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Decontamination of alfalfa and mung bean sprouts by ultrasound and aqueous chlorine dioxide

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ABSTRACT

The antimicrobial effect of ultrasound, aqueous chlorine dioxide (ClO_2) and the combination of both treatments was assessed on *Escherchia coli* and *Salmonella enteritidis* inoculated on alfalfa and mung bean sprouts were performed for 5 min. This work presents the first approach regarding the using of the previously mentioned technologies on the decontamination of inoculated sprouts. Ultrasound (26 kHz, 90 μm, 200 W) process was able to reduce by 1.40 ± 0.40 and 1.89 ± 0.51 log CFU/g *Salmonella* and by 1.06 ± 0.32 and 1.23 ± 0.40 log CFU/g of *E. coli* on alfalfa and mung bean sprouts, respectively. ClO_2 (3 ppm) reduced the population of the studied bacteria in similar levels (≈1.5 log CFU/g) both in alfalfa and mung bean sprouts. However, ClO_2 treatment was more effective than the ultrasound process in terms of *E. coli* and *Salmonella* total inactivation (i.e., water and sprouts) both in alfalfa and mung bean sprouts. Additionally, an additive effect was observed in the reduction of both *E. coli* and *Salmonella* and the total inactivation when ultrasound was combined with ClO_2 in all tested food products.

1. Introduction

Sprouting is the process of germination of sprouts seeds for consumption. The sprouting process where the seed is located in a warm and moist environment, is an ideal environment for the growth of some pathogens such as *Escherichia coli* or *Salmonella* (Erdozain, Allen, Morley, & Powell, 2013). Since these products are consumed raw in order to retain all their nutritional value, they can be potential health hazards for consumer. In fact, numerous outbreaks caused by several pathogens (i.e., *E. coli* and *Salmonella*) due to the consumption of these products have been reported the past years (Erdozain et al., 2013). The outbreak that occurred in Germany in 2011 when mung bean sprouts were contaminated with *E. coli* O104:H4 and caused around 4000 hospitalizations and 47 deaths (EFSA, 2011).

Different decontamination technologies have been applied on sprout seeds to control the microbial contaminants. These include the application of ozonated water (Sharma, Demirci, Beuchat, & Fett, 2002), pulsed ultraviolet light (Sharma & Demirci, 2003), gamma radiation (Thayer, Rajkowski, Boyd, Cooke, & Soroka, 2003), supercritical carbon dioxide (Mazzoni, Sharma, Demirci, & Ziegler, 2001), high hydrostatic pressure (Ariefdjohan et al., 2004) or ultrasound (Scouten & Beuchat, 2002). The ultrasound process is a non-thermal technology able to inactivate bacteria present on food products by cavitation (generation of micro-areas of high temperature and pressure) and sonolysis (formation of free radicals) (Millan-Sango, McElhatton, & Valdramidis, 2015). In order to enhance its antimicrobial efficacy, ultrasound can be combined with other decontamination technologies (Sango, Abela, McElhatton, & Valdramidis, 2014). Some studies have suggested that ClO_2 can be an effective alternative to chlorine (Owusu-Yaw, Toth, Wheeler, & Wei, 1990) due to its antioxidant capacity which has been reported to be 2.5 times greater than chlorine (Benarde, Snow, Olivieri, & Davidson, 1967). Besides, ClO_2 has been classified as a non-carcinogenic product by several international agencies (Lopez-Galvez, Gil, Truchado, Selma & Allende, 2010).

The antimicrobial activity of ClO_2 is related to the disruption of the bacteria membrane, causing loss of permeability (Berg, Roberts, & Matin, 1986), protein synthesis (Benarde, Israel, Olivieri, & Granstrom, 1965) and damage the DNA (Alvarez & O'Brien, 1982). A study carried out by Jin and Lee (2007) reported a reduction of 3.0 and 1.5 log CFU/g for *S.* Typhimurium and L. *monocytogenes*, respectively, on mung bean sprouts when they were

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decontaminated with 100 ppm of aqueous ClO_2 for 5 min. However, some international legislation (e.g., USA) allows a maximum concentration of 3 ppm of aqueous ClO_2 for contact with whole produce (FDA, 2015). In EU there are no clear regulations regarding the use of aqueous ClO_2 for fresh produce washing (López-Gálvez et al., 2010) as each individual member state can establish enforcement levels at the national level until a risk management assessment takes place based on European Food Safety Authority (EFSA) scientific opinion and monitoring data (Banach, Sampers.I, Van Haute, & van der Fels-Klerx, 2015).

Previous studies have reported the potential antimicrobial effect of the combination of ultrasound and ClO_2 on fresh produce. However, these studies either have been focused on inactivation of natural microflora (Chen & Zhu, 2011) or the concentration of ClO_2 tested have been over the limits (i.e., 5, 10, 20 and 40 ppm) of FDA regulation (Huang et al., 2006). Additionally, to the knowledge of the authors none of these studies have assessed the effectiveness of these (combined) technologies on the decontamination of sprouts.

