Research Note

Combination Treatments for Killing *Escherichia coli* O157:H7 on Alfalfa, Radish, Broccoli, and Mung Bean Seeds

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ABSTRACT

In this study, the effectiveness of prolonged dry-heat treatment (50°C) alone or in combination with chemical treatments (1% oxalic acid, 0.03% phytic acid, 50% ethanol, electrolyzed acidic water, and electrolyzed alkaline water) in eliminating *Escherichia coli* O157:H7 on laboratory-inoculated alfalfa, radish, broccoli, and mung bean seeds was compared with that of dry-heat treatment in combination with irradiation treatment. Dry-heat treatment for 17 or 24 h alone could reduce *E. coli* O157:H7 numbers to below detectable levels in radish, broccoli, and alfalfa seeds, but was unable to reduce the pathogen numbers to below the detectable level in mung bean seeds. In addition, dry-heat treatment for 17 h plus sanitizer treatments were effective in greatly reducing pathogen populations on radish, broccoli, and alfalfa seeds, without compromising the quality of the sprouts, but these treatments did not eliminate the pathogen from radish and alfalfa seeds. Seventeen hours of dry heat followed by a 1.0-kGy dose of irradiation completely eliminated *E. coli* O157:H7 from radish and mung bean seeds, whereas only a minimum radiation dose of 0.25 kGy was required to completely eliminate the pathogen from broccoli and alfalfa seeds. Dry heat in combination with radiation doses of up to 1.0 kGy did not negatively impact the seed germination rate or length of alfalfa, broccoli, and radish seeds or the length of alfalfa, broccoli, and radish sprouts, but did decrease the length of mung bean sprouts.

The recent shift toward healthy living and healthier foods has increased the popularity of raw sprouts, mostly in salads and sandwiches, in many parts of the world, including Europe and the United States. Sprouts are low in calories and fat, and provide substantial amounts of key nutrients, such as vitamins, minerals, proteins, enzymes, folate, and fiber (35). Despite being a popular health food, multiple outbreaks linked to the consumption of raw sprouts have occurred. Most of these outbreak origins have been traced to seeds contaminated with *Salmonella* and *Escherichia coli* O157:H7, which are followed by *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Aeromonas hydrophila* (5, 11, 30, 31, 37, 41). The largest outbreak linked to bean sprouts contaminated with *Salmonella* occurred in Ontario, Canada (8), and resulted in more than 600 cases of illness.

The popularity of sprouts has actually decreased due to the ongoing food safety issues. Sprouted seeds, as raw agricultural products, may become contaminated with a variety of bacteria, including pathogens (16). The common microbial load ranges between 10³ and 10⁶ CFU/g, which includes mainly pseudomonads, coliforms, and lactic acid bacteria (20, 21, 24, 29). As with many other crops, the seeds used for sprouting are obtained from plants grown in open fields without special measures, with subsequent commercial seed sprouting conditions favoring microbial growth, including that of pathogens (15, 33). Therefore, in sprout production, assuring the absence of pathogens on seeds is regarded as a critical control point, as defined by the Codex Alimentarius Commission (39).

Many seed decontamination methods have been investigated over the years (12, 14). These include chemical treatments (single chemical compound and/or combination of several chemicals) (6, 7, 17, 19, 28, 32, 40), natural antimicrobials (13), ozone (27, 36), electrolyzed water (18), UV light (26), irradiation (4, 34), high pressure processing (2, 42), dry-heat treatment (16, 38), hot water treatment (3, 10), and ultrasound (25).

In our previous studies (3), we reported that dry heat for 1 h that was followed by irradiation was effective in eliminating *E. coli* O157:H7 on laboratory-inoculated alfalfa, radish, and mung bean seeds. Dry heat (50°C for 1 h) in combination with 2.0-kGy doses of irradiation completely eliminated *E. coli* O157:H7. While this treatment did not unacceptably decrease the percent germination or size of the sprouted alfalfa seeds, radish and mung bean sprouts were shorter in length.

