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Inactivation of *Listeria innocua* by a combined treatment of low-frequency ultrasound and zinc oxide

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ABSTRACT

Microbial contamination of fresh produce remains a food safety issue in the US, with recent outbreaks linked to alfalfa sprouts and packaged salads. Chlorine-based sanitizers widely used on produce have limited efficacy and pose health risks. This study determined efficacy of combined low-frequency ultrasound with zinc oxide (ZnO) in inactivating *Listeria innocua*. ~ 6 log(CFU/mL) *L. innocua* were treated with either 20 or 40 mM ZnO and sonication (20 kHz, 43–45 W, 120 μm amplitude) at room temperature for 0–30 min. 40 mM ZnO and ultrasound resulted in > 5 log CFU/mL *L. innocua* reduction within 8 min, while individual treatments caused < 1 log CFU/mL reduction. The inactivation rate was ZnO concentration-dependent. L-histidine, a known quencher of hydroxyl radicals and singlet oxygen, significantly attenuated bacterial inactivation, suggesting a Reactive Oxygen Species (ROS)-mediated antimicrobial mechanism. ZnO nanoparticle size decreased from ~250 nm to ~100 nm after sonication, potentially allowing ZnO to more easily penetrate cell membranes, while scanning electron microscopy (SEM) images of *L. innocua* suspended in 40 mM ZnO imply ZnO adheres on the surface of bacteria. ZnO-bacterial surface interactions can cause cell membrane damage and even cell death. The proposed technology has potential for sanitizing fresh produce industry wash-water.

1. Introduction

Between 2004 and 2013, fresh and freshly cut produce were responsible for the majority of foodborne disease outbreaks in the United States. The deadliest outbreak since 1990 took place in 2011, when *Listeria monocytogenes*-contaminated cantaloupes resulted in 147 illnesses and 33 deaths across 28 states (CSPI, 2015). Minimal processing of fresh fruits and vegetables makes it difficult to fully eliminate pathogenic organisms (FDA, 2015), which can contaminate produce at the growth, harvesting, postharvest handling, processing, or distribution stage (Birmpa, Vasiliki, & Vantarakis, 2013). Washing with chlorine-based sanitizers is almost universally used in the fresh produce industry to remove harmful bacteria and other microorganisms. However, this sanitation method is not completely effective, and also poses several health risks (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). Wash water rapidly acquires a high organic load during produce washing, due to the introduction of soil, leaves, and other debris along with the produce. Free chlorine reacts with this organic matter, which both decreases the amount of chlorine available to kill harmful microorganisms, and results in the formation of undesirable by-products. These by-products include trihalomethanes, haloacetic acids, haloketones, and chloropicrin, all of which are potentially carcinogenic (Gil, Selma, López-gálvez, & Allende, 2009). While toxicity studies show that the concentration of by-products formed is negligible, the use of chlorine-based sanitizers is increasingly less popular with consumers, and has already been banned in several European countries, such as Germany and Switzerland (Gil et al., 2009). Additionally, chlorine poses other processing challenges such as frequent monitoring of temperature and pH of water (Boyette, Richie, Carballo, Blankenship, & Sanders, 1993, p. 8). Thus, there is a growing need to develop sanitation techniques for wash-water that can address some of these concerns.

Low-frequency (20–100 kHz) ultrasound, as an alternative antimicrobial treatment, has the advantages of being considered safe, non-toxic, and environmentally friendly (Bermúdez-Aguirre, Mobbs, & Barbosa-Cánovas, 2011). The ultrasonic waves create changes in pressure, which results in cavitation bubbles that, upon bursting, kill bacteria (Piyasena, Mohareb, & Mckellar, 2003). However, low-frequency ultrasound alone has limited application to the food industry because of time-constraints. The performance standard for food-contact surfaces is a 5-log reduction of microbial growth within 30 s (AOAC International, 2009). However bacteria, particularly spores, are very

