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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Synergism between high hydrostatic pressure and glutaraldehyde for the inactivation of *Staphylococcus aureus* at moderate temperature

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Abstract

The sterilization of transplant and medical devices should be effective but not detrimental to the structural properties of the materials used. In this study, we examined the effectiveness of chemical and physical agents for inactivating *Staphylococcus aureus*, a gram-positive bacterium and important cause of infections and biofilm production. The treatment conditions in this work were chosen to facilitate their subsequent use with sensitive materials. The effects of temperature, high hydrostatic pressure, and glutaraldehyde disinfectant on the growth of two strains of *S. aureus* (ATCC 25923 and BEC 9393) were investigated individually and/or in combinations. A low concentration of glutaraldehyde (0.5 mM), high hydrostatic pressure (300 MPa for 10 min), and moderate temperature (50 °C), when used in combination, significantly potentiated the inactivation of both bacterial strains by > 8 orders of magnitude. Transmission electron microscopy revealed structural damage and changes in area that correlated with the use of pressure in the presence of glutaraldehyde at room temperature in both strains. Biofilm from strain ATCC 25923 was particularly susceptible to inactivation. The conditions used here provided effective sterilization that can be applied to sensitive surgical devices and biomaterials, with negligible damage. The use of this experimental approach to investigate other pathogens could lead to the adoption of this procedure for sterilizing sensitive materials.

Keywords Biofilms · Glutaraldehyde · High hydrostatic pressure · Nosocomial infections · *Staphylococcus aureus* · Sterilization

Introduction

The continuing increase in the occurrence of antimicrobial-resistant bacteria continues to be a major health problem worldwide. In this context, biomaterial sterilization is always an important consideration, with a need to ensure the efficiency of the process and its effect on the biomaterials being sterilized prior to medical interventions (Park et al. 2012). The decontamination of medical materials is essential for the control and prevention of diseases caused by pathogenic microorganisms (Cozad and Jones 2003; Rivalain et al. 2010). Several conventional methods of cleaning and sterilization, such as gamma radiation, steam autoclaving, oxygen plasma, and ultraviolet (UV) light, can compromise the properties of biomedical implants by changing the surface properties of the material, leading to the deposition of harmful substances and the stimulation of an exacerbated cellular response (Park et al. 2012). The reuse of medical devices raises additional difficulties for sterilization, such as the presence of biofilm that may require more drastic conditions for efficient sterilization (Ntsama-Essomba et al. 1997; Rutala and Weber 2016). In view of these concerns, it is important to investigate new sterilization methods that cause minimal damage to the target materials.

Glutaraldehyde (GA) is a strong disinfectant that is commonly used in hospital settings for surface cleaning and sterilization, as well as for tissue fixation before transplantation. GA acts by cross-linking with amine, amide, and thiol groups of proteins (Takigawa and Endo 2006; Reddy et al. 2015). This

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fixation results in toxicity and sensitization of the eyes, skin, and respiratory tract that make it difficult to manage GA-induced damage (Mcdonnel and Russell 2005; Takigawa and Endo 2006). GA also leaves residues on material surfaces that can cause the calcification of implants treated using this agent (Kim et al. 1999; Yang et al. 2017).

For materials sensitive to high temperature, alternative physical and/or chemical methods of disinfection and sterilization can be used, e.g., vaporizing hydrogen peroxide, ozone, peracetic acid vapor, ionizing radiation, and light pulses (Rutala and Weber 2016). The use of high hydrostatic pressure (HHP) causes less damage to materials and therefore has important advantages for surgical materials, biopharmaceuticals, hemo-derivatives, and implants (Gollwitzer et al. 2009; Rivalain et al. 2010; Durães-Carvalho et al. 2012). HHP can be used in association with other conditions, such as moderate temperatures (up to 60 °C), for more general pathogen inactivation involving sporulated and more resistant bacteria (Naal et al. 2008; De Souza et al. 2013).

In this work, we examined the impact of sterilization processes on strains of *Staphylococcus aureus*, an important pathogen that causes a wide range of clinical infections (Tong et al. 2015). Staphylococci are non-sporulating, gram-positive facultative aerobic cocci that occur in clusters and are generally resistant to desiccation and several antibiotics; these bacteria also tolerate high salt concentration in artificial growth medium (Parfentjev and Catelli 1964). Several *S. aureus* strains can form biofilms, an important resistance barrier to external stressors such as antibiotics, the host's immune defense and the disinfection of materials by antimicrobials and biocides (Götz F. 2002; Shin et al. 2013; Zapotoczna et al. 2016). There is a correlation between strains with a higher capacity for forming biofilm and greater density of *S. aureus* (Shin et al. 2013), as well as unfavorable evolution of clinical infections (Bendouah et al. 2006). Here, we investigated the effectiveness of the inactivation of two strains of *S. aureus* in suspension and in biofilm by HHP in combination with very low concentrations of GA and moderate temperature. The results demonstrate the high efficacy of a combination of conditions used to sterilize medical-surgical supplies and biopharmaceuticals.

