0.01 BCb	4.62 ± 0.15 Db	5.23 ± 0.09 Cb	4.63 ± 0.24 Db	5.21 ± 0.03 Cb
	DW + Vacuum heat	6.25 ± 0.18 Ab	6.90 ± 0.73 *Aa*	4.22 ± 0.65 Bde	4.73 ± 0.80 *Bde*	2.89 ± 0.15 Cd	3.33 ± 0.49 *Cc*	1.94 ± 0.06 De	2.29 ± 0.28 *CDd*	1.75 ± 0.21 De	2.19 ± 0.51 *De*
	H_2O_2 + Dry heat	5.58 ± 0.27 Ac	5.88 ± 0.09 Ab	4.59 ± 0.11 Bcd	5.10 ± 0.36 Bcd	4.31 ± 0.09 Bbc	4.89 ± 0.30 Bb	3.81 ± 0.18 Cc	4.45 ± 0.06 *Cc*	3.87 ± 0.33 Cc	4.43 ± 0.14 *Cc*
	H_2O_2 + Vacuum heat	5.58 ± 0.27 Ac	5.88 ± 0.09 Ab	3.64 ± 0.34 Be	4.20 ± 0.30 *Be*	1.98 ± 0.85 Ce	2.65 ± 0.58 Cd	ND[c] Df	1.84 ± 0.40 *De*	ND De	ND *Ef*

Values represent means and standard deviations of three replications.
Within the same row, different uppercase letters indicate significant differences ($P < 0.05$) in populations relative to dry heat exposure time.
Within the same column, different lowercase letters indicate significant differences ($P < 0.05$) in populations relative to each treatment.
Italic font indicates significant differences between populations on overlaid XLD medium.
[a] Control *Salmonella* population is 7.08 log CFU/g and samples were treated with distilled water or 2% hydrogen peroxide for 10 min and followed by drying in a laminar flow hood for 14 h. After drying, levels of *Salmonella* on DW or H_2O_2 treated sample were 6.25 and 5.58 log CFU/g, respectively.
[b] Vacuum heat indicates that the sample was vacuum packaged and dry-heated.
[c] ND indicates 'not detected'.

were 6.14 log and 5.49 log CFU/g, respectively. *Salmonella* Typhimurium populations on alfalfa seeds sequentially treated with H_2O_2, drying, and dry heat at 70 °C for 8 h decreased to below the detection limit (1.00 log CFU/g). Considering dry heat treatment alone, untreated (control), DW treated, and H_2O_2 treated alfalfa seeds yielded a maximum of 1.32 log, 2.62 log, and 3.13 log CFU/g reductions of *S.* Typhimurium, respectively, compared to the control. When alfalfa seeds were vacuum packaged, dry heat treatment of untreated, DW treated, and H_2O_2 treated alfalfa seeds engendered 4.33, 5.05, 5.99 log CFU/g reductions of *S.* Typhimurium, respectively, compared to that of the control. With regard to injured cells, *S.* Typhimurium levels using the agar overlay method were slightly higher than those on simple XLD across all treatment levels, and the control population was about 7.20 log CFU/g. The sequential treatment of H_2O_2, drying, and dry heat at 70 °C for 8 h caused populations of *S.* Typhimurium, including injured cells, to be reduced to below the detection limit.

Table 2 shows the change of *S.* Typhimurium levels when alfalfa seeds were subjected to sequential treatment at 73 °C. As seen in Table 1, vacuum packaged samples resulted in further reductions compared to simply dry heated samples. Before dry heat treatment, *S.* Typhimurium levels of untreated, DW treated, and H_2O_2 treated alfalfa seeds were 7.08, 6.25, and 5.58 log CFU/g, respectively. Dry heat treatment at 73 °C resulted in an additional maximum reduction of 4.29, 4.50, and 4.58 log CFU/g, respectively, and in the case of H_2O_2 treated alfalfa seeds, *S.* Typhimurium levels diminished to below the detection limit in 3 h. Populations including injured cells were reduced below to the detection limit in 4 h.