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resistant and would require hours of sonication to be inactivated (Sala, Burgos, Cóndon, Lopez, & Raso, 1995). In one study, complete sterilization of an *Escherichia coli* film grown for 14 h was achieved after 6 h of exposure to low-frequency ultrasound (Johnson, Peterson, & Pitt, 2016). In another study, low-frequency ultrasound alone was found to kill less than 1 log (< 90%) of the foodborne pathogens *E. coli*, *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Bacillus subtilis* (Scherba, Weigel, & O'Brien, 1991). Ultrasound in combination with pressure (Mañas, Pagán, Raso, Sala, & Condón, 2000) and/or heat (Ordoñez, Aguilera, Garcia, & Sanz, 1987) has been shown to enhance microbial inactivation (Piyasena et al., 2003). Studies have been done examining the combined effect of ultrasound and heat/chemicals on alfalfa seeds (Scouten & Beuchat, 2002), and of ultrasound and heat/pressure on fruit and vegetable juices (Kuldiloke, 2002). A recent study demonstrated that low frequency ultrasound together with the antimicrobial peptide melittin significantly reduced the cell density of *Listeria monocygotenes* (Wu & Narsimhan, 2017). One combination that has not been explored is ultrasound treatment of microorganisms in the presence of sonochemicals, such as zinc oxide (ZnO) and titanium dioxide (TiO_2). ZnO is an inorganic compound with known antibacterial properties (Brayner et al., 2006; Jones, Ray, Ranjit, & Manna, 2008; Jalal et al., 2010; Seil & Webster, 2012; Emami-Karvani & Chehrazi, 2011; Padmavathy & Vijayaraghavan, 2008; Raghupathi, Koodali, & Manna, 2011). It is generally recognized as safe (GRAS) by the Food and Drug Administration in CFR Title 21 (FDA, 2016), and is currently used as a food additive. ZnO nanoparticles have known antibacterial ability, and have already been incorporated into food packaging material to improve its antibacterial activity (Espitia, Soares, Coimbra, de Andrade, & Cruz, 2012). However, little is known about its synergistic interaction with ultrasound to enhance microbial inactivation.

The overall goal of this study is to offer a proof-of-concept of an antibacterial treatment that is based on synergistic interaction between low-frequency ultrasound and ZnO particles. To that end, we characterized the antimicrobial effect of the combined treatment against *Listeria innocua*, and attempted to understand the mechanism(s) by which the treatment accelerates bacterial inactivation.

2. Materials and methods

2.1. Microorganisms and growth conditions

L. innocua Clip11262 was obtained from Prof. Robert Buchanan at the Department of Nutrition and Food Science at the University of Maryland-College Park. Bacterial suspensions were prepared as follows: stock cultures were kept in Tryptic Soy Broth (TSB, Difco, Becton Dickinson, Sparks, MD), containing 25% glycerol at −80 °C. A loopful of stock culture was streaked onto tryptic soy agar (TSA, Difco, Becton Dickinson, Sparks, MD) and incubated at 37 °C for 24 h. An individual colony was taken from the inoculated TSA and transferred to 9 mL of sterile TSB. The broth was subsequently incubated overnight at 37 °C for 12–14 h, and streaked onto new TSA plates, which were incubated for 24 h at 37 °C. TSA plates were refrigerated and kept for experiments up to three weeks. For the antimicrobial treatments with ultrasound and ZnO, an individual colony of *L. innocua* was taken from the TSA plates, transferred to 9 mL of sterile TSB, and incubated overnight at 37 °C. After 14–15 h incubation, the cells were diluted 100-fold in sterile TSB, and incubated at the same temperature an additional 5 h, until the middle of the exponential phase. After this second incubation period, the cells were subjected to the antimicrobial treatments described in section 2.3.