Materials and methods

Bacterial strains, culture conditions, and quantification

Staphylococcus aureus strains ATCC® 25923 MINIPACK™ and Brazilian epidemic clone (BEC) 9393 were kindly provided by the Laboratory of Biotechnology of the Institute of Biology at UNICAMP. The cells were initially cultured in 5 mL of tryptic soy broth (TSB; Difco-BD) at 37 °C for 24 h. The bacteria were sub-cultured by inoculation in TSB followed by incubation for 16 h, with subsequent centrifugation (Fanem® 206R centrifuge) at 4000*g* for 15 min; the resulting supernatant was discarded. Pellet bacterial cells were suspended in 0.9% (*w*/*v*) saline to achieve an estimated concentration of 10^9 cells/mL, which corresponds to an optical density of 1.5 (Beckman DU640, Beckman Instruments, CA, USA), to be used in the experiments.

Quantification of bacteria was done by serial dilution in 0.9% saline (1:10) followed by plating on TSB agar plates. Bacterial growth was expressed as colony-forming units (CFU/mL) after a 24-h incubation at 37 °C in an incubator.

Treatment at different temperatures, GA, and HHP

Bacterial suspensions were subject to different temperatures and GA conditions typically for 10 min in a water bath. Glutaraldehyde from a 25% stock solution (J.T. Baker®) was diluted to 0.21 M (2% *v*/*v*) in 0.10 M phosphate-buffered saline (PBS), pH 7.0. A bacterial suspension and biofilm in carrier material (see next section) were treated in 0.1 M Tris-HCl, pH 8.0, with different concentrations of GA up to 8 mM (Mcdonnel and Russell 2005; Sehmi et al. 2016), typically for 10 min. GA was neutralized by adding 0.4 M (3%, *v*/*v*) glycine (Sigma®) for 2 min in a 9:1 ratio of glycine solution (Cheung and Brown 1982) and subsequently quantified.

The HHP equipment and water bath supply as well as the experimental method used in this study have been described before (Silva et al. 1989; Santos et al. 2004; Bispo et al. 2007; De Souza et al. 2013). The time required to increase the pressure from atmospheric pressure to 300 MPa was 1.5 min and that required to return to atmospheric pressure was 1 min. A polyethylene bag (Polisilk®) filled with the sample was sealed at high temperature and placed in the high-pressure chamber. The samples treated with HHP, GA, and temperature were exposed to the combination of treatments for 10 min.

All results were expressed as mean values ± standard deviation of at least triplicate independent experiments. All data analyses were done using OriginPro 8 software.

Carrier materials and applications for sterilization

Previous studies (Fux et al. 2004; Wells et al. 2011) have shown that *S. aureus* ATCC 25923 strain is a biofilm producer. This strain was therefore used in experiments to examine biofilm formation on carrier materials in vitro. Sterilized contact lenses (SoftLens®, Sauflon Pharmaceuticals Ltd., Twickenham, UK) and catheters (Jiangsu Jichun Medical Devices Co. Ltd., Jiangsu Province, China) were used as carrier materials. For biofilm formation in vitro, carrier materials were incubated with the ATCC 25923 strain (10^8 CFU/mL) for 24 h at 37 °C in TSB with 1% (*w*/*v*) glucose (Marques et al. 2007; Chaieb et al. 2011). The appearance of turbidity in the medium and thick polysaccharide material on the surface of the carrier material

confirmed bacterial proliferation and biofilm formation. The carrier materials were subsequently removed, washed with sterile distilled water, and then exposed to different conditions. For HHP treatment, the experiments were done using polyethylene bags (Polisilk®), in a manner similar to the experiments with cell suspensions. The treated carrier materials were again incubated in fresh TSB for 24 h at 37 °C, with visual monitoring of turbidity. The presence of bacteria was confirmed by collecting 100 μL of the treated or untreated samples, followed by plating and incubation (24 h at 37 °C). The positive control corresponded to contaminated materials without treatment. After the treatments, the materials were transferred to new tubes containing fresh TSB under sterile conditions and bacterial growth was monitored at 37 °C for 24–48 h.