3.3. The effect of sequential treatment on viability of alfalfa seeds

Fig. 1 depicts the germinability of DW or H_2O_2 treated (2%, 10min), dried (21, 14 h), and dried heated (70 or 73 °C) alfalfa seeds. The germination rate of seeds subjected to the most drastic treatment (H_2O_2, drying, and dry heat) was not significantly different ($P > 0.05$) from that of DW treated seeds. This indicates that H_2O_2 does not have an adverse impact on seed germination.

Table 3 shows the germination rate of alfalfa seeds subjected to the harshest treatment of H_2O_2, drying and dry heat according to heat exposure temperature and time. Across all temperature levels and time intervals, the germination rates were not significantly different ($P > 0.05$) from that of the control.

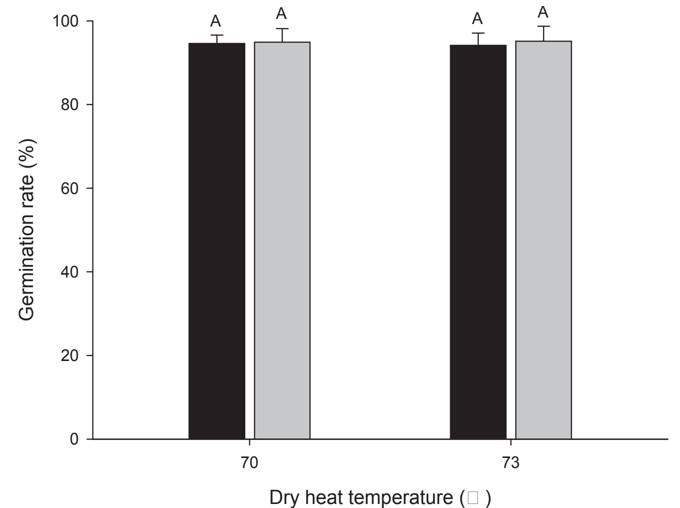

Fig. 1. Germination rate of alfalfa seeds after sequential treatment. Alfalfa seeds were submerged in distilled water (black bars) or 2% hydrogen peroxide (gray bars) for 10 min, dried at 21 °C for 14 h, and heat-dried at 70 or 73 °C for 8 h or 4 h. Uppercase letters on the bars represent significant differences ($P < 0.05$) within the same dry heat temperature.

Table 3
Germination rate of alfalfa seeds after sequential treatment of hydrogen peroxide, drying, and dry heating with vacuum packaging.

Dry heat temperature (°C)	Dry heat treatment time (hours)	Germination rate (%)
Control		94.69 ± 2.04 A
70	2	95.51 ± 1.81 A
	4	95.04 ± 1.78 A
	6	95.57 ± 2.30 A
	8	94.91 ± 3.24 A
73	1	94.48 ± 2.72 A
	2	94.32 ± 2.10 A
	3	94.69 ± 0.70 A
	4	95.12 ± 3.59 A

Within the same dry heat temperature, uppercase letters to the right of values indicate significant differences ($P < 0.05$) relative to dry heating time.

3.4. The effect of vacuum packaging in preserving moisture and inactivating *Salmonella Typhimurium*

To demonstrate that vacuum packaging preserves the moisture content of seeds, and that dry heat treatment of alfalfa seeds while maintaining their moisture content would yield more reduction, two different experiments were conducted. Fig. 2 shows the effect of maintaining moisture content of alfalfa seeds for dry heat treatment. The initial moisture content of simple dry-heated alfalfa seeds was 6.38 ± 0.33% and it was drastically reduced to 1.07 ± 0.26% following treatment, whereas moisture content of alfalfa seeds dry-heated in the oven with saturated K_2SO_4 solution stayed constant (Fig. 2A). *Salmonella* Typhimurium levels on simple dry-heated alfalfa seeds were reduced by 0.60 log CFU/g in 4 h; however, in the case of moisture-maintained seeds, the *S.* Typhimurium population was reduced by 2.48 log CFU/g in the same time interval (Fig. 2B).