2.2. Ultrasound equipment and parameters

The sonic dismembrator is a model FB505 (Fisher Scientific, Pittsburgh, PA), with a maximum power of 500 W and a frequency of 20 kHz. The probe has a replaceable tip (12.7 mm diameter), and experiments were performed at 50% amplitude, or 120 μm (Qsonica, LLC., Newtown, CT), and at a wattage of 43–45 W. The probe was immersed in bacterial suspensions to a depth of 1 cm. Bacterial (50 mL) suspensions were placed in a 100 mL jacketed glass beaker (Kimble Chase, Vineland, NJ) with circulating cold water at 4 °C and stirred continuously via a magnetic rod.

Both probe and suspensions were housed within a sound enclosure chamber. For all treatments, the bacterial suspensions' temperature was maintained at 22.0 ± 1.0 °C using a refrigerated bath.

2.3. Antimicrobial treatments

Bacterial suspensions of ~6 log(CFU/mL) *L. innocua* in 40 mM ZnO were prepared by transferring mid-exponential phase *L. innocua* to 50 mL of 40 mM ZnO in deionized (DI) water. The ZnO-bacteria suspension was subsequently sonicated for 0, 4, 8, and 12 min in triplicate. Similarly, bacterial suspensions were prepared in 20 mM ZnO, and subsequently sonicated in triplicate, but at different time intervals each experiment run, for 0–30 min. After treatment, bacterial suspensions were serially diluted in 0.1% peptone water, plated on TSA, and plate-counted after 24 h incubation at 37 °C. Controls consisted of ~6 log (CFU/mL) *L. innocua* in 40 mM ZnO without sonication at 0 min and at last kinetic timepoint (12 min), as well as ~ 6 log(CFU/mL) *L. innocua* sonicated without addition of 40 mM ZnO at 0 and 12 min.

2.4. Histidine treatments

To determine whether bacterial inactivation was Reactive Oxygen Species (ROS) mediated, 10 mM L-histidine was added to ~ 6 log(CFU/mL) *L. innocua* in 40 mM ZnO (total volume: 50 mL), prior to sonication for 0, 4, 8, and 12 min. Histidine's inhibition of the combined ZnO and sonication treatment's antimicrobial effect was enumerated via plating serial dilutions of bacterial suspensions in 0.1% peptone water onto TSA, and plate-counting after 24 h incubation at 37 °C.

2.5. Particle size measurements

ZnO nanoparticle size measurements were taken to determine the effect of sonication, using a BI-200 SM Goniometer Version 2 Dynamic Light Scattering instrument (Brookhaven Instruments, Holtsville, NY), equipped with a 35 mW He-Ne laser. The following parameters were adopted: detection wavelength of 637 nm and scattering angle of 90°. 2 mL samples of sterile 40 mM ZnO sonicated for 0, 4, 8, and 12 min were analyzed, as well as 2 mL samples of 40 mM ZnO and 10 mM histidine sonicated for the same time intervals.

2.6. Scanning electron microscopy

Control samples (20 mL, ~7 log(CFU/mL)) of 40 mM ZnO were filtered through a 0.2 μm pore filter (25 mm, EMD Millipore Co., Billerica, MA). The filter was then immersed in 10 mL of 0.25% Glutaraldehyde in DI water (Sigma-Aldrich, St. Louis, MO) for 1 h. The filter was next rinsed three times in DI water, then immersed in 10 mL aqueous solutions with increasing concentration of ethanol (v/v: 10%, 25%, 50%, 75%, 90% and 100%). The filter was kept in anhydrous calcium sulfate until analysis. Finally, the filter was sputter-coated with gold for 1 min, after which SEM analysis was performed with a TESCAN XEIA FEG Scanning Electron Microscope (TESCAN, Ltd., Brno, Czech Republic) at 5 kV (Kihm, Leyer, An, & Johnson, 1994; Sousa, Sequeira, Kolen, Pinto, & Petrovykh, 2015).

2.7. Statistical analysis & survival curve fitting

Differences between sample means for bacteria treated with combined ultrasound and ZnO versus bacteria treated with either treatment