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Synergistic effect of sonication and high osmotic pressure enhances membrane damage and viability loss of *Salmonella* in orange juice

E. Wong [a], F. Vaillant-Barka [b,*], E. Chaves-Olarte [c]

[a] Centro Nacional de Ciencia y Tecnología de Alimentos, Universidad de Costa Rica, San José, Costa Rica
[b] UMR QUALISUD, Centre International de Recherche Agronomique pour le Développement (CIRAD), Avenue Agropolis, TA50/PS4, 34398 Montpellier Cedex 5, France
[c] Centro de Investigación en Enfermedades Tropicales, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

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ABSTRACT

The efficacy of using sonication (50 ± 0.2 W, 20 kHz), combined with subsequent concentration and storage at high osmotic pressure, has been evaluated to reduce levels of *Salmonella* bacteria in different solutions (PBS, sucrose and orange juice) at varying concentrations. To visualize the impact on cell membranes, we used a staining protocol (propidium iodide [PI] and 4′,6′-diamidino-2-phenylindole [DAPI]). Sonication alone did not cause significant membrane damage. Storage alone, for 48 h and at high osmotic pressure (10.9 MPa), affected membrane permeability in 20% of cells. However, sonication, combined with storage, considerably increased loss of membrane integrity, resulting in a significant logarithmic reduction of microorganisms. When the combination was applied to contaminated orange juice, a 5 $\log_{10}$ cfu ml^{-1} reduction of *Salmonella* spp. was obtained. "Osmosonication"—the synergistic combination of sonication and subsequent storage at high osmotic pressure—is an innovative alternative for the non-thermal decontamination of liquid foods.

1. Introduction

Of all fruits, orange is the most processed by the beverage industry worldwide (Tiwari, Muthukumarappan, O'Donnel & Cullen, 2008), with more than 50% of juices in international commerce corresponding to citrus fruits (Varnam & Sutherland, 1999). Probably because of the reduced time spent in preparing food in modern households, the consumption of fresh citrus in recent years has decreased while the consumption of processed juice has increased (Ros-Chumillas, Belissario, Iguaz, & López, 2007). Meanwhile, consumers still seek a healthy life style and demand ever more natural products that are minimally processed.

Unfortunately, as the consumption of minimally processed fruit juice increased so has the number of outbreaks of foodborne illnesses (CDC, 2000). Minimally processed orange juice has frequently been identified as the source of pathogenic bacteria in several of these outbreaks, most related to *Salmonella* species (Birkhead, Morse, & Levine, 1993; CDC, 1999; Cook & Dobbs, 1998; Duncan, Doull, Millar, & Bancroft, 1946; Eisenstein, Aach, Jacobson, & Goldman, 1963; Krause, Terzagian, & Hammond, 2001; Singh, Kulshreshtha, & Kapoor, 1996; Tabershaw, Schmelzer, & Bruyn, 1967).

The fruit juice industry therefore faces the challenge of developing and using alternative minimal processes that guarantee food safety and food quality and freshness. These quality aspects are crucial for orange juice mainly consumed for its contribution to the daily intake of essential vitamins that must be preserved during processing (Meléndez-Martínez, Escudero-Gilete, Vicario, & Heredia, 2010; Penicaud, Peyron, Bohuon, Gontard, & Guillard, 2010). Consequently, agro-industries are increasingly interested in innovative non-thermal processing alternatives. The main requirement for such alternatives is that they reduce the pathogen load in food to a minimum of 5 $\log_{10}$, as recommended by the U.S. Food and Drug Administration (FDA, 2001).

One potential alternative—sonication—is an emerging technology that has already been used in combination with other processing steps to reduce microbial loads in food products (Levandowsky, 1981; Piyasena, Mohareb, & McKellar, 2003). Combined with heat (thermosonication), pressure (manosonication) or both (thermomanosonication), sonication acts synergistically to reduce the time, temperature and pressure required in processing (Lee, Zhou, Feng, & Martin, 2009; Pagan, Manas, Alvarez, & Condon, 1999; Walkling-Ribeiro, Noci, Cronin, Lyng, & Morgan, 2009; Wu, Gamage, Vilkhu, Simons, & Mawson, 2008). Sonication appears to weaken microbial membranes through cavitation induced by ultrasonic shock waves (Butz & Tauscher, 2002), thereby making microorganisms more vulnerable to external stresses (Levandowsky, 1981; Pagan, Manas, Raso, & Condon, 1999; Piyasena et al., 2003; Ulusoy, Colak, & Hampikyan, 2007).