Also, Fig. 3 shows the reduction level of *S.* Typhimurium relative to moisture content. The average moisture content of each level was 6.32 ± 0.63, 9.38 ± 0.50, and 12.45 ± 0.36% respectively, and these moisture levels were maintained throughout the experiment (data not shown). While the *S.* Typhimurium population on alfalfa seeds which had the highest moisture content level declined by 5.97 log CFU/g in 3 h, the population of the lowest moisture level sample yielded a 3.23 log CFU/g reduction. These results indicate that seeds of higher moisture content experience greater reduction of *S.* Typhimurium.

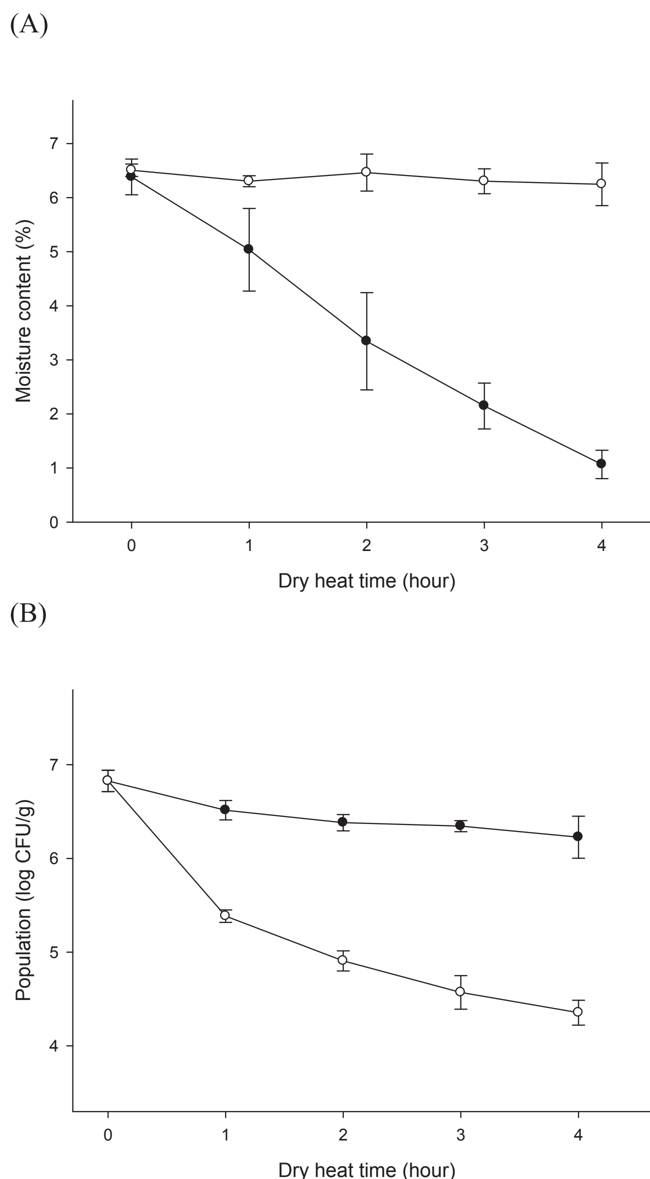

Fig. 2. Effect of maintaining moisture content of alfalfa seeds for dry heat treatment. (A) Moisture content of alfalfa seeds dry heated in the oven. One set of alfalfa seeds (●) was simply dry heated, the other set (○) was dry heated while maintaining its moisture content by placing saturated aqueous potassium sulfate solution in the oven. (B) Population of *Salmonella* Typhimurium on alfalfa seeds after 70 °C dry heat treatment with (○) or without (●) maintaining moisture content.

