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Effectiveness of different antimicrobial washes combined with freezing against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* inoculated on blueberries

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ABSTRACT

To ensure the safety of produce, including blueberries, elimination of potential pathogens is critical. This study evaluated the efficacy of antimicrobial washes when coupled with frozen storage against *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* on wild blueberries. Inoculated blueberries were sprayed with antimicrobial solutions at different concentrations for various contact times (chlorine dioxide ~2.5, 5, 10, and 15 ppm for 10 s, 1, 5, and 10 min; chlorine ~100, 150, and 200 ppm for 10s, 1, 5, and 10 min; lactic acid 1 and 2% for 5, 10 and 20 min) and following treatment, stored at −12 °C for 1 week. Compared to antimicrobial washing alone, the additional freezing significantly reduced pathogens ($P < 0.05$). Concentrations of all three antimicrobials combined with freezing reduced *L. monocytogenes* to undetectable levels (detection limit < 1 log CFU/g). The greatest reduction of *E. coli* O157:H7 (4.4 log CFU/g) and *Salmonella* (5.4 log CFU/g) was achieved by 2% lactic acid or 200 ppm Cl_2 followed with frozen storage. These antimicrobials maintained the visual quality of blueberries and did not leave detectable residues. In conclusion, antimicrobial washes, when combined with frozen storage, effectively reduce the risk of pathogen contamination on blueberries.

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1. Introduction

The consumption of raw fruits and vegetables has increased in recent years. However, raw produce can be a source of foodborne pathogens and subsequently cause illness (Yeni et al., 2016). For example, an outbreak of listeriosis in 1984 was associated with contaminated blueberries (Ryser and Marth, 2007) and other berries, such as raspberries or strawberries, also have been sources foodborne illness and are commonly linked to outbreaks of hepatitis A and cyclosporiasis (Palumbo et al., 2014). Although the presence of wildlife in blueberry fields has previously been identified as a potential source of *Escherichia coli* O157:H7 and *Salmonella* contamination of berries (Jones et al., 2015; Wu et al., 2017), pathogen introduction can occur anywhere in the farm-to-fork continuum. Therefore, treatment steps that sufficiently reduce levels of potential microbial contaminants from berries need to be utilized to help ensure consumer safety.

Chlorine (Cl_2) solutions (e.g. sodium hypochlorite), in concentrations ranging from 50 to 200 ppm, are the most widely used antimicrobial in produce processing, including berries (Beuchat et al., 2004; Yeni et al., 2016). However, due to inadequate decontamination effectiveness and concerns regarding consumer and environmental safety of chlorinated washes, alternative antimicrobial washes such as chlorine dioxide (ClO_2) and lactic acid have become research focuses (Goodburn and Wallace, 2013; Trevisani et al., 2017; Wu, 2016). Previous research has demonstrated that chlorine washes would typically not achieve greater than a 2 log reduction of microorganisms on blueberries (Crowe et al., 2005) while chlorine dioxide washes of blueberries, comparatively, have been reported to be capable of > 4 log CFU/g reductions of *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Native yeasts and molds, *Salmonella* ser. Typhimurium, and *Yersinia enterocolitica* were all reduced by > 2 log CFU/g as well (Wu and Kim, 2007). Organic acids, such as lactic acid, are also frequently used in the food industry to reduce microbial populations and previous studies with produce models such as chicory,

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tomatoes, and lettuce have demonstrated that lactic acid is an effective antimicrobial treatment (del Carmen Velázquez et al., 2009; Trevisani et al., 2017). With 2% lactic acid, more than 6 log CFU/cm^2 of *E.* coli O157:H7 was reduced from surface of cantaloupes (Materon, 2003). However, no studies yet have demonstrated antimicrobial effectiveness of lactic acid treatment of berries.

Berries, especially wild blueberries are often sold in a frozen state. In fact, the vast majority (>99%) of wild blueberries from Maine are sold frozen (Yarborough, 2012). While washing with an effective antimicrobial solution can act as a first barrier against pathogens and other microorganisms on produce, an additional freezing step to slow or even stop microbial growth may act as a second hurdle to reduce levels of microbial pathogens. There have not been any studies on the efficiency of different antimicrobial washes combined with freezing on frozen produce so far. Therefore, the objective of this study was to 1) evaluate the effectiveness of various antimicrobial washes including Cl_2, ClO_2, or lactic acid against *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* on inoculated blueberries and 2) investigate how decontamination efficacy increased when Cl_2, ClO_2, or lactic acid washes were combined with a freezing step as an additional hurdle.

2. Materials and methods

2.1. Bacterial strains

Two strains of *E. coli* O157:H7 (ATCC 35150 and NCTC 129000), *S.* Typhimurium (ATCC 6962 and ATCC 14028) *L. monocytogenes* (ATCC, 19115 and ATCC 49594) were obtained from the Pathogenic Microbiology Laboratory (University of Maine, Orono, ME). Beads from a cryogenic (−80 °C) stock were initially cultured in brain heart infusion broth (BHI: Difco, Becton Dickinson, Sparks MD) and further maintained on trypticase soy agar (TSA) slants at 4 °C throughout the study. A cocktail mixture of two strains for each pathogen was used in this study and each species was studied individually.