In the present study, we determined (i) the effectiveness of a prolonged dry-heat treatment in combination with different sanitizers and/or irradiation to eliminate *E. coli* O157:H7 from laboratory-inoculated alfalfa, broccoli, radish, and mung bean seeds, (ii) the presence of any surviving

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pathogens after the treatment, and (iii) the effect of the treatment on seed and sprout length.

MATERIALS AND METHODS

Test strains. Enterohemorrhagic E. coli O157:H7 strains CR-3, MN-28, MY-29, and DT-66 (isolated from bovine feces) used in this study were obtained from the Laboratory of Zoonosis, National Institute of Animal Health, Tsukuba, Japan. Rifampin-resistant strains were prepared in our laboratory by chemical mutagenesis according to the method of Adelberg et al. (1), and the resistant strains were stored in 20% glycerol at −80°C pending further use. To minimize the presence of naturally occurring microorganisms from sprouts on the enumeration medium, all E. coli O157:H7 was adapted to grow in tryptic soy broth (TSB, pH 7.3; Nissui Seiyaku, Tokyo, Japan) supplemented with rifampin (50 μg/ml).

Preparation of inocula. Each strain of E. coli O157:H7 was cultured in TSB containing 50 μg/ml rifampin at 37°C, with loop transfer at three successive 24-h intervals immediately before use as an inoculum. Cells of each strain were collected by centrifugation (3,000 × g for 10 min at 20°C) and resuspended in 10 ml of sterile distilled water. Equal volumes of these suspensions were combined to produce a four-strain cocktail containing approximately equal populations of each strain. The inocula were maintained at room temperature (21 ± 1°C) and applied to seeds within 1 h of preparation.

Seed inoculation. Mung bean seeds were obtained from Sanwa Norin Co., Ltd. (Saitama, Japan), and radish, broccoli, and alfalfa seeds were obtained from Daisey Machinery Co., Ltd. (Saitama, Japan). Five hundred grams of each seed type was soaked separately in the four-strain cocktail of E. coli O157:H7 (ca. 10⁸ CFU/ml) for 5 min, while undergoing gentle agitation. After decanting the inoculum, the seeds were placed on a sterile, perforated tray lined with four layers of cheesecloth and dried in a biosafety cabinet at room temperature (21 ± 1°C) for 6 to 8 h. Dried seeds containing approximately 10⁸ to 10⁶ CFU of E. coli O157:H7 per g were stored at 4°C for 8 to 10 weeks.

Sanitizer used. Chemical sanitizers included electrolyzed water (acidic and alkaline; Hoshizaki Co., Ltd., Sizuka, Japan), 0.03% phytic acid (Wako Chemical Co., Osaka, Japan), 1% oxalic acid (Wako Chemical Co.), and ethanol (Wako Chemical Co.).

Seed treatments. The inoculated seeds (25 g) were placed in closed 50-ml tubes and dried overnight for 17 or 24 h in a drying oven at 50°C. After drying, the seeds were placed in a sterile netted bag (Tokiwa Kogyo, Ehime, Japan), which was immersed in the different chemical sanitizers for 20 s, while undergoing shaking.

Irradiation treatment. Sealed Ziploc bags containing 25 g of seeds were irradiated at 21.6 ± 2.8°C and received doses of 0.25, 0.50, 0.75, and 1.0 kGy at 1.4 kGy/h, with a 16,841 Ci cobalt-60 gamma source (Gamma Cell-220, Nordion International, Inc., Kanata, Ontario, Canada). The absorbed dose was confirmed with an radiochromic film dosimeter and a cellulose triacetate film dosimeter (FTR-125, Fuji Photo Film Co., Ltd., Tokyo, Japan) that were attached to the surface of the plastic bag according to McLaughlin et al. (22).