Kirby-Bauer disc diffusion method for antibiotic susceptibility

The antibiotic susceptibility of the *S. aureus* strains was assessed using the Kirby-Bauer disc diffusion method (DDM). Primary brain heart infusion (BHI) broth (Neogen-Acumedia) was prepared and *S. aureus* were allowed to grow for 12–14 h overnight at 37 °C, followed by sub-culturing in BHI broth until a turbidity of 0.5 MacFarlane units was achieved. Mueller Hinton agar (MHA) (Difco-BD) plates were prepared by dissolving 38 g of MHA in 1 L of distilled water, sterilized and cooled to 45 °C, and 20 mL of the molten agar was poured into pre-sterilized petri plates. The plates were checked for sterility by incubating them at 37 °C for 6–7 h before use. Approximately 10^6 cells of *S. aureus* were spread on the plates followed by the introduction of antibiotic discs and incubation at 37 °C for 16–18 h to allow zone development. The inhibition zones were classified into one of three categories based on the criteria of the "Clinical and Laboratory Standards Institute" (CLSI), namely, susceptible (S), intermediate (I), and resistant (R). The antibiotic concentrations were kept accordingly for the same standards of CLSI and the results were interpreted by measuring the clear inhibition zone (Alagumaruthanayagams et al. 2009).

Transmission electron microscopy

For transmission electron microscopy (TEM), treated and non-treated bacterial pellets were initially incubated for 3 h at room temperature in 1 M sodium cacodylate, pH 7.2, containing 2.5% glutaraldehyde and 1% tannic acid and centrifuged for 15 min at 7000*g*. The pellets were then washed and the samples were prepared as previously described (Durães-Carvalho et al. 2012).

Morphometric analysis

For morphometric analysis, bacterial samples that had or had not been treated with 300 MPa HHP, 0.5 mM GA at 25 °C for 10 min were subjected to TEM and five images of treated and non-treated *S. aureus* ATCC 25923 and BEC 9393 strains were selected using the same magnification (× 46,460). Fifty bacterial cells were selected from the images for measurement of the surface area using ImageJ software. Polygonal measurements of each cell were used to determine the area (Watanabe et al. 2013) and graphs were plotted using GraphPad Prism v.6 software. Statistical comparisons were done using Student's paired *t* test with $p < 0.05$ indicating significance.

Results

Effect of temperature

Figure 1 shows the inactivation patterns of *S. aureus* strains BEC 9393 and ATCC 25923 at different temperatures. The sensitivity of both strains was very similar: significant inactivation occurred at > 55 °C and total inactivation at ≥ 65 °C. There was also a significant reduction in the colony sizes of both strains after incubation for 24 h and 72 h at 55 °C compared to lower temperatures (Supplementary Fig. S1); this finding may reflect a significant phenotypic change in these experimental conditions.

Combined effect of GA and temperature

The combination effect of a very low GA concentration with temperature and HHP on bacterial inactivation was investigated. The potentiation of GA inactivation would be highly useful because the presence of residual disinfectant from cleaning and sterilization of some materials in hospitals represents a risk factor for toxicity. The GA concentrations used here

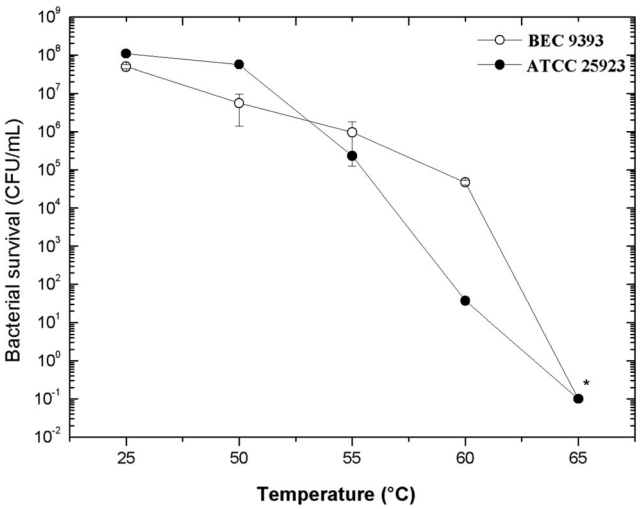

Fig. 1 Survival curves of *S. aureus* strains ATCC 25923 (*closed symbols*) and BEC 9393 (*open symbols*) after exposure to different temperatures for 10 min in the absence of GA. Asterisk: no bacteria detected. The error bars represent standard deviations ($n = 3$)

was about 100 times lower than those currently used for disinfection, which may reach up to 2% (212 mM). Figure 2 shows that *S. aureus* strains ATCC 25923 and BEC 9393 were inactivated at a GA concentration of 2 mM and 3 mM, respectively, at 25 °C. At higher temperatures, the inactivation of both strains occurred at significantly lower GA concentrations, whereas total inactivation of both strains was seen at 65 °C, even in the absence of GA (Fig. 1).

