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Determination of the efficacy of ultrasound combined with essential oils on the decontamination of *Salmonella* inoculated lettuce leaves

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ABSTRACT

Salmonella is one of main pathogenic bacteria present in fresh produce. Ultrasound has been reported to be effective at inactivating food-borne pathogens. Moreover, ultrasound can be combined with essential oils to enhance its efficacy. This study evaluates the reduction and inactivation of *Salmonella enterica* Abony inoculated on lettuce leaves by the application of continuous and pulsed ultrasound as well as ultrasound combined with the essential oil of oregano and thyme. The physicochemical properties of these essential oil nanoemulsions are characterised while the structural damage of treated leaves is determined by the electrolyte leakage. Ultrasound combined with essential oils enhanced the microbial reduction on lettuce leaves and inactivation on the treated water, resulting on significant differences at concentrations higher than 0.018% (v/v) compared to control. Particle size, zeta potential and pH varied between 35 and 133 nm, −26 to −36 mV and 5.67 to 5.38, respectively. Electrolyte leakage was similar for both the control and the treated samples, increasing when essential oils were applied.

1. Introduction

Salmonella is one of major bacterial challenges of food safety and it is responsible for numerous foodborne illnesses around the world. In the United States, *Salmonella enterica* causes 1.2 million illnesses each year (Jackson, Griffin, Cole, Walsh, & Chai, 2013). In Europe, the European Food Safety Authority estimates that over 100,000 human cases of *Salmonella* illnesses are reported each year. The presence of *Salmonella* on these products is an important health issue related with food safety issues as fresh fruits and vegetables are consumed raw. Hence, they have been catalogued as high risk food products. Moreover, in recent years, there has been an increment of consumption of fresh vegetables and fruits due to their health benefits linked to the prevention of chronic diseases, such as heart disease, cancer, diabetes, and obesity (Callejón et al., 2015). Therefore, the food industry has to implement sound and safe decontamination technologies to ensure high availability of safe fresh produce to the consumers.

Washing with sanitizers, especially chlorine-based products, is the most common decontamination technology applied by the food industry. However, increasing public health concerns about the possible formation of trihalomethanes, which have been reported for possible carcinogenic effects (Dunnick & Melnick, 1993) and legislation about controlling the use of chlorine, makes the need to find new decontamination technologies imperative (Fernandez, Noriega, & Thompson, 2013).

Recent studies have shown the efficacy of several new decontamination technologies in order to assess the microbial safety of the fresh produce (Allende, Tomás-Barberán, & Gil, 2006; Gil, Selma, López-Gálvez, & Allende, 2009; Gómez-López, Rajkovic, Ragaert, Smigic & Devlieghere, 2009; Rico, Martin-Diana, Barat, & Barry-Ryan, 2007; Ölmez & Kretzschmar, 2009). Among them, ultrasound is a non-thermal technology, able to inactivate microorganisms, which is acquiring importance within the food industry because is reasonably inexpensive and energy saving (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). . Its antimicrobial efficacy is related to two main mechanisms, namely, cavitation and sonolysis. During cavitation, bubbles generated by ultrasound collapse, producing areas of high temperature (approximately 5500 °C) and pressure (approximately 50 MPa) (Mukhopadhyay & Ramaswamy, 2012). The high temperatures and pressures created within the bubble are responsible for the generation of hydrogen atoms and hydroxyl radicals, resulting in the phenomenon called

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sonolysis (Riesz, Berdahl, & Christman, 1985). These mechanisms damage the cellular and functional components of microorganisms and therefore result in cell lysis (Chemat & Khan, 2011). However, ultrasound alone is not very effective in inactivating bacteria on food (Piyasena, Mohareb, & Mckellar, 2003). Ultrasound can be combined with other decontamination technologies for enhancing microbial inactivation (Sango, Abela, McElhatton, & Valdramidis, 2014). The effect of cavitation generated by the ultrasound appeared to enhance the removal of attached or entrapped cells on the surfaces of fresh produce, rendering the bacteria more susceptible to antimicrobial sanitizers (Seymour, Burfoot, Smith, Cox, & Lockwood, 2002). Essential oils such as oregano or thyme are natural sanitizers and they are considered as Generally Recognized as Safe (GRAS) food ingredients and can be used as postharvest treatment for controlling bacteria growth. Their antimicrobial efficacy is related to their chemical composition, where the phenolic components are the main compounds responsible for bacterial inactivation (Burt, 2004, Cosentino et al., 1999). Different mechanisms have been described to explain this antimicrobial mechanism: degradation of the bacterial wall (http://www.sciencedirect.com/science/article/pii/S0963996914006905 Helander, Alokomi, Latva-kala, Mattila-Sandhlom, Pol, Smid, Gomis, & Von Wright, 1998), damage of the cytoplasmic membrane (http://www.sciencedirect.com/science/article/pii/S0963996914006905Ultee, Kets, & Smid, 1999), coagulation of the cytoplasm (http://www.sciencedirect.com/science/article/pii/S0963996914006905Gustafson et al., 1998), depletion of the proton force (Ultee & Smid, 2001) and increase of the cell membrane permeability, producing leaking of the intracellular constitutes (http://www.sciencedirect.com/science/article/pii/S0963996914006905Lambert, Skandamis, Coote, & Nychas, 2001).

