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Short communication

Comparison of the growth of *Escherichia coli* O157: H7 and O104: H4 during sprouting and microgreen production from contaminated radish seeds

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ABSTRACT

Both sprouts and microgreens are popular tender produce items, typically grown and harvested in indoor facilities which allow a higher degree of control compared to open field production. While sprouts, which have frequently been implicated in foodborne illness outbreaks, are the subject of numerous national and international standards for their production and distribution, there is a lack of data pertaining to the microbiological safety of microgreens. In this study, sprouts and microgreens were produced from radish seeds inoculated with *Escherichia coli* O157: H7 or O104: H4 and *E. coli* populations on the harvested products compared to assess the potentials of product contamination from contaminated seeds during sprouting and microgreen production. Both *E. coli* O157:H7 and O104:H4 grew rapidly during sprouting, reaching levels of 5.8–8.1 log cfu/g and 5.2–7.3 log cfu/g, respectively, depending on the initial inoculation levels of the seeds (1.5–4.6 log cfu/g and 0.8–4.3 log cfu/g on radish seeds, respectively). In comparison, *E. coli* O157:H7 and O104:H4 populations on harvested microgreens ranged from 0.8 to 4.5 log cfu/g and from 0.6 to 4.0 log cfu/g, respectively. Although harvested microgreens carried significantly less ($P < 0.001$) *E. coli* than sprouts germinated from seeds inoculated at the same levels, proliferation of *E. coli* O157:H7 and O104:H4 occurred during both sprouting and microgreen growth.
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1. Introduction

As consumers' demand for healthy and convenient foods increases, raw seed sprouts have gained popularity worldwide as they are perceived as healthier sources of carbohydrates, proteins, minerals, and vitamins (Martínez-Villaluenga et al., 2008). However, sprouts consumption has been implicated in several high-profile foodborne illnesses outbreaks. An outbreak of enterohemorrhagic *Escherichia coli* (EHEC) O157: H7 which affected over 6000 people in Japan in 1996, was linked to the consumption of contaminated radish sprouts (Taormina et al., 1999). More recently, sprouts from an organic farm in Germany were determined to be the source of an outbreak of enteroaggregative *E. coli* (EAEC) O104:H4, which infected nearly 4000 people, and caused 53 deaths in 2011 (Uphoff et al., 2014). These outbreaks have heightened public health concerns over the safety of sprouts from consumers and from officials at federal and local regulatory agencies, and prompted many food retail and service establishments to institute policies restricting the availability of sprouts.

As a new class of specialty fresh produce, microgreens have gained increasing popularity with consumers in recent years (Xiao et al., 2012). Microgreens are tender cotyledonary plants with seed leaves fully developed and the first pair of true leaves emerged or partially expanded. Compared to more mature leafy produce, microgreens exhibit more attractive colors, intense flavors and tender textures. A recent study showed that microgreens generally contained higher concentrations of phytonutrients than their mature counterparts (Xiao et al., 2012).

In most cases both sprouts and microgreens are grown in indoor facilities that can restrict the access of insects and wild animals, and minimize other factors of environmental contaminations compared to field grown leafy greens. Contaminated seeds were generally recognized as the main source of bacterial pathogens in most sprout-related outbreaks reported by the National Advisory

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Committee on Microbiological Criteria for Foods (1999), which could also be true for microgreens. For commercial production, sprouts typically germinate from seeds in rotary drums or other types of containers with high humidity and frequent or constant watering. The conditions for sprouting, including relatively high temperature, high humidity, low (or no) light, and abundance of nutrients from sprouting seeds, are conducive of rapid bacterial growth. It has been reported that the *E. coli* population on the final products of sprouts could exceed 7 log cfu/g without negatively affecting the appearance of products (Taormina et al., 1999). In addition, the high volumes of water used during sprout production offers opportunities for pathogens to be spread between sprouts within the production batch, especially in systems where sprouts are exposed to a common "water bath" and frequently or continuously mixed, such as in a rotary drum.

