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Antibacterial activity of acidified sodium benzoate against *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in tryptic soy broth and on cherry tomatoes

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ABSTRACT

Concerns about undesirable by-products from chlorine sanitation of fresh produce and the limited efficacy with the presence of organic matter, have led to studies on alternative washing solutions. The aim of this study was to evaluate the antibacterial activities of acidified sodium benzoate (NaB) solutions against *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* in growth medium and on cherry tomatoes. Experimentally, the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs, > 3 Log reduction) of NaB against *E. coli* O157:H7 ATCC 43895, *S.* Enteritidis, and L. *monocytogenes* Scott A were determined at pH 7.0–4.0 using micro-broth dilution method and agar plating method, respectively. The reduction of the three bacteria in tryptic soy broth (TSB) by 500 and 1000 ppm NaB at pH 2.0, 2.5 and 3.0 for 30 min at 21 °C was compared. Residual bacterial cocktails inoculated on cherry tomatoes were determined after soaking in 3000 ppm NaB solution adjusted to pH 2.0 for 3 min at 21 °C. Results showed that the MBC of NaB reduced from > 10,000 ppm at pH 7.0 to 1000 ppm at pH 4.0 and was identical for the three bacteria. The log reduction of bacteria in TSB indicated that 1000 ppm NaB at pH 2.0 was the most effective in killing the three pathogens. The respective reduction of *E. coli* O157:H7 and *S. enterica* cocktails inoculated on cherry tomatoes immersed in 3000 ppm NaB (pH 2.0) at 21 °C for 3 min was 4.99 ± 0.57 and 4.08 ± 0.65 log CFU/g, which was significantly higher ($p < 0.05$) than the treatments of 200 ppm free chlorine at pH 6.5. Conversely, the reduction of L. *monocytogenes* on tomatoes by 3000 ppm NaB (4.88 ± 0.73 log CFU/g) was similar ($p > 0.05$) to 200 ppm chlorine. Furthermore, the reduction of bacterial cocktails on tomatoes by 3000 ppm NaB at pH 2.0 was not affected after adding 1% tomato puree, and bacteria were not detected in NaB washing solutions with and without 1% tomato puree and on following un-inoculated tomatoes. This study showed that acidified NaB solution may be used as an alternative post-harvest wash of produce.

1. Introduction

Chlorine is the most widely used chemical disinfectant in the fresh produce industry due to its low cost and bactericidal activities (Beuchat, 2000). However, chlorine-based sanitizing solutions face several challenges. First, at typical sanitation conditions with 50–200 ppm free chlorine at pH 6.0–7.5 for 1–2 min, only a 1–2 log reduction is obtained on many produce commodities, and even high concentrations of chlorine are not able to eliminate pathogens on produce (USDA, 2001). Second, organic matter, such as produce debris, soil, and microorganisms from fresh produce may react and cause the oxidation of chlorine and reduce the sanitation effectiveness (Richardson et al., 1998). The reactions can produce harmful toxic compounds such as chloroform, chloramines and trihalomethanes, which may cause negative impacts on the quality of produce and human health (Huang and Chen, 2011; Richardson et al., 1998; Rodgers et al., 2004). Trace chlorine residues can also cause sensory defects (Singh et al., 2002). Third, the reduced effectiveness of chlorine can lead to cross-contamination, i.e., the transfer of pathogens from contaminated produce to wash solution and then to uncontaminated produce, which may result in outbreaks of foodborne illnesses (Luo et al., 2011). In view of these concerns, alternative low-cost but effective washing solutions are gaining interest in the scientific community and produce industry.

