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● *Original Contribution*

INACTIVATION OF PLANKTONIC *ESCHERICHIA COLI* BY FOCUSED 2-MHz ULTRASOUND

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Abstract—This study was motivated by the desire to develop a non-invasive means to treat abscesses, and represents the first steps toward that goal. Non-thermal, high-intensity focused ultrasound (HIFU) was used to inactivate *Escherichia coli* ($\sim 1 \times 10^9$ cells/mL) in suspension. Cells were treated in 96-well culture plate wells using 1.95-MHz ultrasound and incident focal acoustic pressures as high as 16 MPa peak positive and 9.9 MPa peak negative (free field measurements). The surviving fraction was assessed by coliform culture and the alamarBlue assay. No biologically significant heating was associated with ultrasound exposure. Bacterial inactivation kinetics were well described by a half-life model, with a half-time of 1.2 min. At the highest exposure levels, a 2log inactivation was typically achieved within 10 min. The free field-equivalent peak negative acoustic pressure threshold for inactivation was ~7 MPa. At the highest acoustic pressures used, inactivation efficacy was insensitive to reciprocal changes in pulse length and pulse repetition frequency at constant duty factor. Although treated volumes were very small, proof of principle was provided by these experiments. (E-mail: brayman@apl.washington.edu) **© 2017 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Acoustic bacterial inactivation, *Escherichia coli* inactivation, Focused ultrasound, High-intensity focused ultrasound, Planktonic bacterial inactivation, Ultrasonic bacterial inactivation.**

INTRODUCTION

Acoustic fields have been known to inactivate bacterial cells for nearly 90 y (Harvey and Loomis 1929). A substantial literature on the topic has been amassed, largely in response to the food industry's desire to reduce microbial contamination of food without the requirement for quality-altering high-temperature treatments (see reviews: Chemat et al. 2011; Cullen et al. 2012; Earnshaw et al. 1995, Knorr et al. 2004; Piyasena et al. 2003) and a means to decontaminate surfaces. Other applications have included attempts to decontaminate septic water (see, *e.g.*, Antoniadis et al. 2007; Broekman et al. 2010, Dehghani et al. 2008, Drakapoulou et al. 2009; Jin et al. 2013, Scherba et al. 1991) and to reduce "hitchhiker" species in ship ballast water (Holm et al. 2008). Medical applications of ultrasound to address microbial biofilms in dentistry, and in the decontamination of implanted prostheses, were reviewed recently (Erriu et al. 2014). We have not found examples in the literature of therapeutic applications of ultrasound to control bacteria in fluid collections *in vivo*. It is the long-range goal of our research program to devise high-intensity focused ultrasound (HIFU) therapies for non-invasive *in vivo* treatment of fluid-filled abscesses as an alternative to standard-of-care drainage. We report here initial steps toward that goal using a relatively simple bench-top system and monomicrobial cell suspensions.

Microbial inactivation by ultrasound depends on a variety of non-acoustic factors, including type of organism, pH and temperature, as well as the acoustic factors of frequency, intensity, treatment time, nature of the suspending medium (*e.g.*, composition and viscosity), presence or absence of overpressure (the latter increasing efficacy at sufficient acoustic intensities) and heating (also increasing efficacy, although at temperatures that would be lethal to mammalian tissues). The rate of acoustic *Escherichia coli* cell inactivation depends strongly on the ultrasound frequency over the range 0.2–1.1 MHz and

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at a constant energy density, decreasing as frequency increases (Hua and Thompson 2000). Inactivation results from inertial cavitation, either *via* direct mechanical effects related to the shear forces associated with bubble collapse (Gao et al. 2014c) or *via* sonochemical effects (Joyce et al. 2003). Although it has been argued that sonochemical production is not the principal mechanism responsible for acoustic microbe inactivation (Hua and Thompson 2000); it appears that both mechanical and sonochemical effects are involved in the lysis of gram-negative *E. coli* and gram-positive *Streptococcus mutans* by 0.5-MHz ultrasound *in vitro*, as evidenced respectively by empty bacterial "shells" seen on electron microscopy (see also Chandler et al. 2001) and by diminution of the cell inactivation effect by inclusion of the free radical scavenger t-butanol (Koda et al. 2009).