* Corresponding author. Tel.: +33 4 67 61 55 19; fax: +33 4 67 61 55 15.
E-mail addresses: eric.wong@ucr.ac.cr (E. Wong), fabrice.vaillant@cirad.fr (F. Vaillant-Barka), esteban.chaves@ucr.ac.cr (E. Chaves-Olarte).

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High osmotic pressure also affects bacterial survival (Bayer, 1967; Del Valle, 1983; Levandowsky, 1981; Mille, Beney, & Gervais, 2005; Poirier, Marechal, Evrard, & Gervais, 1998; Walter, Morris, & Kell, 1987). Abrupt changes of osmotic pressure in the medium are well known to affect bacterial survival, whether through osmotic shock (immediate effect) or long exposure to high osmotic pressure (Bayer, 1967; Mille, Beney, & Gervais, 2002; Poirier et al., 1998). High osmotic pressure causes metabolic perturbation, presumably by inhibiting the glucose phosphotransferase system through which microorganisms take up glucose (Walter et al., 1987).

However, in natural juices, neither sonication nor high osmotic pressure alone sufficiently reduces the presence of microorganisms. A previous study that introduced, for the first time, osmosonication (Wong, Pérez, & Vaillant, 2008) showed that orange juice stored at high osmotic pressure (12.6 MPa) for 7 days at −18 °C reduced microorganism levels by only 2.68 $\log_{10}$ cfu ml^{-1}. But when it was combined with batch sonication (15 to 60 min, 46 kHz) before storage, the target of 5 $\log_{10}$ of *Salmonella* spp. was met in less than 3 days. The combination of both hurdle technologies may therefore induce a synergistic effect that reduces bacterial counts. However, more knowledge about the mechanism that induces such reductions is needed even though membrane damage is strongly suspected.

This research aimed at evaluating the extent to which in-line sonication, followed by concentration and storage at high osmotic pressure, would affect membrane integrity and reduce the presence of *Salmonella* spp. in different solutions including orange juice.

2. Materials and methods

2.1. Solutions and orange juice

Solutions used for *Salmonella typhimurium* viability assays included sterilized PBS (Phosphate buffered saline) (pH = 7.2) and two sucrose solutions at 115 (pH = 7.1) and 650 (pH = 6.4) g TSS kg^{-1}, prepared with commercial sugar cane sucrose and sterilized water. The liquid food comprised commercial, pasteurized, frozen, concentrated orange juice at 650 g TSS kg^{-1} (pH = 3.4) (FCOJ; TICOFRUT S.A., San Carlos, Costa Rica), which was stored at −20 °C. The orange juice was diluted when required to 115 g TSS kg^{-1} (pH = 3.9) with sterilized water under aseptic conditions. Osmotic pressure for samples of the solutions and orange juice was estimated according to the following equation:

$$\Pi \approx \frac{-RT \ln a_w}{V_w}$$

where,

Π	is the osmotic pressure expressed in MPa
R	is the universal gas constant (8.3144×10^{-6} MPa m^3 mol^{-1} k^{-1})
T	is absolute temperature (°K)
a_w	is water activity
V_w	is the partial molar volume of water (m^3 mol^{-1}) in solution, calculated from the density value of solutions

