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Evaluation of resistance development and viability recovery by toxigenic and non-toxigenic *Staphylococcus aureus* strains after repeated cycles of high hydrostatic pressure

Inês Baptista [a, b], Rui P. Queirós [b], Ângela Cunha [a], Sílvia M. Rocha [b], Jorge A. Saraiva [b], Adelaide Almeida [a, *]

[a] *Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal*
[b] *Department of Chemistry and QOPNA, University of Aveiro, 3810-193 Aveiro, Portugal*

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A B S T R A C T

In this work, the development of resistance and the recovery of growth after several consecutive cycles of high hydrostatic pressure (HPP) were for the first time evaluated in different strains of *Staphylococcus aureus*. Three strains of this important and highly resilient to HPP foodborne pathogen were used: a non-enterotoxigenic ATCC 6538 strain, treated with 600 MPa for 30 min at 20 °C, and the toxigenic strains 2153 MA (with enterotoxin A) and 2065 MA (with the enterotoxins A, G and I), treated with 600 MPa for 15 min at 20 °C. After the first treatment, surviving colonies were used to produce new bacterial cultures. This procedure was repeated nine times more for each bacterium or until total inactivation occurred. The inactivation profile of non-enterotoxic strain and the two enterotoxic strains did not change after consecutive cycles, but the toxic strain with three enterotoxins was completely inactivated after the fourth cycle. The three strains did not recover their viability after 14 days. The results indicate that HPP effectively inactivates non-toxigenic and toxigenic strains of *S. aureus* after a single treatment. The surviving bacteria did not develop resistance after 10 cycles of pressurization and did not recover their viability after 14 days of incubation.

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1. Introduction

High pressure processing (HPP) treatment is an emerging food preservation method able to meet not only the increasing consumer demand for microbiologically safe foods, but also to produce foods with fresh-like appearance and with minimal modification of nutritional and organoleptic properties (Daryaei et al., 2007; Neetoo et al., 2011; Pulido et al., 2008; Sharma et al., 2002; Syed et al., 2012). HPP treatments of food are carried out with intense pressure (commercially ranging from 100 to 600 MPa) with or without heat, inactivating efficiently microorganisms and, consequently, extending food products shelf-life (Campus, 2010; Hugas et al., 2002). The susceptibility of microorganisms to high hydrostatic pressure varies considerably depending on the pressure range applied, temperature and duration of the treatment (Considine et al., 2008; Fonberg-Broczek et al., 2005; Karatzas and Bennik, 2002; Rodriguez et al., 2005), but also depends on each microorganism characteristics, growth phase and suspending medium (Alpas et al., 1999; Jofré et al., 2010; Pulido et al., 2008; Rodriguez et al., 2005). It is generally assumed that Gram-positive bacteria and cells in the stationary growth phase are more resistant than Gram-negative and cells in the exponential growth phase (Campus, 2010). It has also been stated that bacterial protective effect against HPP may be given by carbohydrates, proteins, lipids and other constituents of the suspending medium or food (which, when caught up by the bacteria decrease their sensitivity to HPP) (Garriga et al., 2004; Pulido et al., 2008; Van Opstal et al., 2005).

HPP affects bacterial cellular processes such as protein and DNA synthesis, membrane-associated processes, macromolecular quaternary structures (e.g., protein denaturation) and cellular membrane structure. Cell external structures are the primary sites that are damaged by pressure, altering cell permeability, transport systems, osmotic pressure and ability to preserve pH, resulting in leakage of cell contents (Bowman et al., 2008; Hauben et al., 1997; Karatzas and Bennik, 2002; Pulido et al., 2008; Ritz et al., 2002; Yang et al., 2012). As the main targets of HPP are the external

* Corresponding author.
E-mail addresses: mines.baptista@ua.pt (I. Baptista), rui.queiros@ua.pt (R.P. Queirós), acunha@ua.pt (Â. Cunha), smrocha@ua.pt (S.M. Rocha), jorgesaraiva@ua.pt (J.A. Saraiva), aalmeida@ua.pt (A. Almeida).

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structures, microorganisms seem to have low chance to develop resistance. However, it has been shown that some cells can repair the sub-lethal damage induced by HPP, allowing them to proliferate once they have restored the injury. Injury recovery of Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* O157:H7 933 and *Salmonella enteritidis* FDA) foodborne pathogens in HPP treated milk during storage was observed (Bozoglu et al., 2004). Recovery of *E. coli* B in phosphate saline buffer after treatment with HPP was also reported (Koseki and Yamamoto, 2006). Nevertheless, the bacterial development of resistance and the growth recover after several cycles of HPP have not yet been evaluated.

The aim of this study was to evaluate if toxigenic and non-toxigenic *S. aureus* strains that survived HPP treatment can develop resistance to repeated cycles and recover their viability. *S. aureus* was selected as a bacterial model because this bacterium is an opportunistic human pathogen that causes a broad spectrum of infections (Bore et al., 2007), being the fourth most common causal agent in bacterial food poisoning outbreaks (Clarisse et al., 2013; EFSA, 2010). *S. aureus* has no particular nutritional and environmental requirement for growth and has also the ability to grow in a vast range of temperature, pH and NaCl concentration (Dengremont and Membre, 1995; Normanno et al., 2005; Medveďová and Valík, 2012). Besides, some strains produce heat-stable enterotoxins that are powerful gastrointestinal toxins, causing frequently severe disease (Bien et al., 2011; Bore et al., 2007; Kérouanton et al., 2007; Ortega et al., 2012; Paulin et al., 2012).