In contrast, microgreens are typically grown hydroponically or in soil/soil substitutes based growth media. *E. coli* O157:H7 was shown capable of rapid proliferation on hydroponically grown radish sprouts when seeds were artificially contaminated or when the roots were soaked in contaminated water (Hara-Kudo et al., 1997), and internalization of *E. coli* O157:H7 in hydroponically grown radish sprouts was also observed (Itoh et al., 1998). However, although hydroponic sprouting shares some similarity with some of the microgreen production systems, the growth conditions used in these studies were very different from current practices of microgreen production. To date, there is still a lack of scientific information relative to the microbiological safety risks of microgreens. The objective was to investigate the survival and proliferation of *E. coli* O157: H7 and O104: H4 on radish sprouts and microgreens cultured under laboratory conditions simulating commercial sprout and microgreen production.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

E. coli O157: H7 strains ATCC 43888 harboring a stable plasmid that encoded for green fluorescence protein (GFP) and ampicillin-resistance (pGFP) (Fratamico et al., 1997), and ATCC 43895 were from Environmental Microbial and Food Safety Laboratory (EMFSL) collections. *E. coli* O157:H7 strain EC415, which was isolated from spinach outbreak in 2006, was provided by Dr. M. Mammel (Food and Drug Adminstration (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Laurel, MD, USA). *E. coli* O104:H4 strain TW16133, which was isolated from the Germany sprout outbreak in 2011, was obtained from Michigan State University STEC Center (Al Safadi et al., 2012). This *E. coli* O104:H4 strain is resistant to several antibiotics, including ampicillin (Gault et al., 2011). Both ATCC 43895 and EC415 were transformed with pGFP extracted from ATCC 43888/pGFP to facilitate the isolation and confirmation of these strains in ensuing inoculations. Plasmid stability of the transformed strains was evaluated by two consecutive overnight subculturings (approximately 60 generations) in the absence of selective antibiotic (ampicillin) followed by plating on non-selective agar plates. All the colonies examined expressed GFP, indicating stable maintenance of the plasmid.

All *E. coli* strains were grown in tryptic soy broth (TSB, BD Biosciences, Sparks, MD, USA) containing 100 μg/mL ampicillin overnight at 37 °C and harvested by centrifugation at 2300× *g* for 10 min at 4 °C, followed by resuspension with sterile distilled water. Equal volumes of cell suspensions of the three O157:H7 strains were combined as a cocktail and further diluted in sterile distilled water to obtain desired cell concentration for seed inoculation. Cell suspension of the O104:H4 strain was used separately for the inoculation of a parallel batch of seeds.

2.2. Seeds and inoculation

Daikon radish (*Raphanus sativus* var. *longipinnatus*) seeds were purchased from a commercial provider (Living Whole Foods, Springville, UT, USA) and stored in the dark at 4 °C until use. For inoculation, a portion of 100 g seeds was immersed in 200 mL of appropriate inoculum suspension with gentle swirling for 5 min at room temperature. The concentrations of inoculum suspensions were 10^2~10^3 and 10^5~10^6 cfu/mL, respectively, for achieving targeted low (~1 log cfu/g) and high (~4 log cfu/g) levels of inoculation. After draining, inoculated seeds were spread over sterile absorbent sheets and air-dried overnight under a laminar flow biological safety hood at room temperature and stored in a 4 °C refrigerator for up to 48 h before being used for sprouting or growing microgreens. In addition to seeds with low and high level inoculations, a portion of radish seeds with high inoculum density and un-inoculated seeds were also mixed at a ratio of 1:99 (w/w) to simulate sporadically contaminated seeds. The same batches of seeds for each inoculation levels were used for sprouting and growing microgreens.

2.3. Sprouting and microgreen growth

Inoculated radish seeds (10 g) were placed in a sterile glass sprouting jar and soaked in sterile distilled water for 4 h at room temperature (22 ± 1 °C). After draining, sprouting jars were inverted and kept at an angle in a tray that ensured proper drainage during the incubation. The sprouting jars were incubated at 25 °C with a relative humidity of 70 ± 5% in the dark for 3 days. Germinating seeds were rinsed with 200 mL sterile distilled water twice daily. Radish sprouts were exposed to light on day 4 and harvested on day 5 as intact plants, including underdeveloped seed leaves, stems and roots, for microbial enumeration.