Organic acids are naturally present in fruits and vegetables and are known for their antimicrobial activities with a potential to disinfect

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produce (Joshi et al., 2013). Huang and Chen (2011) studied the effectiveness of lactic, acetic, tartaric, citric, and malic acids in reducing bacteria inoculated on baby spinach. Washing solutions with 1–2% of these organic acids had a pH between 2.0 and 2.7, and a maximum of 1.9 log (CFU/g) reduction was obtained at room temperature (Huang and Chen, 2011). Peroxyacetic acid is also an effective organic acid that is environmentally friendly and has good efficacy of sanitizing produce (Bang et al., 2017). Rinsing tomatoes for 10 min at room temperature with 50 ppm peroxyacetic acid resulted in a reduction of *E. coli* O157:H7 by 4.48 log (CFU/g) (Keeratipibul et al., 2011). However, other acids such as ascorbic, caprylic, levunilic, sorbic, and benzoic acids have not been studied as a disinfectant of fresh produce.

Sodium benzoate (NaB), the sodium salt of benzoic acid, is more soluble in water than benzoic acid and is preferred in many cases. NaB is a commonly used food preservative that is listed among the generally-recognized-as-safe (GRAS) additives by the United States Food and Drug Administration, and can be present in foods at a concentration up to 1000 ppm (Chipley, 2005). NaB has a relatively low cost (Chipley, 2005). Additionally, benzoic acid is considered as a natural compound since it has been identified as a major compound in extracts of fresh tomato and some other fruits (Chipley, 2005). Both NaB and benzoic acid exhibit activities against a wide range of microorganisms, especially fungi, yeasts and molds, which are targeted microorganisms for applying NaB to preserve foods such as fruit juice, soda, soy sauce, and ketchup (Chipley, 2005). The activity of NaB inhibiting pathogens is dependent on acidity. At 35 °C, 500 or 1000 ppm NaB at pH 5.0 inhibited the growth of L. *monocytogenes* in tryptic soy broth (TSB) for at least 40 h, and the bacterium grew in the presence of 2000 ppm NaB at pH 5.6 after 8 h incubation (Elshenawy and Marth, 1988). In another study, 85,000 ppm NaB at pH 7.0 was reported to only reduce L. *monocytogenes* by 2 log (CFU/mL) after 1 h treatment (Buazzi and Marth, 1992). There are reports about applying NaB together with other preservatives such as sodium chloride (Seman et al., 2008), sodium acetate (Seman et al., 2008), and lactic acid (Ozdemir et al., 2006) to inhibit pathogens. However, the application of NaB as an antimicrobial disinfecting fresh produce has not been reported. We hypothesize that acidified NaB solutions can reduce *E. coli* O157:H7, *S. enterica*, and L. *monocytogenes* by > 3 log in microbial growth media and on fresh produce in a short time.

The first objective of this study was to determine antimicrobial activity of NaB in TSB adjusted to different pHs. This was evaluated for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against pathogens at their optimum growth temperatures and log reductions after ambient incubation for 30 min. The second objective was to evaluate the potential of acidified NaB solutions for post-harvest washing of fresh produce. Cherry tomatoes were used as a model fresh produce commodity because tomatoes have been associated with several multi-state outbreaks of foodborne illnesses that accounted for about 17.1% of all produce related outbreaks between 1996 and 2006 (Mukhopadhyay et al., 2015). In addition to characterizing the reduction of pathogens inoculated on cherry tomatoes, the presence of pathogens in wash solutions and the transfer of pathogens to following un-inoculated tomatoes were evaluated. NaB treatments were also compared to 200 ppm free chlorine at pH 6.5, which is the highest chlorine concentration commonly used in industrial production (Huang and Chen, 2011; Stopforth et al., 2008). The impact of organic matter on sanitation was studied by adding 1% tomato puree.

2. Materials and methods

2.1. Chemicals

NaB (> 98% purity) and agar were procured from Sigma-Aldrich Corp. (St. Louis, MO, USA). HCl (12 N), nalidixic acid (NA), calcium hypochlorite (~65% available chlorine), Tween 80, peptone, TSB, yeast extract, and blender bags (105 mm × 155 mm) were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA).