Ultrasound has been used at relatively high powers (often several hundred watts) from horn-type sonicators (see Piyasena et al. 2003; Ugarte-Romero et al. 2007). However, in some cases, low intensities have inactivated significant numbers of microbes; for example, at 26 kHz, spatial peak temporal average (SPTA) intensities of 1–3 W/cm^2 were sufficient to kill about half of populations of *E. coli*, *Staphylococcus aureus* and *Bacillus subtilus* (Scherba et al. 1991). At relatively low power and kilohertz frequencies, inactivation of *Bacillus subtilis* had what appeared to be a threshold exposure time, with ≥6 min of exposure required to diminish coliform counts. At still lower power and frequencies of 0.5–0.8 MHz, ultrasound exposure did not inactivate the bacteria; coliform counts instead increased, apparently because of disaggregation of clusters by ultrasound exposure (Joyce et al. 2003, 2011). In other cases, the energetic requirements to achieve modest levels of inactivation can be extreme; for example, about 1.5 kW-h per liter treated (Drakapoulou et al. 2009). At these low frequencies, bacterial sensitivity to ultrasound exposure appears to be influenced by whether the organism is gram positive, such as *Shigella boydii*, *Listeria monocytogenes* and *Listeria seeligeri,* or gram-negative, such as *E. coli*, with gram-negative species being more resistant to inactivation than gram-positive species (Lee et al. 2003; Ugarte-Romero et al. 2007; see also Drakapoulou et al. 2009). Ultrasound exposure of the gram-negative bacterium *E. coli* and the gram-positive bacterium *Lactobacillus rhamnosis* at 20 kHz inactivated both species, but *E. coli* was more resistant than was *Lactobacillus*. However, ultrasound exposure permeabilized the outer membrane of *E. coli* cells to a normally impermeant fluorescent dye precursor, which was then taken up and metabolized by the cells, indicating mechanical damage to the outer membrane with apparent preservation of the inner membrane; that is, the outer membrane apparently shielded the inner one from lethal damage in some cases (Ananta et al. 2005). Low pH, overpressure and elevated temperatures greatly improve bacterial cell inactivation; high viscosity and proteinaceous media reduce efficacy (Piyasena et al. 2003). Bacterial growth phase may also influence bacterial stress responses to stimuli, with stationary phase cells generally being more resistant to stressors than are cells in the exponential growth phase (Gao et al. 2014b; Hengge-Aronis 1996; Siegele et al. 1996; Vollmer et al. 1998). Bacterial inactivation by ultrasound may depend qualitatively on acoustic frequency and the nature of the host fluid: *Enterobacter aerogenes* was more sensitive to low-frequency inactivation in water than in reconstituted skim milk, and could not be inactivated by 0.85-MHz ultrasound in the protein-rich milk (Gao et al. 2014a). Similar results have been obtained for other host fluids (Utsunomiya and Kosaka 1979).

Relatively few studies have been conducted on the application of low-megahertz-frequency ultrasound on microbes, as might be required for clinical applications to treat fluid collections. An early exploration of the relationship between bacterial inactivation and passively detected and quantified inertial cavitation activity using 1-MHz HIFU was published by Vollmer et al. (1998). These authors exposed suspensions of *E. coli* to 1000 cycle pulses at 20-Hz pulse repetition frequency (PRF), with a spatial peak pulse average (SPPA) intensity of 500 W/cm^2, and used a 20-MHz passive transducer to detect noise emissions associated with symmetric cavitation bubble collapse. The suspensions were enriched in cavitation nuclei by use of a microbubble-based contrast agent. Cell inactivation correlated poorly with cavitation "dose" as determined by the passive cavitation detector. This was attributed in part to the close proximity of many bacterial cells around each contrast agent microbubble initially present, which may produce undetected asymmetric bubble collapse.

Others have used megahertz-frequency ultrasound to exploit collection of cells at pressure nodes in standing wave fields to concentrate and remove them from suspensions (see, *e.g.*, Limaye and Coakley 1998; Miles et al. 1995). Still others have focused on biofilm treatment (Erriu et al. 2014). As an example of the latter, Xu et al. (2012) conducted *in vitro* studies of the destruction and removal of biofilms composed of the gram-negative bacterium *Pseudomonas aeruginosa*, motivated by the problem of biofilm growth on implanted prostheses. With 1.1-MHz HIFU of pressure amplitudes +30 MPa and −13 MPa peak positive and peak negative, respectively, treatment with 10 cycle pulses at a PRF of 167 Hz for 30 s was sufficient to kill the bacteria in exposed areas of the biofilms.