For microgreen seeding, 10 g portions of inoculated seeds were evenly spread on top of a 2.5 cm layer of moisturized Fafard Super Fine Germination Mix (Griffin Greenhouse & Nursery Supplies, Bridgeton, NJ, USA) in flat plastic culture trays (28 cm W × 54 cm L × 6 cm D, Growers Supply, Dyersville, IA, USA). Seeded trays were incubated in a walk-in growth chamber set at 25 °C/18 °C (day/night) with 12 h photoperiods, with daily irrigation with sterile distilled water. During the first three days, trays were covered to allow seeds germination in the dark. On day 4, the seedlings were exposed to white fluorescent light (light irradiance = ~150 μmol/s/m^2, determined by LI-1000 datalogger, LI-COR, Lincoln, NE, USA). On day 7, microgreens were harvested by cutting stems at 1 cm above the substrate surface with sterile scissors. A 5-g sample of randomly selected microgreen plants from each tray (replicate) was collected for microbial enumeration.

2.4. Microbial enumeration

Five grams of seeds, sprouts, or microgreens were pummeled with 45 mL sterile phosphate buffered saline (PBS) in filtered bags using a stomacher for 2 min, and *E. coli* cells in the filtrate enumerated using a combination of spiral plating and most probable number (MPN) methods (Luo et al., 2011). Sorbitol MacConkey agar (SMAC) supplemented with 200 μg/mL ampicillin was used for selective growth of both *E. coli* O157:H7 (colorless colonies) and O104:H4 (red/pink colonies). The microbiological profiles of germination mix and un-inoculated radish seeds were determined using 3M Petrifilm™ plates (3 M Inc., St. Paul, MN, USA) for aerobic plate count (30 °C for 48 h), yeast and mold count (25 °C for 5 days), *Enterobacteriaceae* count (37 °C for 24 h) and *E. coli*/Coliform count (37 °C for 48 h) following the manufacturer's instructions. The filtrates of germination mix and of radish seeds were also plated on

SMAC containing 200 μg/mL of ampicillin to screen for the presence of bacteria in the germination mix and radish seeds that might form colonies indistinguishable from that of *E. coli* O104:H4. No such colonies were observed.

2.5. Experimental design and statistical analysis

Same batches of seeds inoculated at the indicated levels were used for both sprouting and microgreen growth. Four replications were conducted for seed inoculation, sprouting, microgreen growth, and microbiological enumeration. For sprouts and microgreens, one sample was taken from each individual production unit (i.e. the sprouting jar for sprouts or the growth tray for microgreens). All microbiological data were log transformed and expressed as the mean ± standard error (SE). Univariate analysis of variance (ANOVA) was performed with SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, USA). The statistical significance of the data was determined by performing Tukey's honestly significant difference (HSD) tests at an experiment-wise significance level of 0.05.

3. Results and discussion

The main microbiological group in the germination mix was yeast and mold at 5.7 log cfu/g, followed by total aerobic bacteria (recoverable at 30 °C) at 3.7 log cfu/g, and *Enterobacteriaceae* and total *E. coli*/Coliform, both at 3.5 log cfu/g. Indigenous microbial populations on raw radish seeds were mainly composed of aerobic mesophilic bacteria and yeast & mold, at 3.3 and 2.5 log cfu/g, respectively. *Enterobacteriaceae* or *E. coli*/Coliform bacteria were not detected on radish seeds. When plated on SMAC plates, filtrate from the germination mix but not from the seeds gave raise to colonies that were resistant to 200 μg/mL of ampicillin. However, these colonies had morphology (slimy) very different from that of either *E. coli* O157:H7 or O104:H4, and therefore did not interfere with the enumeration of the targeted bacteria populations.

The survival and growth of *E. coli* O157: H7 (Fig. 1) and O104:H4 (Fig. 2) during sprouting and microgreen growth were examined using radish seeds with different inoculation levels. Populations of *E. coli* O157: H7 and O104:H4 were expressed as log cfu per gram of inoculated seeds, resultant sprouts, and microgreens. For the purpose of comparing *E. coli* counts on inoculated seeds to levels on finished sprouts and microgreens, data were also reported as log cfu per "gram seed equivalent" (gse), which represents cell counts on sprouts or microgreens produced from 1 g of seeds, by factoring in the average yields (8.0 ± 0.3 and 10.0 ± 0.5 for sprouts and microgreens, respectively). *E. coli* O157: H7 and O104:H4 were not detected on either un-inoculated radish seeds or the resultant sprouts and microgreens. By the end of 5-day sprouting, sprouts germinated from radish seeds inoculated at the low level (1.5 log cfu/g) carried *E. coli* O157: H7 population of 7.6 log cfu/g (8.5 log cfu/gse). Sprouts grown from radish seeds inoculated at the high level (4.6 log cfu/g) carried a higher *E. coli* O157:H7 population (8.1 log cfu/g or 9.0 log cfu/gse). For sporadically contaminated seeds (1% seeds inoculated at the high level, 2.6 log cfu/g), *E. coli* O157: H7 populations on sprouts reached 5.8 log cfu/g (6.7 log cfu/gse). Although no attempt was made to specifically quantify the growth of *E. coli* O157:H7 during sprouting, such as by quantifying pathogens removed by twice daily rinsing, the above data indicated that *E. coli* O157:H7 was capable of proliferation by at least 4.1 to 6.0 logs during sprouting.