Therefore, the aim of this study is to determine the efficacy of three different decontamination processes (i.e., ClO_2, ultrasound and their combination) for the reduction and total inactivation (referring to the processed product and the water menstruum) of pathogens (i.e., *E. coli* and *Salmonella*) on inoculated mung bean sprouts *(Vigna radiata)* and alfalfa sprouts (*Medicago sativa).*

2. Materials and methods

2.1. Bacterial strain and inoculum preparation

Salmonella enterica, serotype Abony 6017 and *Escherichia coli* NCTC 12,900 were obtained from the National Collection of Types Cultures (Health Protection Agency, Salisbury, England) in lyophilized form in a glass vials. The strain NCTC 12,900 of *E. coli* O157:H7 is non-pathogenic and devoid of the ability to produce verotoxins. However, it possesses similar phenotypic characteristics with the toxigenic strain of *E. coli* O157:H7. The bacteria cultures were prepared in beads and kept in vials at − 70 °C. The stock cultures were re-activated by inoculation onto Tryptic Soya Agar (TSA) (Oxoid, UK) plates, which were incubated for 24 ± 2 h at 37 °C, in order to obtain single colonies that were stored at 4 °C for a maximum of a month.

The inoculum was prepared selecting a single colony from the stock culture and incubating it in Tryptic Soya Broth without dextrose (Scharlau, Spain) (TSB-D) at 37 °C for 24 ± 2 h. A subculture was also prepared in TSB-D at the same temperature and for 17 h allowing the bacteria to reach the stationary phase (10^8–10^9 CFU/mL). After incubation, 5 mL of the suspension was centrifuged (6400×*g*) for 20 min (Benchtop Centrifuge 2–16P, Sartorious, Goettingen, Germany) and washed with 10 mL of Ringer's solution (Scharlau, Sentmenat, Spain), Final preparation was resuspended in 10 mL of Ringer's solution, resulting in a concentration of 10^6 CFU/mL.

2.2. Sprouting

In order to obtain raw alfalfa and mung bean sprouts, a sprouter (Bavicchi, Italy) and sprouting seeds (Bavicchi, Italy) were purchased. Sprouting process was carried out following these steps: seeds were soaked into tap water overnight and rinsed with tap water before they were placed on a sprouter on a single layer. The seeds were regularly watered until they grew to a large enough size to be harvested from the sprout container. Then, the sprouts were well rinsed in water for a period of 2–3 min in order to remove the seed husks. The seeds were placed in a plastic container (15 × 10 × 10 cm) and kept at refrigeration temperature.

2.3. Bacteria attachment

Samples (10 g) of alfalfa and mung bean sprouts were immersed in a ethanol (30% v/v) solution for 15 min in order to reduce the background microflora. The corresponding sprouts were gently dried with paper and transferred to a biosafety cabinet for 30 min in order to allow a completely dried out. A dip inoculation procedure was carried out in a biosafety cabinet. Samples were immersed into a 300 mL of sterile distilled water which contained 10 mL of the previously prepared *E. coli* or *Salmonella* inoculum. Samples were introduced into the microbial solution for 15 min. The samples were then recovered from the solution through a sterilize strainer and were kept on the strainer for 1 h in biosafety cabinet to allow the bacterial attachment on the produce prior to any decontamination process.

2.4. Decontamination treatments

Inoculated sprouts (10 g) were treated by different decontamination processes. Additionally, uninoculated controls (samples immersed in sterilize distilled water (500 mL)) were also assessed. Ultrasound treatment was applied by an ultrasonic system (UP 200ST, Hielscher Ultrasonic, Germany) operating at 26 kHz, 90 μm, 200 W which was attached with a probe of 14 mm Ø that was submerged (3 cm) into the 500 mL of sterilized distilled water with the samples (conditions were chosen based on previous studies assessing the efficacy of ultrasound on fresh produce) (Millan-Sango et al., 2015; Millan-Sango et al., 2016). For ClO_2 treatment, 500 mL of an aqueous ClO_2 solution (3 ppm) was prepared from the stock solution. The stock solution of ClO_2 (Accepta, UK) was prepared by mixing two components (A-Sodium chlorite and B-Sodium hydrogen sulphate) according to the manufacturer protocol in 500 mL of sterilize distilled water. The final concentration of the stock solution was 3000 ppm. Dilutions were performed in order to achieve a 3 ppm concentration of ClO_2. Same ultrasound configuration was applied on samples simultaneously with the combination of aqueous ClO_2 solution at 3 ppm. Control and decontamination treatments (i.e., ultrasound, ClO_2 and the combination of ultrasound and ClO_2) were run for 5 min. Immediately after the decontamination process, treated and control samples were transferred by using a sterile tweezers into a stomacher bag under sterile conditions for further analysis.

2.5. Microbial analysis

50 ml of Ringer's solution was added to stomacher bags containing the samples and the mixture was homogenized for 2 min in a stomacher device (BagMixer 400P, Interscience, France). The microbial load of the washed water after all experiments was determined. Serial dilutions of homogenized sprouts samples and washed water were performed and the appropriate dilutions were spread plated onto Xylose Lysine Deoxycholate agar (XLD) (Roth, Germany) for *Salmonella* and Sorbitol McConkey Agar (Biolife, Italy) supplemented with cefixime and tellurite (SMAN-CT) (Abtek Biologicals, UK) for *E. coli* O157:H7 enumeration. Samples were incubated at 37 °C for 24 h. Low microbial population counts were assessed by plating 1 mL of the sample over three corresponding agar plates according to ISO 7218:2007.

Total inactivation levels of the different studied decontamination technologies and the control samples were determined. For this purpose, the survival population on sprouts and in washed water was calculated. The obtained value was subtracted from the initial population inoculated on the sprouts, resulting in the total inactivation level.