2.2. Media preparation

TSB medium was prepared by dissolving 30.0 g powder in 1000.0 mL water. TSA was prepared by adding 12 g agar into the TSB medium. A NA stock solution was prepared at a concentration of 1600 ppm in ethanol. Twenty five milliliters of the NA stock solution were added to 1 L sterile TSB or TSA to obtain a TSBN or TSAN medium with 40 ppm NA that was used as a selective medium to prepare bacteria inocula and enumerate bacteria in the tomato washing treatments (Harness III, 2015).

2.3. Test microorganisms.

E. coli O157:H7, *L. monocytogenes*, and *Salmonella* strains were obtained from the culture collection of the Department of Food Science at the University of Tennessee (Knoxville, TN, USA). All strains were maintained at −20 °C in 40% glycerol. Each strain was transferred consecutively 2 times in TSB with an interval of 24 h before use. *E. coli* O157:H7 ATCC 43895, *S. enterica* serovar Enteritidis, and *L.monocytogenes* Scott A were used to determine MICs and MBCs of NaB and log reduction in TSB. These commonly-studied strains were used so as to compare characteristics of other antimicrobials in the literature. *E. coli* O157:H7 strains (H1730, F4546, K3995, CDC658 and 932), five *S. enterica* serovars (Agona, Montevideo, Gaminara, Michigan and Saintpaul), and five L. *monocytogenes* strains (ENV2011010804-1 (390-1), ENV2011010804-2 (390-2), 310, Scott A, and V7) were used for cherry tomato study. These strains were chosen in this set of treatments because they were associated with outbreaks of illnesses due to contaminated produce. The bacteria used for tomato study were individually made resistant to 40 ppm NA after culturing in the above TSBN medium.

2.3. Determination of MIC and MBC in TSB

The MIC was determined using the microbroth dilution method (Branen and Davidson, 2004). The stock culture was diluted to about 5 log CFU/mL in TSB pre-adjusted to a test pH as the working culture right before transferring to a microtiter plate. NaB stock solutions at pH 7.0, 6.0 and 5.0 were prepared at a concentration of 20,000 ppm, while the pH 4.0 stock solution had 16,000 ppm NaB because precipitation was observed at 20,000 ppm. The acidification was conducted using 6.0 N HCl. The stock solutions were then diluted to 16,000, 8000, 4000, 2000, and 1000 ppm NaB using TSB pre-adjusted to the same pH. A 120 μL aliquot of a diluted NaB solution was mixed with the same volume of the working culture in each well to obtain a NaB concentration of 10,000, 8000, 4000, 2000, 1000 and 500 ppm and ~4.5 log (CFU/mL) bacteria. The 96-well plates were incubated at 32 °C (for L. *monocytogenes*) or 37 °C (for *E. coli* O157:H7 and *S*. Enteritidis) for 24 h. The MIC was defined as the lowest antimicrobial concentration that had an optical density change at 630 nm (ΔOD_{630nm}) below 0.05. The MBC was determined by pipetting 100 μL aliquots from wells with ΔOD_{630nm} of < 0.05 and spreading on tryptic soy agar (TSA), followed by incubation for another 48 h at 32 °C or 37 °C. MBC was defined as the lowest antimicrobial concentration corresponding to ≥ 99.9% (~3 log) reduction of viable cells.

2.4. Evaluation of log reductions in TSB

A total of 6 treatments, combinations of two NaB concentrations (500 and 1000 ppm) and three acidity conditions (pH 2.0, 2.5, and 3.5), were studied for log-reductions of pathogens in TSB at room temperature (RT, ~21 °C). The stock solution with 20,000 ppm NaB in distilled water was mixed with an appropriate volume of TSB to an overall NaB concentration of 0, 500, and 1000 ppm and adjusted to pH 2.0–3.5 with 6.0 N HCl. The bacterial culture was diluted to 8 log (CFU/mL) in TSB