Microbiological analysis. The treated seeds (25 g) were placed in a stomacher bag containing 225 ml of peptone water and stomached for 2 min. The homogenate was serially diluted in 0.1% peptone water and plated onto tryptic soy agar (Nissui) and sorbitol MacConkey agar (Nissui) supplemented with 50 μg/ml rifampin. The plates were incubated at 37°C for 24 to 48 h, after which the surviving colonies were counted. Presumptive colonies of E. coli O157:H7 were randomly picked and confirmed as E. coli O157, using a direct immunoassay test kit (Universal Health Watch, Columbia, Md.).

Determination of percent germination. Percent germination was determined as described by Hu et al. (16). Six 5-g samples of the control and treated seeds were placed in a sterile container (Eiken Kizai Co., Ltd., Tokyo, Japan) in a darkened room at 25 ± 1°C for 3 days, with sterile water applied periodically to maintain a high-moisture environment. The total number of seeds and germinated seeds left in the containers were then counted, with percent germination defined as the number of germinated seeds/total number of seeds.

Statistical analyses. All trials were replicated three times. Reported plate count data represent the mean values obtained from three individual trials, with each of these values being obtained from duplicated samples. Data were subjected to analysis of variance using the Microsoft Excel program (Redmond, Wash.). Significant differences in plate count data were established by the least-significant difference at the 5% level of significance.

RESULTS AND DISCUSSION

Dry-heat treatment of mung bean, radish, broccoli, and alfalfa seeds. Mung bean, radish, broccoli, and alfalfa seeds were inoculated to contain an average of 5.0 log CFU/g of E. coli O157:H7, and then incubated in sealed tubes at 50°C for 17 or 24 h. After exposure to dry heat for 24 h, E. coli O157:H7 populations decreased 3.0 log CFU/g in mung bean seeds and 5.0 log CFU/g in radish seeds (Fig. 1). However, numbers of E. coli O157:H7 in broccoli and alfalfa seeds decreased below the detectable limit within 17 h of incubation at 50°C. This milder heat treatment did not affect the germination rate of the seed types examined, nor was there any visual difference in the finished sprouts (data not shown). After 24 h of heating, E. coli O157:H7 population numbers were below the detectable limit; however, the pathogen reached a final population of 7.0 log CFU/g in fully sprouted radish, broccoli, and alfalfa seeds, indicating that this heat treatment alone was unable to completely eliminate the pathogen.

Dry-heat treatments plus ethanol soaking. Dry-heat treatments that were followed by a soaking in >50% eth-
anol reduced the \( E. coli \) O157:H7 population below the detectable limit in mung beans and radish seeds. However, \( E. coli \) O157:H7 population numbers in broccoli and alfalfa seeds decreased below detectable levels irrespective of soaking in ethanol (Fig. 2). Exposure to dry heat for 17 h and then followed by soaking in <50% ethanol did not affect seed germination or the appearance of the fully sprouted seeds. However, \( E. coli \) O157:H7 was detected in the germination study for all the seeds tested. While these results suggest that a 17-h dry-heat treatment at 50°C followed by a soaking in ethanol can reduce \( E. coli \) O157:H7 populations below the detectable limit in mung bean, radish, broccoli, and alfalfa seeds, complete inactivation was not possible.

**Dry-heat treatments plus sanitizer soaking.** Dry-heat treatments that were followed by a soaking in 1% oxalic acid, 0.03% phytic acid, 50% ethanol, electrolyzed acidic water (EOAc), and electrolyzed alkaline water (EOAl) solutions could inactivate \( E. coli \) O157:H7 in broccoli and alfalfa seeds (Fig. 3). Inactivation of \( E. coli \) O157:H7 in mung beans seeds was achieved by soaking in 1% oxalic acid, 0.03% phytic acid, or 50% ethanol (Fig. 3), but not by soaking in EOAc and EOAl (Fig. 3). Dry heat in combination with 1% oxalic acid and EOAc reduced \( E. coli \) O157:H7 population numbers below detectable levels in radish seeds. However, other sanitizer treatments were unsuccessful for radish seeds.

