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Thermal inactivation responses of acid adapted and non-adapted stationary phase Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* spp. and *Listeria monocytogenes* in orange juice

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ABSTRACT

All published *D*-values for Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella*, and *Listeria monocytogenes* in orange juice were obtained using strain cocktails. The objective of this study was to evaluate the heat resistance of individual strain of stationary phase non-adapted and acid adapted STEC, *Salmonella* spp., and *L. monocytogenes in* orange juice. Three STEC and *Salmonella* isolates were grown in TSB, and three *L. monocytogenes* strains grown in BHI, supplemented with 1% glucose for acid adaption. Sealed microcapillary tubes with inoculated single-strength pasteurized orange juice without pulp were treated at 56, 58, and 60 °C for STEC and *L. monocytogenes* and at 55, 58, and 60 °C for *Salmonella*. Thermal tolerance was increased significantly ($P < 0.05$) for acid adapted STEC strains, however, no improvement was observed for *Salmonella* spp., and *L. monocytogenes* strains at most temperatures tested. *Salmonella* serotypes are less heat resistant, at all temperatures tested, than *L. monocytogenes* and STEC. STEC, especially strain O111, are the most heat resistant at 56 and 58 °C; *L. monocytogenes* strains are the most thermal tolerance at 60 °C. Combining individual results of all pathogens tested, the formula of log $D = 8.2167 - 0.1356\ T(°C)$ was used to calculate a general process for orange juice at 71.1 °C. Using this equation, a 5-log reduction of all three pathogens in single strength orange juice requires 11 s at 71.1 °C, with a *z*-value of 7.1 °C.

1. Introduction

A total of 21 outbreaks of *Escherichia coli* O157:H7, *E. coli* O111, *Salmonella* spp., and *Crytosporidium,* associated with the consumption of unpasteurized fruit juice were reported to the US Centers for Disease Control and Prevention (CDC) between 1995 and 2005 (Vojdani, Beuchat, & Tauxe, 2008). Several confirmed outbreaks of similar pathogens occurred related to unpasteurized juice in various states from 2006 to 2012 (CDC, 2014; Danyluk, Goodrich-Schneider, Schneider, Harris, & Worobo, 2012). Outbreaks of Shiga-toxin producing *E. coli* (STEC) have been associated with the consumption of unpasteurized apple cider and apple juice. Outbreaks of *Salmonella* serotypes have been linked mainly to unpasteurized orange juice consumption. *Listeria monocytogenes* has not been implicated in any outbreak related to fruit juice consumption (CDC, 2015; Vojdani et al., 2008). The routes of contamination of these pathogens have not been conclusively identified in any of the juice outbreaks. The use of dropped fruit, non-potable water, and the presence of cattle or wildlife in, or close to, the production or processing environment are included as likely sources of juice contamination (Harris et al., 2003). Inactivation of pathogens before distribution of fruit juices has become mandatory in the United States as a result of these outbreaks associated with fruit juice. The U.S. Food and Drug Administration (FDA) published its juice final rule (66 FR 6137) in January 2001, requiring that all the juice processors must comply with the Hazard Analysis Critical Control Points (HACCP), and achieve a 5-log reduction of the pertinent pathogenic microorganism (FDA, 2001).

Acidic foods, like most fruit juices, were not recognized as a vehicle of foodborne pathogens until the first confirmed outbreak of *E. coli* O157:H7 related to unpasteurized apple cider in 1991 (Besser et al., 1993). Some foodborne pathogens can develop acid adaptation systems that induce cross-protection, and makes them more resistant against other environmental stresses (Bearson,

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Bearson, & Foster, 1997; Leyer & Johnson, 1992; Ryu & Beuchat, 1998; Ryu, Deng, & Beuchat, 1999), thus, increasing their ability to survive in juice. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., and *Cryptosporidium parvum*, can tolerate low pH values and survive in fruit juices and juice concentrates longer than non-adapted cells (Gahan, O'Driscoll, & Hill, 1996; Hsin-Yi & Chou, 2001; Oyarzabal, Nogueira, & Gombas, 2003). The acid adaptation of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* spp, also increases the heat resistance of these bacteria in apple, orange, white grape juices, apple cider, juice blends, cantaloupe, and watermelon juice (Mazzotta, 2001; Ryu & Beuchat, 1998; Sharma, Adler, Harrison, & Beuchat, 2005; Usaga, Worobo, & Padilla-Zakour, 2014).

The inactivation of the "pertinent microorganism" is targeted by processors in the determination of pasteurization parameters under the US FDA juice HACCP rule. To inactivate these pathogens, thermal pasteurization is one of the most commonly applied and effective techniques (MacGregor & Farish, 2000). Determination of the appropriate thermal inactivation parameters constitutes essential variables for the designation of juice pasteurization to produce safe, stable, and quality juices. Current thermal treatment of fruit juices and apple cider appears to be efficient in the prevention of microbial spoilage and elimination of pathogens. Pasteurization parameters for fruit juice with the use of cocktails of target microorganisms have been studied by numerous researchers (Mak, Ingham, & Ingham, 2001; Mazzotta, 2001; Singh, Mullins, Simpson, & Dickson, 2008), and appropriate time and temperature parameters in pasteurization of orange juice have been recommended as 3 s at 71.1 °C by FDA (2004).