4. Discussion

The objective of this study was to demonstrate the effectiveness of vacuum packaging for enhancing the efficacy of dry heat to inactivate *Salmonella* Typhimurium on contaminated alfalfa seeds. We designed this study to follow a sequence of hydrogen peroxide treatment, drying, vacuum packaging, and dry heat so that packaged alfalfa seeds could be the final product of the procedure and easily distributed for commercial use. As a result, sequential treatment could yield a reduction of *S.* Typhimurium on alfalfa seeds to below the detection limit which is greater than the FDA's recommendation of 5 log CFU/g.

According to a previous epidemiological study, seeds are known to be the main cause of sprout-associated outbreaks (Mahon et al., 1997). A proper control intervention for seeds is needed, but the unique and difficult problem seeds present is that they are alive and must germinate after treatment. For a long time, the feasibility of thermal treatment has been evaluated by many researchers, and many studies have substantiated it, especially dry heat, but the effect of dry heat treatment has been reported to be limited. Bari et al. (2003) reported dry-heat treatment at 50 °C for 1 h reduced populations of *Escherichia coli* O157:H7 on mung bean seeds by 1.00 log CFU/g. Feng et al. (2007) reported dry heat treatment at 55 °C for up to 6 days was needed to inactivate *Salmonella* on alfalfa seeds without affecting germination significantly. Similarly, when inoculated seeds were subjected to only dry heat, *S.* Typhimurium populations reduced slightly for the first few hours, but a tailing effect was observed as demonstrated in our results. *Salmonella* populations which went through 70 °C dry heat did not show a significant difference ($P > 0.05$) by treatment time after 2 h.

Bang et al. (2011) evaluated the lethality of sequential treatments with aqueous ClO_2 (500 μg/ml) for 5 min, drying (45 °C, 23% RH) for 24 h, and subsequent dry heating at 70 °C for 48 h. Although this sequential treatments eliminated *E. coli* O157:H7 on radish seeds without decreasing the germination rate, a long dry heating time is required. On the other hand, *Salmonella* levels of vacuum packaged alfalfa seeds which underwent the same conditions with simple dry-heated seeds decreased drastically. This is because vacuum packaging helps alfalfa seeds preserve their moisture content by putting the seeds into a hermetic environment, and also vacuum packaging conforms the seeds into

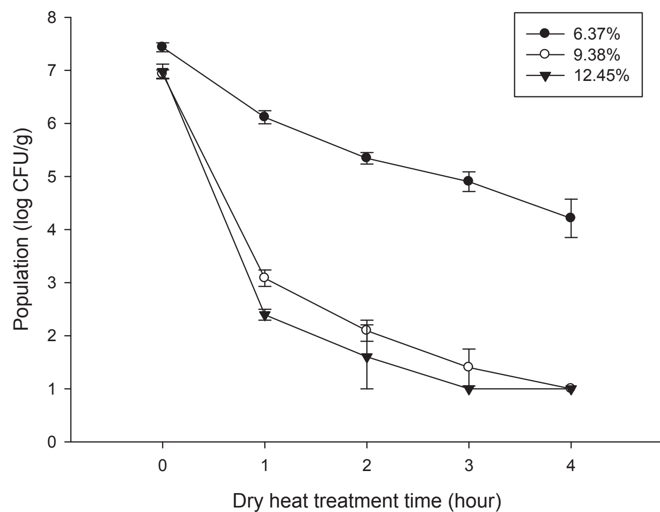

Fig. 3. Effect of dry heat treatment on *Salmonella* Typhimurium populations on alfalfa seeds relative to moisture content. Moisture content of alfalfa seeds was adjusted (about 6, 9, or 12%), vacuum packaged, and 70 °C dry heat treatment was applied on alfalfa seeds for up to 4 h.