Effect of HHP and GA on *S. aureus* inactivation at different temperatures

The effect of HHP (300 MPa) on both *S. aureus* strains at different temperatures and GA concentrations (10-min exposure) is shown in Fig. 3. There was negligible inactivation by HHP at 25 °C and was not affected by increasing the length of treatment to 60 min. At moderate temperature (50 °C), HHP caused inactivation in both strains that was 4–5 orders of magnitude greater than at 25 °C (Fig. 3). At 25 °C, GA (up to 1 mM) did not significantly inactivate either strain, but the effect of GA was significantly potentiated at moderate temperature and/or by HHP. At 50 °C and 300 MPa, 0.16 mM GA totally inactivated both strains, whereas, when tested separately, these conditions caused little or no inactivation.

Effect of GA, HHP, and moderate temperature on *S. aureus* in biofilm

Staphylococcus aureus strain ATCC 25923 was used to screen for biofilm eradication because of its ability to produce biofilm. Table 1 shows the results for the lenses and catheter fragments treated with HHP, moderate temperature, and different concentrations of GA, compared with bacterial suspensions. Overall, the biofilm did not significantly protect *S. aureus* strain ATCC 25923 against inactivation by HHP and glutaraldehyde at moderate temperatures. Supplementary Figures S2 and S3 show representative images on which Table 1 is based. Figure S2 shows that the exposure of lenses with biofilm to 0.5 mM GA and 300 MPa at 50 °C prevented bacterial growth after 24 h (tube 2 and plate 2), compared with the positive control (lens without treatment that showed turbidity; tube 1 and plate 1). Figure S3 shows the catheter fragments treated with different concentrations of GA at 50 °C and HHP, and the respective untreated control. Total inactivation was observed in catheter with the same conditions of lenses.

Antibiotic susceptibility

The disc diffusion method (DDM) was used to assess the antibiotic susceptibility of the two strains of *S. aureus*. Strain BEC 9393 was significantly resistant to most of the antibiotics tested, in contrast to strain ATCC 25923 that was not (Fig. S4). BEC 9393 was completely susceptible to vancomycin but showed intermediate resistance to tetracycline and rifampicin and complete resistance to the other tested antibiotics. ATCC 25923 strain, which is used as a quality control strain by the CLSI, showed intermediate resistance to amikacin, gentamycin, ampicillin, oxacillin, and vancomycin, and complete susceptibility to the other antibiotics.

Transmission electron microscopy

Figure 4a–d shows the morphological alterations induced by HHP in synergism with GA at room temperature. TEM

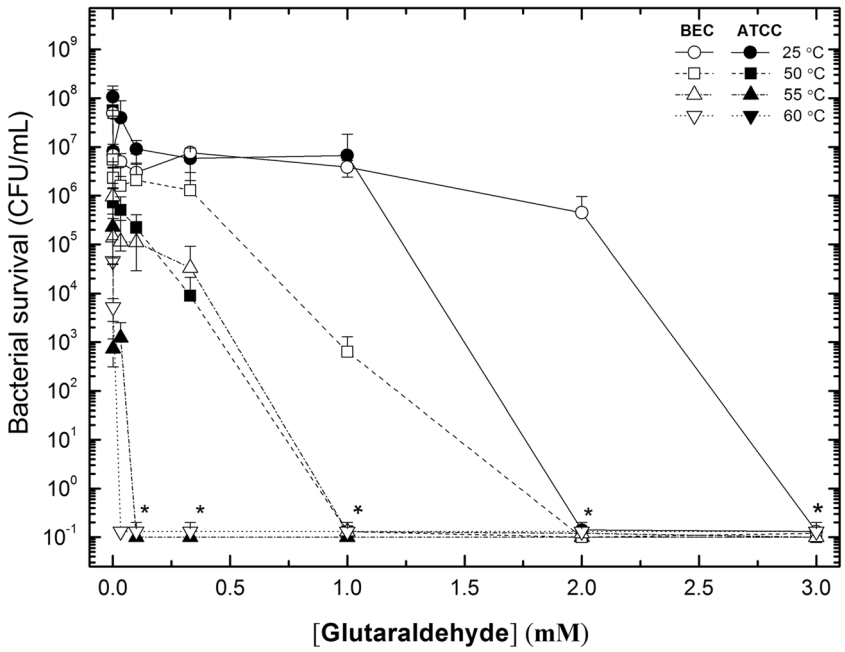

Fig. 2 Effect of GA on the inactivation of *S. aureus* strains ATCC 25923 and BEC 9393 at different temperatures (10-min exposure, pH 8.0). Asterisk: no bacteria detected. The error bars represent standard deviations (n = 3)