2.2. Microbial strains and culture and/or inoculum preparation

For the viability assays, a pure culture of *S.* Typhimurium strain ATCC 14028 was used. For the logarithmic reduction experiments, a mixed culture of *Salmonella* spp. was used, prepared by combining equal parts of pure cultures of *S.* Typhimurium strain ATCC 14028, *S.* Typhi strain ATCC 6539 and *S.* Enteritidis strain ATCC 13076 (American Type Culture Collection, Manassas, VA, USA). These strains were available in our laboratory and were used to account for variability among *Salmonella* strains and to better represent the *Salmonella* group. The three strains were grown from frozen stocks (brain heart-infusion broth, containing 15% glycerol; Oxoid Ltd, Basingstoke, UK) and cultured onto xylose lysine deoxycholate agar (XLD, Oxoid). Each strain was then aseptically transferred into 250 ml of sterilized trypticase soy broth (TSB; Oxoid) and incubated for either 12 h (viability assays) or 18 h (logarithmic reduction experiments) at 35 °C in an Orbit Shaker Waterbath (model 3540; Lab-Line Instruments, Inc., Melrose Park, IL, USA). A 12 h culture provides cells in logarithmic growth where none or very few dead cells are expected. An 18 h culture provides more mature cells in stationary phase, that can better survive different applied stress conditions, however, a proportion of dead cells is present. When required, the starting bacterial concentration was determined by serial dilutions in 0.1% peptone solution (Scharlau Chemie S.A., Sentmenat-Barcelona, Spain), plated on XLD and incubated for 24–48 h at 35 °C. Typical colonies were then counted and the results expressed as $\log_{10}$ cfu ml^{-1}.

2.3. Fluorescent dyes and viability assays

Viability assays were conducted using a pure *S.* Typhimurium culture. Treatments were analyzed, using the vital stain technique for all combinations of all levels of the following three factors: solution (PBS, sucrose at 115 g TSS kg^{-1} or sucrose at 650 g TSS kg^{-1}), sonication time (0 or 15 min) and storage time (0 or 48 h). 1-ml samples of the 12 h incubated culture (non-sonicated or sonicated) were centrifuged at 7700 × *g* for 3 min, using a 16 K microcentrifuge (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to obtain a pellet, which was resuspended in either of the three solutions. Half of the samples were immediately stained, and the rest were stained after 48 h of storage at 4 °C. This temperature was used to avoid freezing which can cause cell disruption. Also, preliminary tests showed that centrifugation at 7700 × *g*, part of a classical protocol extensively used in research with bacteria, causes no important membrane damage.

A positive control for dead cells was prepared by treating a pellet of non-sonicated culture with 1 ml of 3.5% paraformaldehyde (PF) in PBS (Merck & Co., Inc., NJ, USA) and incubating at 23 °C for 20 min. For the vital stain, 1 ml of PBS was added to all samples. These were centrifuged at 7700 × *g* for 3 min and the supernatant discarded. The pellet was resuspended in 1 ml of PBS. The stains DAPI and PI (Sigma-Aldrich Corporation, St. Louis, MO, USA) were then added simultaneously to final concentrations of 2 μg ml^{-1} and 20 μg ml^{-1}, respectively. Samples were incubated at 23 °C for 20 min and washed twice by centrifuging, discarding the supernatant and resuspending the pellet in 1 ml of PBS. Then 10 μl of the sample were put on a microscope slide, dried at 37 °C, fixed with 10 μl of Mowiol mounting medium (Merck & Co., Inc., NJ, USA) and left in the dark at 23 °C for 1 h.

Samples were observed, using an Olympus fluorescent microscope BH2-RFL coupled to a camera (model HKHO27243; Olympus Optical Co. Ltd, Japan). Cell counts were performed directly under the microscope for fixed focused regions in both DAPI and PI channels at 100×. Microphotographs were taken at 40×. Percentage of dead cells was calculated in the same focused region according to the number of PI-stained cells (red colour) and of DAPI-stained cells (blue colour).

2.4. Determining the absence and/or presence of Salmonella spp. or their logarithmic reductions

Samples of 250 ml of orange juice (115 g TSS kg^{-1}) were each inoculated with *Salmonella* spp. to obtain final concentrations of 6–7 $\log_{10}$ cfu ml^{-1}. The inoculum was evenly distributed, using a homogenizer (IKA® Works, Inc., Malaysia). Initial counts were calculated, taking into account inoculum concentration, inoculum volume and final sample volume. Final counts of treated samples were