No significant difference was seen in percent germination or appearance of the finished sprouts irrespective of treatment conditions. However, \( E. coli \) O157:H7 populations were detected in the germination study, indicating that a 17-h dry-heat treatment at 50°C that was followed by soaking in different sanitizers could not completely inactivate the pathogen completely.

**Dry-heat treatments plus irradiation.** Dry heat (50°C for 17 h) alone was able to reduce the pathogen population by ca. 2.0 log CFU/g in mung bean seeds, without affecting percent germination or average sprout length, whereas dry heat in combination with 1.0-kGy doses of irradiation completely eliminated the pathogen in mung bean seeds and the sprouted seeds (Fig. 4). Although percent germination was unaffected, a significant decrease in average sprout length was observed throughout the study (Fig. 5). Dry heat in combination with a 1.0-kGy dose of irradiation was required to eliminate the pathogen on radish seeds, and at this dose, percent germination and sprout length of radish seeds did not significantly decrease. Using dry heat for 17 h, \( E. coli \) O157:H7 population decreased 5.2 log CFU/g in the seeds, with percent germination and average sprout length of broccoli and alfalfa seeds remaining unaffected. However, dry heat in combination with a 0.25-kGy dose of irradiation completely eliminated the pathogen from broccoli and alfalfa seeds, with the average sprout length and percent germination not decreasing significantly (Fig. 5). Radish, broccoli, and alfalfa sprout length remained acceptable after a dose of 1.0 kGy, whereas mung bean
sprouts were sensitive to irradiation, with a dose of even 1.0 kGy decreasing their length by 50% (4.8 cm) compared with radish, broccoli, and alfalfa sprouts (Fig. 5).

The inability of sanitizers to completely eliminate pathogens without affecting germination is probably the result of cells being trapped in cracks or crevices and thus being protected from direct exposure to active sanitizer components. Microscopic examination of the mung bean seed showed that while the seed surface is relatively smooth, the stem scar is sufficiently porous to permit bacteria to penetrate deep into the seed (9). The drying of seeds after inoculation would in effect protect E. coli O157:H7 cells that had entered the seeds through cracks and crevices against inactivation by sanitizers. Even with liquid disinfection of the seed, there is still no guarantee that the resulting sprout will be pathogen free. The U.S. Food and Drug Adminis-

![Graph showingurvival of E. coli O157:H7 on different seeds](image1)

**FIGURE 4.** Dry-heat treatment at 50°C for 17 h, which was followed by different doses of irradiation to reduce E. coli O157:H7 populations on alfalfa, radish, broccoli, and mung bean seeds.

![Images of sprouted seeds](image2)

**FIGURE 5.** Five-day-old sprouted seeds after exposure to dry heat at 50°C for 17 h, which was followed by different doses of irradiation. (A) Mung bean sprouts, (B) radish sprouts, (C) broccoli sprouts, (D) alfalfa sprouts. In each photograph from the left: control, dry heat plus 0.25 kGy, dry heat plus 0.5 kGy, dry heat plus 0.75 kGy, and dry heat plus 1.0 kGy.
tration has approved the use of irradiation at doses of up to 8.0 kGy to control bacterial pathogens on seeds to be used for sprouting (23).

The results of this study indicate that dry heating and then soaking in 1% oxalic acid, 0.03% phytic, 50% ethanol, EOAc, and EOAl of E. coli O157:H7–contaminated mung bean, radish, broccoli, and alfalfa seeds is not effective in eliminating the pathogen, although a significant reduction in the numbers of viable cells can be achieved. Dry heating of radish seeds for 17 h and subsequent 20-s exposure to 75% ethanol is lethal to E. coli O157:H7 but might also decrease germination, leading to a commercially unacceptable sprout yield rate. These results also indicate that as the radiation dose increases to 1.0 kGy, the percent germination for mung bean seeds is unaffected, but the sprout length decreases. However, dry heating of radish, broccoli, and alfalfa seeds, with a subsequent 1.0-kGy dose of irradiation was able to eliminate the pathogen on seeds without compromising the quality of the finished sprouts.

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