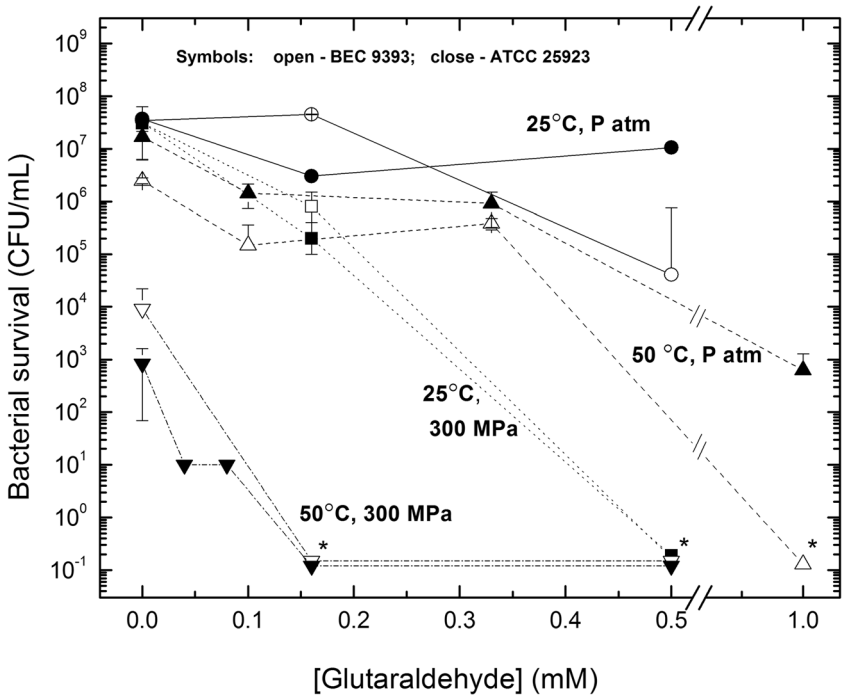

Fig. 3 Combined effect of HHP, GA, and moderate temperature (50 °C) on the inactivation of *S. aureus* strains ATCC 25923 and BEC 9393 after 10-min incubation. Asterisk: no bacteria detected. The error bars represent standard deviations ($n = 3$). P_{atm} atmospheric pressure

analysis of non-treated samples of *S. aureus* (ATCC 25923 and BEC 9393) revealed an intact cell walls and membranes with no alterations suggestive of morphological damage. In contrast, the exposure of both strains of *S. aureus* to 0.5 mM GA plus 300 MPa HHP at 25 °C for 10 min resulted in substantial cellular damage that included the disruption of cellular structures, the leakage of cytoplasmic content to the surrounding environment, disrupted cell division, intracellular vacuole formation, and a change in cell shape.

Table 1 Comparison of the inactivation of *S. aureus* strain ATCC 25923 present in biofilm in carrier materials (lens or catheter) with the inactivation of a bacterial suspension of the same strain by different concentrations of GA at moderate temperatures and an atmospheric pressure of 300 MPa

	Treatment conditions						
	50 °C, Patm	50 °C, 300 MPa (up to 24 h)		55 °C, Patm (up to 24 h)		55 °C, 300 MPa (up to 24 h)	
GA (mM)	BS	Lens	BS	Catheter	BS	Catheter	BS
8	−	N.D.	−	−	−	−	−
4	−	N.D.	−	−	−	−	−
2	−	N.D.	−	−	−	−	−
1	+	−	−	−	−	−	−
0.5	+	−	−	+	+	−	−
0.25	+	N.D.	−	+	+	−*	−
0.16	+	+	−	+	+	+	−
0.125	+	+	+	+	+	+	+
0.08	+	+	+	+	+	N.D.	N.D.
0.06	+	+	+	+	+	+	+
0.04	+	+	+	+	+	N.D.	N.D.