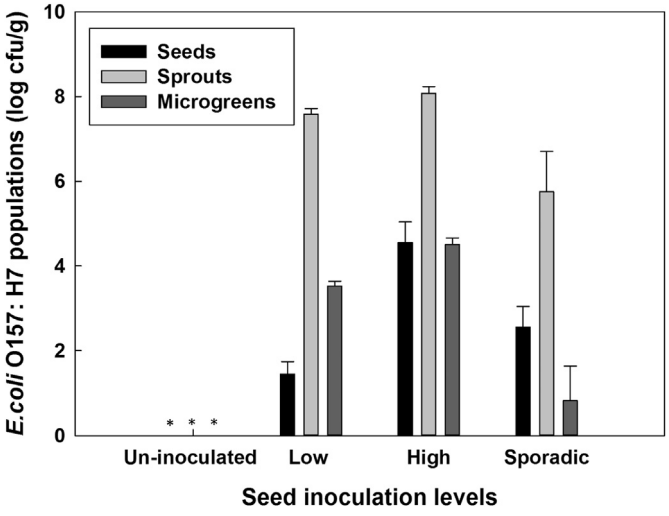

Fig. 1. Populations of *E. coli* O157: H7 on radish seeds, sprouts, and microgreens. * Not detected at detection limit of −0.4 log cfu/g. Vertical bars represent standard errors ($n = 4$).

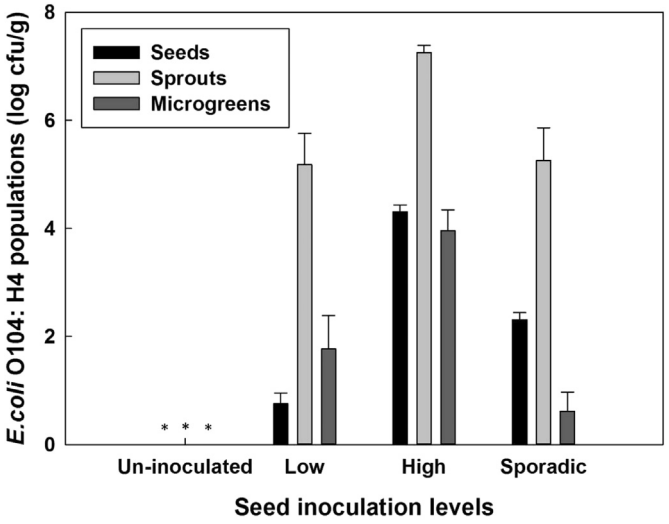

Fig. 2. Populations of *E. coli* O104:H4 on radish seeds, sprouts, and microgreens. * Not detected at detection limit of −0.4 log cfu/g. Vertical bars represent standard errors ($n = 4$).

Similar trends were observed for *E. coli* O104:H4. Starting with inoculated radish seeds (0.8 log cfu/g for low level, 4.3 log cfu/g for high level and 2.3 log cfu/g for sporadic inoculation), *E. coli* O104:H4 populations on sprouts reached 5.2, 7.3 and 5.3 log cfu/g (6.1, 8.2 and 6.2 log cfu/gse), respectively. These results represented a proliferation of *E. coli* O104:H4 during sprouting by 4.8, 3.9 and 3.9 logs from seeds inoculated at different levels indicated above, without counting *E. coli* O104:H4 cells removed from sprouts by daily rinsing. Previous studies showed that low levels of *Salmonella* species inoculated on alfalfa seeds increased by as much as 4–5 logs in the germinated sprouts (Andrews et al., 1982) and *E. coli* O157: H7 inoculated alfalfa seeds increased by 2–4 logs in the sprouts (Stewart et al., 2001). Prokopowich and Blank (1991) previously reported that microbial populations on sprouts obtained from retail stores reached as high as 10^9 cfu/g.