2. Materials and methods

2.1. Preparation of bacterial cultures

Three *S. aureus* strains were used in this study: one non-enterotoxic strain (ATCC 6538) and two enterotoxic strains isolated from food (2153 MA, with enterotoxin A, and 2065 MA, with enterotoxins A, G, I). *S. aureus* cultures were grown in Brain–Heart Infusion (BHI, LIOFILCHEM, Italy) at 37 °C for 22 h at 170 rpm to achieve a concentration of approximately 10^8–10^9 colony forming units mL^{-1} (CFU mL^{-1}) reaching the stationary phase. Cells were harvested by centrifugation (15,000 × *g* 10 min), washed twice and resuspended in the same volume of sterile phosphate buffer solution (PBS) (pH 7.0) in order to achieve concentrations of 10^8–10^9 CFU mL^{-1}.

2.2. HPP treatments

Prior to HPP treatments, cell suspensions were transferred with sterile glass Pasteur pipettes to 0.4 mL polyethylene tubes. For each strain, three independent samples were used and for each one, three sub-samples were prepared ($n = 9$). The tubes containing each of the 3 sub-replicates set were placed in low permeability polyamide–polyethylene bags (PA/PE-90, Albipack – Packaging Solutions, Portugal) sterilized distilled water which were then thermo-sealed and pressurized. According to the results of preliminary assays (Baptista et al., unpublished data) for the establishment of the pressurization conditions required to achieve a bacterial inactivation corresponding to *ca*. 5 to 6 log reductions (from ~9 initial logs), ATCC 6538 strain was pressurized at 600 MPa for 30 min at 20 °C and the strains 2065 MA and 2153 MA were pressurized at 600 MPa for 15 min at 20 °C in a hydrostatic press (High pressure system U33, Institute of High Pressure Physics, Warsaw, Poland). The pressurization liquid was a mixture of 60% water and 40% propylene glycol (DOWCAL™, Dow). With such a procedure, it was intended to obtain a sizeable number of colonies, which were used to study the possible development of resistance and bacterial recovery. Non-pressurized controls were also included in the experiments.

2.3. Enumeration of viable cells

For each sub-sample treated and for untreated samples 10-fold serial dilutions (10^{-1}–10^{-8}) were made in sterile PBS. One milliliter of each dilution was plated on plate count agar medium (PCA, LIOFILCHEM, Italy) in duplicate. The plates were incubated at 37 °C for 48 h and the number of colonies was counted for each sample. The number of survivors was reported as log CFU mL^{-1}. The reduction efficiency (RE) of high pressure inactivation of the pathogen strains was calculated using the equation RE = log N_0 − log N, where N_0 represents the average number of viable cells in the untreated suspensions and N the number of viable cells in the pressurized suspensions.

2.4. HPP resistance assays

To verify the development of resistance to HPP, a new set of toxigenic and non-toxigenic bacterial cultures was produced from an isolated colony obtained after each cycle of exposure to HPP treatment. In order to obtain a bacterial inactivation corresponding to *ca*. 5 log of reductions, the bacterial suspension was exposed to HPP in the same conditions of the aforementioned HPP assay. This allowed to test if the bacteria affected by HPP were able to develop resistance to HPP. After each cycle, survivor colonies were removed from PCA and incubated in BHI at 37 °C for 22 h at 170 rpm. Non-pressurized controls were also included in the experiments. This procedure was repeated for ten consecutive cycles. For each strain, and for each of the 10 cycles, three independent samples were used and for each sample three sub-samples were done ($n = 9$).

2.5. HPP viability recovery assay

In order to evaluate if pressurized cells could recover viability after HPP treatments, toxigenic and non-toxigenic bacterial suspensions were subjected to HPP in the conditions described above. After the initial enumeration (48 h), the plates used to count viable cells were incubated for 14 days at 37 °C and the colonies were recounted after 5, 8, 11 and 14 days of incubation. This counting strategy was used after each pressurization cycle and the concentration of viable bacteria was determined. For each strain, three independent samples were counted, each one with three sub-samples ($n = 9$).

2.6. Statistical analysis

HPP inactivation data from the resistance and recovery assays were statistically analyzed using analysis of variance (ANOVA) and the post-hoc Tukey test, with the SPSS 20.0 (IBM, New York, USA). Statistical significance was considered for $p < 0.05$.

3. Results

3.1. HPP resistance assays

The application of HPP treatment produced reductions in the counts of the three strains, being the magnitude dependent on each strain under study ($p < 0.05$). In each cycle, no significant differences among the 3 independent samples (ANOVA, $p > 0.05$) was observed for the three strains. Though, the efficiency of bacterial inactivation after the first treatment was almost the same ($p > 0.05$), the HPP treatment time was different for the non-

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