A positive sign for the carrier materials indicates turbidity in TSB medium after 24 h and BS assayed on TSB plates, with inactivation > 8 log CFU/mL, if negative. The responses were assessed after a 10-min exposure to the above indicated conditions. Obs: All experiments in lens and catheter with absence of growth at 24 h were monitored up to 48 h to check the sterilization

BS bacterial suspension, *Patm* atmospheric pressure, *N.D.* experiments not done

*Appearance of bacterial growth after 48 h

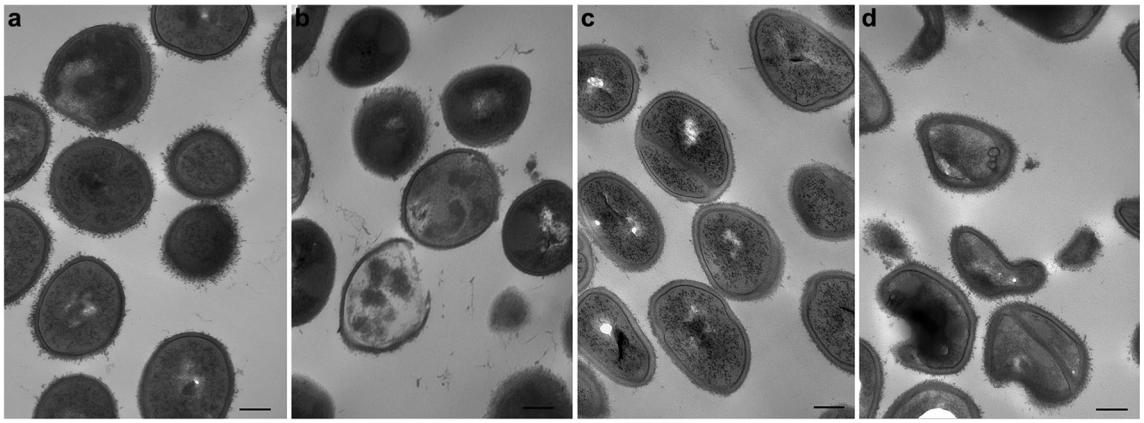

Fig. 4 TEM images of *S. aureus* ATCC 25923 (control (**a**) and pressurized (**b**) samples) and BEC 9393 (control (**c**) and pressurized (**d**) samples). The pressurization conditions for both strains were 300 MPa HHP at 25 °C for 10 min in the presence of 0.5 mM GA. The arrows indicate morphological changes on the bacteria. The *scale bars* correspond to 200 nm

Surface area measurements

Surface area measurements allowed the conversion of qualitative data to quantitative data, as well as the comparison of bacterial cells before and after treatment with 0.5 mM GA in conjunction with 300 MPa HHP at 25 °C for 10 min; this treatment combination no longer allowed bacteria to grow, even on enriched media such as TSB plates. Morphometric analysis of TEM images revealed a significant difference in the surface area of both strains of bacteria after treatment. In *S. aureus* ATCC 25923, a major decrease in area resulted from the lack of cell wall and cell membrane and the appearance of hair-like structures outside the cells (Fig. 5(a–c)). In *S. aureus* BEC 9393, the treatment produced structural modifications that ensued in bean-shaped cells caused by the release of cytoplasmic content including significant increase in surface area (Fig. 5(d–f)). The data of five images (50 bacterial cells) of treated or non-treated cells of both strains were analyzed with Student's paired *t* test and showed a significant effect of treatment ($p < 0.05$).

Discussion

The use of HHP for microorganism inactivation has been described in several systems and its application in food processing allows preservation of the molecular characteristics of a variety of products, including organoleptic properties (Heinz and Buckow 2009).

The sterilization of medical materials requires the elimination of different pathogenic microorganisms that occasionally demonstrate broad-spectrum resistance to antibiotics. At the same time, preservation of the properties of these materials is an important distress. In this work, we examined the usefulness of combinations of conditions for inactivating microorganisms. For this, we used strains of *S. aureus*, a bacterium that is often the cause of hospital-acquired infections and may show resistance to multiple antimicrobial agents (Korting et al. 1998; Sievert et al. 2013; González-Arenzana et al. 2016; Kpeli et al. 2016). *Staphylococcus aureus* is of clinical importance because it causes opportunistic infections in patients with chronic diseases, immune deficiency and those who undergo surgical interventions leading to infective endocarditis and prosthetic device infections (Tong et al. 2015), hospital-acquired pneumonia (Herkel et al. 2016), and scalded skin syndrome in neonates (Bhavsar et al. 2016).