2.2. Preparation of inoculum

From the stock cultures maintained on TSA, one loopful of each strain was transferred into 20 ml of BHI and incubated at 37 °C for 24 h. The broth was then centrifuged at 5000 ×*g* for 15 min at 4 °C to yield a pellet which was washed twice with 0.1% peptone water (Bacto, Becton Dickinson, Sparks, MD, USA) and finally re-suspended in 20 ml 0.1% peptone water. A 40 ml of intraspecific cocktail mixture was then made by combining the two strains of each pathogen with approximately equal populations. Before inoculation of blueberries, the population of each cocktail mixture was enumerated and determined by serial dilution in 0.1% peptone water and plating on MacConkey sorbitol agar supplemented with cefixime and tellurite (CT-SMAC), xylose lysine deoxycholate agar (XLD), or modified oxford medium (MOX) for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Each cocktail mixture contained approximately 8–9 log CFU/ml prior to berry inoculation.

2.3. Inoculation of blueberries

Frozen blueberries used in this study were kindly provided by the Wild Blueberry Association of North America (Old Town, Maine USA) and stored at (−17 °C) before experimentation. Twenty-five grams of frozen berries were placed on sterile petri dishes and inoculated with a 2.5 ml of bacterial cocktail for each pathogen by a dipping method. The inoculated berries were shaken for 3 min at 160 rpm using a shaker (Barnstead Thermolyne, Roto Mix-Type 50800). To allow for bacterial attachment, inoculated blueberries were dried in a laminar flow hood on sterile glass rods for 2 h (Wu and Kim, 2007). The initial pathogen concentrations on the berries were measured by serial dilution using 0.1% peptone water and plating using selective agar and was found to be approximately 7 log CFU/g after drying.

2.4. Chlorine preparation

Chlorine (Cl_2) solutions were prepared from regular household bleach solution containing 6.0% sodium hypochlorite on the day of experimentation. Bleach solution was diluted with deionized water to obtain 100, 150, or 200 ppm concentrations of free chlorine and measured using a Hach chlorine test kit (Hach Company, Loveland, CO) following the protocol outlined by Harp (2002). The pH of each solution was measured with a pH meter (Model 410A, Thermo Orion, Baverly, MA) and adjusted to 6.5 ± 0.2. The concentrations of Cl_2 selected for this study were selected based on common practices in the blueberry industry (Crowe et al., 2005).

2.5. Aqueous chlorine dioxide preparation

According to manufacturer protocol, a chlorine dioxide (ClO_2) solution sachet (ICA TriNova, LLC, Forest Park, GA) consisting of two components (sodium chlorite and a proprietary "activating acid") was placed in 7.5 l of distilled water and stored at room temperature (21 °C) for 3 days in the dark. This stock solution was then stored at 4 °C for later use. Concentration of ClO_2 in the stock solution was measured by a DPD method (*N*,*N*-diethyl-*r*-phenylenediamine) using a Hach DR/820 Colorimeter (Hach, Loveland,CO) and was diluted to 2.5, 5, 10, and 15 ppm as previously described by Wu and Kim (2007).

2.6. Lactic acid preparation

Lactic acid solutions (1% or 2%) were prepared by diluting 90% L-lactic acid (Acros Organics, New Jersey, USA) with distilled water following the methodology of del Carmen Velázquez et al. (2009). The concentrations selected were based on common practices in published literature (del Carmen Velázquez et al., 2009; Trevisani et al., 2017).

2.7. Blueberry treatment

Twenty-five grams of inoculated blueberries were aseptically spread on sterile wire screens using forceps and sprayed with 250 ml of sterile distilled water (control) or 250 ml of antimicrobial solution and allowed to sit for designated exposure times. Experimental concentrations and exposure times were: ClO_2 (2.5, 5, 10, and 15 ppm for 10 s, 1, 5 and 10 min), Cl_2 (100, 150, and 200 ppm for 10 s, 1, 5 and 10 min), and lactic acid (1% or 2% for 5, 10 and 20 min). To follow industrial setup, home and garden sprayers, similar to those used in blueberry processing in Maine, (RL Flomaster, Root-Lowell manufacturing Co., Lowell, Michigan) modified with Whirljet (1/4 B s 3) nozzles (spraying Systems Co.) were used for treatment spraying (Crowe et al., 2005). At the end of each exposure, one set of treatments was stored at −12 °C in a freezer (VWR cat # 10819-892) for 1 week, which mimicked industrial setup under the allowed experiment setting, and the other was analyzed immediately for microbial enumeration on the previously mentioned selective media. Briefly, samples were diluted using 25 ml 0.1% peptone water and subjected to shaking for 5 min at 200 rpm. Then, a series of dilutions were prepared for plating. Each plate was incubated overnight at 37 °C. The detection limit was <1