In contrast, microgreens grown from same batches of inoculated radish seeds carried significantly lower ($P < 0.001$) populations (in comparison to sprouts) of *E. coli* O157: H7 or O104:H4 cells. *E. coli* O157:H7 population reached 3.5, 4.5, and 0.8 log cfu/g on microgreens grown from low, high, and sporadically inoculated radish seeds, respectively, which corresponded to 4.5, 5.5 and 1.8 log cfu/gse. *E. coli* O104:H4 populations on harvested

microgreens reached 1.8, 4.0 and 0.6 log cfu/g (2.8, 5.0, and 1.6 log cfu/gse), respectively, for seeds inoculated at low level, high level, and those inoculated sporadically. These would represent proliferation of *E. coli* O157: H7 by 3.0, 0.9 and −0.9 logs and *E. coli* O104: H4 by 2.0, 0.7 and −0.7 logs on the microgreens produced from seeds with low, high and sporadic inoculation levels, respectively. It should be noted that only the portion of the microgreen plant above the growth medium were harvested and analyzed. Proliferation of *E. coli* O157:H7 or O104:H4 on the lower stem and roots was not accounted for by analyzing the harvested microgreen tissues, thus the actual proliferation by these pathogenic *E. coli* was likely greater.

Overall, both *E. coli* O157:H7 and O104: H4 on inoculated radish seeds significantly proliferated during sprouting, and to a lesser extent, during the germination and growth of microgreens, regardless of the initial inoculation levels. At the same seed contamination level, cell counts of *E. coli* O157:H7 and O104:H4 on harvested microgreens tend to be 3–5 logs lower than those on sprouts. It's commonly accepted that factors unique to sprouting, such as high humidity and temperature and available nutrients contribute to the higher growth of bacterial pathogens on sprouts. Compared with microgreens, the higher growth of *E. coli* on sprouts could also be due to the frequent rinsing and mixing, which greatly promote the dispersion and redistribution of bacterial cells on various parts of sprouts and between sprouts. Another explanation for the lower bacterial cell counts on microgreens is the fact that only part of the plants was harvested, leaving bacterial cells on seed coats, roots, lower stems and in the growth medium unaccounted. This study compared the growth of *E. coli* O157:H7 and O104:H4 under conditions simulating one system for microgreen growth and one system for sprouting. It is noteworthy that commercial sprouting and microgreen growing practices vary greatly depending on the type of sprouts or microgreens being grown and the facilities and equipments used.

Data presented here indicated that significant proliferation by both *E. coli* O157:H7 and O104:H4 occurred during sprouting at all levels of seed contamination used in this study. Data also indicated that these pathogens could proliferate during microgreen growth, though to a lesser degree. Since neither sprouts nor microgreens generally involve a kill step for bacterial inactivation before consumption, preventing seed contamination is an important step in a multi-hurdle approach for ensuring the safety of sprouts and may also be important for microgreens. The proposed FDA produce rule, Standards for the Growing, Harvesting, Packing and Holding of Produce for Human Consumption (78 FR 3504) for implementing the Food Safety Modernization Act (FSMA) includes a subpart directed to hazards and controls specific to sprouts. Among other things, the proposed produce rule would require sprouters to treat seeds to reduce pathogens immediately before sprouting. Currently there is a lack of scientific data supporting or against the extension of such requirements to the production of microgreens. It is worthy of mention that microgreens shares many characteristics of what FDA termed "soil-grown sprouts" for production and consumption. While acknowledging the unawareness of any outbreaks associated with sprouts grown in soil or other growth media, current FDA regulations make no distinction for sprouts from different production systems.

In conclusion, *E. coli* O157:H7 and O104:H4 on radish seeds proliferated more significantly (by 3–5 logs) during sprouting than during microgreen production. Although microgreens seemed to present relatively low food safety risks in comparison to sprouts germinated from seeds with the same contamination levels, significant proliferation of bacterial pathogens also occurred during microgreen growth. Therefore, it is of great importance to minimize bacterial contamination of seeds for both sprout and microgreen productions.

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