Staphylococcus aureus strains found in medical centers often show multi-resistance to antibiotics that is an important cause of hospital-acquired infections (Poorabbas et al. 2015). The confirmation here that *S. aureus* BEC was resistant to most of the antibiotics tested in this work (Fig. S4) stresses the need for alternative methods for sterilization or bacterial inactivation since contamination by antibiotic-resistant strains can result in severe morbidity. Rochford et al. (2014) have previously shown that the proliferation and propagation of *S. aureus* on surgical material is enhanced by increasing the surface roughness of polyetheretherketone (PEEK) implants through treatment with oxygen plasma. This observation indicates the need to consider the possibility that the surface roughness of the material of interest may be influenced by the sterilization process used. Whereas treatment with HHP (300 MPa) for 10 min did not significantly affect the viability of either strain, however, synergism between a low GA concentration and an HHP of 300 MPa lead to the eradication of *S. aureus*, with a 10-min treatment being sufficient to completely inactivate the bacteria and their biofilm. Additionally, the use of 3% glycine intended to neutralization and removal of GA traces would be beneficial for avoiding its toxicity. Such synergism provided a less time-consuming and more cost-effective means of sterilizing surgical material and biomaterials. Synergism between nitric oxide and HHP has been reported for the inactivation of *Escherichia coli* and *Listeria monocytogenes* prior to food processing and resulted in a ~ 6-log reduction in the bacterial counts (De Alba et al. 2013).

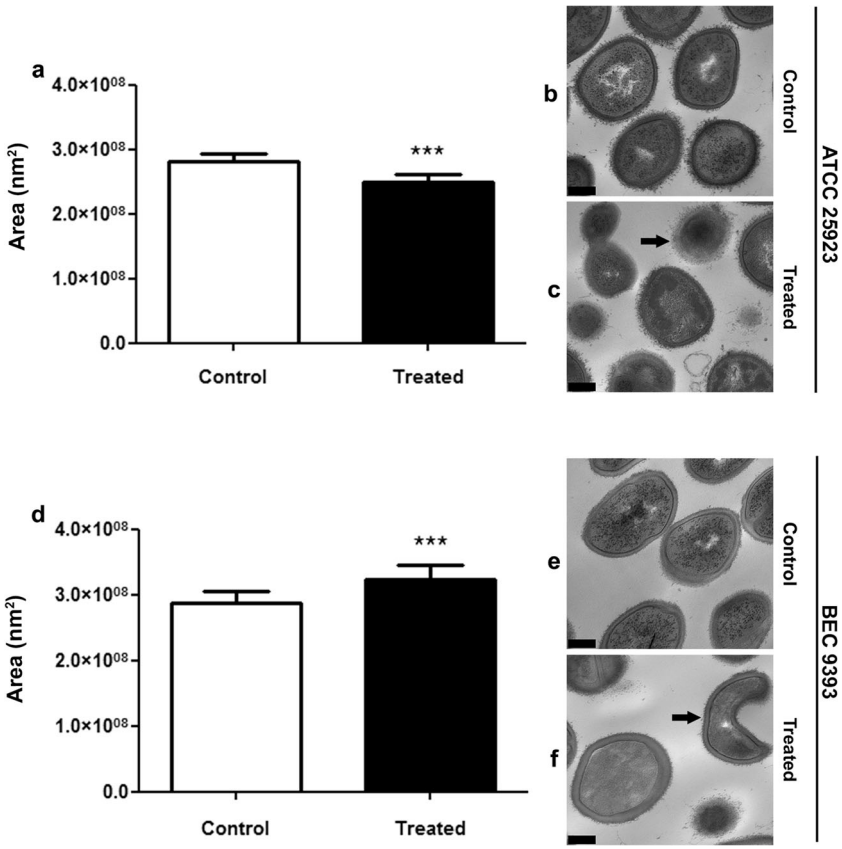

Fig. 5 Morphometric analysis of bacterial TEM images based on the change in bacterial surface area (nm^2) without (control) and with treatment with 0.5 mM GA and 300 MPa HHP and 25 °C for 10 min of *S. aureus* ATCC 25923 (a) and BEC 9393 strain (d). The control and treated images are shown respectively in (b) and (c) for ATCC 25923 strain, and (e) and (f) for BEC 9393 strain. Arrows indicate the altered area in both strains in c and f. The *scale bars* correspond to 200 nm. ***$p < 0.05$ compared to the corresponding control

Recent kinetic work with several strains of *S. aureus* have shown that HHP inactivation was more significant after 20 min of treatment at 450 MPa (Cebrián et al. 2010); another strain tested for 2.5 h at 500 MPa showed total inactivation (> 8 orders of magnitude) (Rigaldie et al. 2007). Mechanistically, HHP affects several cellular targets in *E. coli*, including the barrier properties of the outer membrane, the intactness of the cytoplasmic membrane, the activity of membrane-bound enzymes, and the intactness of ribosomes, as suggested by the TEM analysis of bacteria after treatment (Fig. 4a–d). HHP also stimulates the formation of reactive oxygen species and cell death. The morphometric analysis of images is an appropriate method for assessing the effects of any treatment. A previous study used images to measure the area and volume of bacteria (Massana et al. 1997) and we used a similar approach to examine the effect of GA, HHP, and moderate temperature on bacterial survival (Fig. 5(a–f)). This image analysis revealed clear changes in bacterial area and shape. The significant difference between the two strains in response to the same treatments suggests important biochemical/genetic differences that deserve investigation in the future.