a compact unit. One of the factors that determine heat transfer efficiency is material characteristics. Also, if material is denser, the heat transfer rate is faster. Generally, at 25 °C, water's thermal conductivity (k) value (0.58 W/m/°C) is much higher than that of air (0.024 W/m/°C), which indicates that heat transfer is faster in water than in air. Dry heat led alfalfa seeds to lose their water, so efficiency of heat transfer declined. In addition, simple dry-heated seed samples have more air-filled spaces between seeds, so heat cannot be transferred as effectively as with more dense vacuum packaged seeds. Based on the results of this study, the moisture content of vacuum packaged alfalfa seeds was maintained, so more heat was able to be conveyed to pathogens continuously. Therefore, the result of the present study bespeaks that moisture content of seeds is one of the crucial factors which determine dry heat efficiency.

In this study, efficacy of two different dry heat temperatures was evaluated. Considering dry heat alone, differences in efficacy between the two temperature levels do not exist since there is only a 3 °C difference. The reduction of *Salmonella* populations in seeds subjected to 4 h dry heat treatment was not significantly different ($P > 0.05$). However, in case of vacuum packaged samples, dry heat treatment time was curtailed by one half using a 3 °C higher temperature.

Unlike temperature, which is a key factor influencing the efficacy of vacuum packaging, the chemical used for pretreatment of seeds does not seem to be important at least in our study. As seen in the results above, vacuum packaging led to additional *Salmonella* reductions on untreated, DW treated, and H_2O_2 treated samples of 4.33, 4.20, and 4.49 log CFU/g, respectively, at 70 °C, and 4.29, 4.50, and 4.58 log CFU/g reductions, respectively, at 73 °C. For both temperature levels, there was no significant difference ($P > 0.05$) between pretreatments. We anticipated a further reduction or synergistic effect in H_2O_2 pretreated samples, but it was not observed. We could conclude that this is because H_2O_2 decomposes into water and oxygen overtime, and therefore its efficacy as a bactericidal agent declined. Regardless of this limitation, the reason why we used H_2O_2 in this study is because it is designated as GRAS (Generally Recognized as Safe) and does not produce any harmful byproducts since it degrades into only water and oxygen as already mentioned. Moreover, in our previous study, H_2O_2 did not have an adverse impact on germination, but it actually enhanced germinability of alfalfa seeds; hence additional microbial reduction was anticipated; at the same time, it was less stressful to seeds (Hong and Kang, 2016).

With regard to the study of sprouts and seeds, controlling sub-lethally injured cells is imperative since they can resuscitate and recover under favorable conditions in the surrounding environment and proliferate during sprouting. In addition, xylose lysine desoxycholate agar is a commonly used selective medium; however, selective media have unique agents responsible for their selectivity, including antibiotics, bile salts, and surfactants, and these agents only permit the growth of healthy cells and inhibit the growth of sub-lethally injured cells. Therefore, the use of selective XLD medium tends to underestimate the population of *Salmonella* (Wu et al., 2001). Thus, *Salmonella* populations including injured cells should be investigated. As the present study demonstrates, at 73 °C, *Salmonella* on XLD was not detected after 3 h of dry heat treatment when seeds were treated with H_2O_2 and vacuum packaged. However, 1.84 log CFU/g of *Salmonella* was detected on overlaid medium, and the remaining population was reduced to below the detection limit after 4 h in actuality.

Considering both inactivation level and germinability, sequential treatment of H_2O_2, drying, vacuum packaging, and dry heat at 73 °C for 4 h was the most effective according to this study. It might be plausible that vacuum packaging and dry heat treatment alone could reduce *Salmonella* levels by the same amount as sequential treatment, and thus shorten the whole treatment time since no drying process would be required. However, since we do not know whether further dry heat treatment is detrimental to seed germination, further studies optimizing dry heat temperature and time are needed. Also, studies correlating moisture content of seeds, dry heat temperature, and time are required as well.

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