Additionally, ultrasound is able to generate nanoemulsions. Nanoemulsions are oil-in-water or water-in-oil dispersions with a droplet size in the range of 20–200 nm and showing narrow size distributions (Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005). The small size of the droplets generated during the process enhances the bioactivity of the disperse medium due to an increment of the contact surface. Moreover, nanoemulsions can improve the stability of the dispersion (Solans et al., 2005) which can be determined by the z-potential.

The aim of this study was to assess the efficacy of ultrasound, alone or combined with two essential oils (namely essential oil of oregano (EEO) and thyme (EOT)), on the decontamination of lettuce leaves inoculated with *Salmonella*. Moreover, the presence of the bacteria in water after the studied decontamination processes was evaluated. Reduction levels on the surface of the fresh lettuce and the presence of *Salmonella* in water were used to calculate the total inactivation efficacy of the different decontamination set-ups. The physicochemical characterization (i.e. particle size, z-potential, pH) of the EOS/water nanoemulsions generated by the different ultrasound set-ups was also determined while the damage of lettuce leaves treated by ultrasound was analysed by the electrolyte leakage rate.

2. Materials and methods

2.1. Bacterial strain and inoculum preparation

Salmonella enterica, serotype Abony 6017 was obtained from the National Collection of Types Cultures (Health Protection Agency, Salisbury, England) in a lyophilised form. The bacterial cultures were prepared in beads and kept in vials in a freezer at −70 °C. A bead was taken from the vial and streaked on Tryptic Soya Agar (TSA) (Oxoid, UK) plates, which were incubated for 24 ± 2 h at 37 °C, in order to obtain single colonies. Thereafter, the plates were stored at 4 °C for a maximum of one month. A single colony from the stock culture was picked with a loop under sterile conditions and transferring to 9 mL of Tryptic Soya Broth without dextrose (Scharlau, Spain) (TSB-D) and then incubated at 37 °C for 24 ± 2 h. A subculture was prepared by transferring 10 μL of the initial culture to 100 mL TSB-D, incubated overnight at 37 °C allowing the bacteria to reach stationary phase (10^8-10^9 log CFU/mL). In order to obtain the final inoculum, 5 mL of the suspension was centrifuged (6400×*g*) for 20 min (Benchtop Centrifuge 2–16P, Sartorious, Goettingen, Germany) and washed with Ringer's solution (Scharlau, Sentmenat, Spain) twice, resulting in a concentration of 10^6 CFU/mL.

2.2. Preparation of the lettuce

Fresh Romain lettuces (*Lactuca sativa* L. var. longifolia) were purchased from a local supplier and kept in refrigeration conditions at 4 °C. For the experiments, damaged outer leaves of the lettuce were removed. Medium and inner leaves were washed with tap water for 30–60 s to eliminate any presence of soil or other material, and then they were gently dried with absorbing paper to drain the water excess. Lettuce leaves were cut using a scalpel into pieces of 4 × 4 cm and placed under a UV lamp (λ = 253.7 nm) in a laminar flow cabinet (Microflow Peroxide Class II, Bioquell, Andover, England) for 15 min each side in order to eliminate the lettuce's natural flora.

2.3. Challenge tests

100 μL of the fresh inoculum of *Salmonella* was inoculated on one side of the lettuce samples through spot inoculation. Inoculated samples were dried for 1 h in a laminar flow cabinet in order to allow bacterial attachment. After 1 h samples were turned over and the same bacterial attachment protocol described above was repeated on the other side. Experimental samples consisted of 6 pieces of lettuce inoculated on both sides, giving a total inoculated area of 192 cm^2.

After inoculation, samples were placed in an empty sterilized beaker, together with 500 mL of sterilized distilled water. Ultrasound treatments were performed with an ultrasonic system (UP 200ST, Hielscher Ultrasonic, Teltow, Germany) operating at 26 kHz, 90 μm, 200 W attached with a probe of 14 mm Ø which was submerged (3 cm) into the water with the samples. The same processing time (300 s) was applied through continuous or pulsed mode. Three different set-ups were applied for the pulsed mode (i) 10 s on/6 s off, (ii) 5 s on/5 s off and (iii) 2 s on/8 s off. Therefore, the total treatment time was 5 min for continuous, 8 min for 10 s on/6 s off (300 s on (30 pulsed 'on' of 10 s)/180 s off (30 pulsed 'off' of 6 s), 10 min for 5 s on/5 s off (300 s on (60 pulsed 'on' of 5 s)/300 s off (60 pulsed 'off' of 5 s) and 25 min for 2 s on/8 s off (300 s on (150 pulsed 'on' of 2 s)/1500 s off (150 pulsed 'off' of 8 s)). Control samples were immersed in 500 mL of sterile dissolved water and kept unsonicated for the different treatment times corresponding to the same treatment applied to the ultrasound treated samples (5, 8, 10 and 25 min). The maximum temperature achieved for the different ultrasound treatments was below 45 °C in order to avoid any bacterial heat stress.

Additionally, continuous ultrasound and 2 s on/8 s off of the ultrasound pulsed mode were combined with several concentrations (0.010%, 0.014%, 0.018%, 0.022% and 0.025% v/v) of EOO (Ecopharm Hellas, Thessaloniki, Greece) or EOT (BioAroma, Crete, Greece). Furthermore, control of EOO and EOT were performed at the concentrations described previously for either 5 or 25 min. According to the manufacturer the chemical composition of the main components of the EOO were carvacrol (75–85% v/v) and