Misfolded proteins in inclusion bodies can increase the sensitivity to HHP. The resistance of *E. coli* to HHP may be related to the over-expression of stress proteins (Ganzle and Liu 2015). *Staphylococcus aureus* is the most prevalent pathogenic bacterium in domestic refrigerators and different thermal inactivation schemes for this bacterium in food have been proposed, e.g., 70 °C for 2 min or 75 °C for 1 min (Kennedy et al. 2005). Our temperature experiments showed marked bacterial inactivation between 55 and 60 °C, so we investigated the possible potentiation of HHP at a lower temperature (50 °C) and the use of a very low concentration of disinfectant for the treatment of sensitive medical materials. We have previously shown that the pressure-induced inactivation of *Aeromonas hydrophila* was much more efficient at 40 °C (15-min treatment at 250 MPa) (Durães-Carvalho et al. 2012), whereas *Mycobacterium abscesses* inactivation was achieved by using a combination involving other conditions, such as moderately high temperature (60 °C), or pH 4.0 or pH 9.0, and was less efficient at subzero temperature (− 15 °C) (De Souza et al. 2013). Previously (Bonafe et al. 1998), the dissociation of the classic tobacco mosaic virus by HHP was significantly observed only in the presence of urea or at subzero temperatures (less than − 19 °C). Such report illustrates the potential of synergism between HHP and other favoring condition for an effective dissociation.

HHP and dissolved CO_2 act synergistically to inactivate *S. aureus* and *E. coli* (Wang et al. 2010). We therefore considered that the use of a very low concentration of disinfectant could improve pressure-induced inactivation and be very suitable for sterilizing medical materials. GA is a disinfectant used to sterilize medical equipment and has the advantage of not

being corrosive to metal and of not causing damage to lensed instruments, rubber or plastics. However, the use of GA, even for non-critical surface cleaning, is controversial because of its toxicity (Takigawa and Endo 2006). In the present study, we tested GA at a concentration less than one tenth of that typically used in hospitals, i.e., 53–212 mM (0.5–2%) (Rutala and Weber 2016). Both strains of *S. aureus* were inactivated by 2–3 mM GA at room temperature (25 °C), as also reported by Gorman et al. (1980). The action of GA was very sensitive to an increase in temperature from 50 to 60 °C and with HHP (Figs. 2 and 3). As shown in Fig. 3, total bacterial inactivation was observed in both strains (a reduction of > 8 orders of magnitude) treated with 0.16 mM GA at 50 °C and 300 MPa, even though individually neither of these conditions significantly reduced the bacterial population.

Another important challenge in sterilization is the presence of biofilm, classically present in reused medical devices. The microorganisms in such biofilms are less susceptible to inactivation because of the protective barrier that biofilm provides (Zapotoczna et al. 2016). We have previously reported total inactivation of *M. abscesses* in biofilm present on PVC fragments after treatment for 45 min at 250 MPa and 60 °C (De Souza et al. 2013), indicating a synergistic effect of pressure and moderate temperature. The presence of low concentrations of GA should further enhance bacterial inactivation in this situation. In contrast, HHP 350 MPa alone or in combination with antibiotics did not significantly reduce the number of gram-negative bacteria in cell suspensions or in biofilm on human ossicle explants from cholesteatoma patients (Dommerich et al. 2012).

GA is considered the most practical cross-linking agent and is suitable for treating biomaterials made from biomolecules and synthetic biopolymers. A limitation to its use is the difficulty in handling and its cytotoxicity at high concentrations (Reddy et al. 2015). Thus, protocols involving HHP in the presence of low concentrations of GA could be more effective in inducing cross-linking reactions, with a decrease in the risks associated with handling and cytotoxicity. The successful treatment of materials contaminated with *S. aureus* biofilm suggests the possibility of treating different systems that use biomaterials of biotechnological interest. The synergistic effect observed here represents a powerful tool for sterilization with high efficiency and low damage.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethical statement This article does not contain any studies with animals performed by any of the authors.

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