The number of current studies regarding thermal inactivation of acid-adapted microorganisms in the fruit juices is limited, and compounded by the use of strain cocktails. In thermal fruit juice pasteurization, the success of the process depends on the establishment of appropriate heat application times and temperatures. Validation of these parameters through elaborated studies with appropriate methodologies is essential. The purpose of this study is to evaluate the thermal inactivation responses of acid adapted and non-adapted stationary phase Shiga toxin-producing *E. coli* (STEC), *Salmonella* spp., and *L. monocytogenes* by using individual strains of each organism in single strength orange juice, respectively. This study provides more detailed and clarified information about the effect of acid adaptation of stationary phase microorganisms on thermal inactivation of bacterial survival, and determination of pasteurization parameters for orange juice processors.

2. Material and methods

2.1. Juice

A single lot number of one brand of 100% pure and single-strength pasteurized orange juice without preservatives and pulp was purchased in enough volume from a local supermarket (Winter Haven, FL) to perform the complete experiment. The pH of juice was obtained via lab scale pH meter (accumet® AB15 Basic, Fischer Scientific, Pittsburg, PA USA). The soluble solid content of juice was measured by using The *Leica* Mark II plus digital refractometer (Buffalo, NY, USA). Juice was filled into 50 mL conical centrifuge tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) aseptically, and stored at −20 °C until use. One tube of juice was used for each experimental trial.

2.2. Strains used

Three strains of STEC, three serotypes of *Salmonella,* and three strains of *Listeria monocytogenes* were used. The three STEC strains included *E. coli* O111 (MDD339; clinical isolate from a 2004 apple cider outbreak, New York), *E. coli* O157:H7 (MDD338; clinical isolate from an apple juice outbreak of 1991, Massachusetts), and *E. coli* O157:H7 (F4546; clinical isolate from a sprout outbreak of 1997). The *Salmonella* serotypes included Typhimurium (ATCC 14028; orange juice outbreak of 1999), Gaminara (CDC HO622; orange juice outbreak of 1995), and Muenchen (MDD30; orange juice outbreak of 1999). The three *L. monocytogenes* strains included *L. monocytogenes* (LCDC 81-861; raw cabbage outbreak of 1981), *L. monocytogenes* (Scott A; milk outbreak of 1983), and *L. monocytogenes* (v7; milk associated outbreak of 1985).

2.3. Inoculum preparation

All strains, stored at −80 °C, were converted to working cultures by streaking on non-selective Tryptic Soy Agar (TSA; Becton, Dickinson and Company, Sparks, MD, USA) plates for STEC and *Salmonella,* and on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company, Sparks, MD, USA) plates for *L. monocytogenes*. Plates were incubated at 37 ± 2 °C for 24 ± 2 h.

Inoculua were prepared differently for non-adapted and acid adapted cells. For non-adapted stationary phase inoculum preparation, one isolated colony from each strain of STEC, and *Salmonella* were grown in Tryptic Soy Broth (TSB; Becton, Dickinson and Company) at 37 ± 2 °C for 18 ± 2 h. One isolated colony from each *L. monocytogenes* strain was incubated in BHI broth at 37 ± 2 °C for 18 ± 2 h. One loopful of overnight growth was transferred to a new tube of broth and incubated at 37 ± 2 °C for 18 h ± 2 h. For acid adapted stationary phase inoculum preparation, strains of STEC and *Salmonella* were grown in TSB supplemented with 1% glucose (Fisher Scientific, Lawn, NJ, USA) (10 g/L) TSBG at 37 ± 2 °C for 18 ± 2 h as described by Buchanan and Edelson (1996). For strains of *L. monocytogenes*, BHI supplemented with 1% glucose (10 g/L) BHIG was used at 37 ± 2 °C for 18 ± 2 h. One loopful (10 μL) of overnight growth was transferred to the new tube of broth containing 1% of glucose and incubated at 37 ± 2 °C for 18 ± 2 h. The addition of glucose to the broth results in acid production by STEC, *Salmonella,* and *L. monocytogenes* strains, thus reducing the pH of the media, inducing the acid tolerance response of cells, and the development of acid adaptation (Buchanan & Edelson, 1996; Buchanan, Golden, Whiting, Phillips, & Smith, 1994; Foster, 1995).

Following incubation, cells were collected by centrifugation at 3000 × g for 10 min (Allegra X-12, Beckman Coulter, Fullerton, CA). The supernatant was removed and 10 mL of 0.1% peptone water (Becton, Dickinson and Company) was vortexed with the pellet to wash cells. Cells were centrifuged and the washing step was repeated. After the cells had been washed three times, the pellets were resuspended in 5 mL orange juice to obtain the desired concentration of cells (10^8–10^{10} CFU/mL).

2.4. Thermal treatment of inoculated juice

Each strain of STEC, *Salmonella*, and *L. monocytogenes* was inoculated separately into orange juice as described above. Two sterile microcapillary tubes (1.5–1.8 (ID) 90 mm; Kimble-Kontes, Vineland, NJ, USA) with one head heat sealed, were each injected with 50 μL of inoculated orange juice. The aseptic injection was achieved using a sterile 20 gauge 4 inch deflected-point needle (Popper and Sons, Inc., Hyde Park, NJ, USA), and 1 mL syringe (Luer-Lok Tip, Franklin Lakes, NJ, USA). The open end of the microcapillary tubes was sealed with a Bunsen burner flame. To prevent cracking of the tubes from sudden temperature change before thermal treatment, the microcapillary tubes were held at room temperature for 30 min prior to the beginning of the experiment.

Inoculated, sealed, microcapillary tubes of each strain were immersed into water baths (LAUDA Brinkmann, ECO-Line RE120,