UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

MARRIAM YAMIN

High hydrostatic pressure synergism with various physical and chemical agents for biomaterials treatment and inactivation profile of *S. aureus, P. aeruginosa and K. pneumonia* as superbugs.

Sinergismo de alta pressão hidrostática com vários agentes físicos e químicos para o tratamento de biomateriais e perfil de inativação de *S. aureus, P. aeruginosa e K. pneumoniae* como superbacterias.

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Thesis/Dissertation presented to the Faculty/Institute of the University of Campinas in partial fulfilment of the requirements for the degree of doctoral degree in Functional and Molecular Biology in the area of Biochemistry.

Dissertação/Tese apresentada à Faculdade/Instituto da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Funcional e Molecular, na área de Bioquimica.

ESTE ARQUIVO DIGITAL CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA MARRIAM YAMIN E ORIENTADA PELO PROF. DR. CARLOS FRANCISCO SAMPAIO BONAFE.

Orientador: *Prof. Dr. CARLOS FRANCISCO SAMPAIO BONAFE*

CAMPINAS

2018

Agência(s) de fomento e nº(s) de processo(s): CAPES, 2014/PROEX023208

Agência(s) de fomento e nº(s) de processo(s): CAPES
ORCID: https://orcid.org/0000-0003-2039-6500

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

Y14h

Yamin, Marriam, 1986-
High hydrostatic pressure synergism with various physical and chemical agents for biomaterials treatment and inactivation profile of *S. aureus*, *P. aeruginosa* and *K. pneumoniae* as superbugs / Marriam Yamin. – Campinas, SP : [s.n.], 2018.

Orientador: Carlos Francisco Sampaio Bonafe.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Alta pressão hidrostática. 2. Biomateriais. 3. Biofilme. 4. Farmacorresistência bacteriana. 5. Esterilização. 6. Glutaraldeido. I. Bonafe, Carlos Francisco Sampaio, 1961-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Sinergismo de alta pressão hidrostática com vários agentes físicos e químicos para o tratamento de biomateriais e perfil de inativação de *S. aureus*, *P. aeruginosa* e *K. Pneumoniae* como superbactérias
Palavras-chave em inglês:
High hydrostatic pressure
Biomaterials
Biofilms
Drug resistance, Bacterial
Sterilization
Glutaraldehyde
Área de concentração: Bioquímica
Titulação: Doutora em Biologia Funcional e Molecular
Banca examinadora:
Carlos Francisco Sampaio Bonafe [Orientador]
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Luis Antonio Peroni
Data de defesa: 09-08-2018
Programa de Pós-Graduação: Biologia Funcional e Molecular

Campinas, [9th August, 2018].

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Os membros da Comissão Examinadora acima assinaram a Ata de defesa, que se encontra no processo de vida acadêmica do aluno.

Dedication

I would like to dedicate my thesis to my late father ***Mohammad Yamin Sheikh*** *and to all my family to always keep trust in me. Emotional back up from my husband* ***Khalid Ahmed*** *is always supportive and reviving, without him I would not be able to accomplish this goal.*

Acknowledgments

This PhD work was conducted at Institute of biology in State University of Campinas (UNICAMP) with the financial support from Coordination for the Improvement of Higher Education Personnel (CAPES). I am highly grateful for my selection as PhD candidate and later CAPES grant approval.

Scientific and motivational guidance provided by my supervisors Prof. Dr. Carlos Francisco Sampaio Bonafe was indispensable for moving forward with all stages of the PhD thesis. His humble nature and support in all circumstances always pushed me forward. The liberty he gave me to develop as an independent researcher was appreciable. I would also like to mention and share gratefulness to my other professors, Prof. Dr. Silvia Maria Gatti, Prof. Dr. Paulo Pinto Jozeiro, Prof. Dr. Marcello Bispo de Jesus, Prof. Dr. Hiroshi Aoyama, Prof. Dr. Marcello Brocchi and other members and staff of laboratories from Institute of Biology, UNICAMP and at LNNano, CNPEM. I would like to make special thanks to our Lab. Technician Juliana Mattoso Goncalves for all support and concern, she had for me. Furthermore, I would also like to mention here about Prof. Dr. Mohammad Habib for his precious work of guidance and to he always stands by with us.

I would like to thank all our Muslim community here in Brazil to give another family to us. My colleagues more like friends Luiz Bandeira, Ancelmo R. de Souza, Allan Radiac, Bianca G. Castelucci at UNICAMP and Carlos costa and Evandro at LNNano, I really appreciate their presence around me, during my academic years here in Brazil.

A very special thanks to my friends more than sisters Samia Tariq, Almas Taj Awan and Naheed Bibi to always be with me, for their time and friendship.

Marriam yamin

"God doesn't put the secrets of the world in one box,

instead He distributes them in many boxes."

A lot of innovation and creativity involves combining concepts from different disciplines.

Lim Boon Han

Resumo

A capacidade de aquisição de resistência a antibióticos de várias espécies bacterianas vem ganhando destaque, dia após dia, devido tanto ao aumento nas taxas de incidência quanto as possíveis consequências relacionadas. Dentre as espeícies bacterianas capazes de desenvolver resistência a antibióticos, *Staphylococcus aureus, Pseudomonas aeruginosa* e *Klebsiella pneumonia* são potenciais patógenos oportunistas em infecções hospitalares (IACS). Cada cepa bacteriana relacionado com essa estudo é capaz de gerar diferentes perfis de resistência contra diferentes agentes antimicrobianos, dentre elas, a formação de biofilme por estas bactérias dificulta sua eliminação e pode atuar como barreira protetora contra várias condições de estresse geradas pelo ambiente externo. Biomateriais ou implantes contaminados com essas bactérias geralmente necessitam de condições severas de tratamento para sua esterilização, o que pode levar a perda de integridade. Tendo em mente este agravante, o uso de uma técnica alternativa acoplada à alta pressão hidrostática (HHP) pode ser uma abordagem muito favorável ao processo de esterilização de biomateriais hospitalares. Neste estudo, duas cepas diferentes de bactérias gram-positivas (*S. aureus*) e gram-negativas (*P. aeruginosa e K. pneumoniae*) foram submetidas à HHP, combinadas ou não com Glutaraldeído (GA), a temperatura moderada por 10 min. Após o tratamento de células do fitoplâncto e biofilme em crescimento sob material transportador (fragmentos de catétere e lente), observamos a completa inativação / erradicação das cepas de *S. aureus* e *K. pneumoniae* e de uma cepa de *P. aeruginosa* NM 31 na faixa de 0,01- 0,5 mM de GA, 300 MPa de alta pressão a 50 ℃ por 10 min. Entretanto, quando expostas a essas condições individualmente, tal redução do crescimento bacteriano das cepas não foi observada. A cepa ATCC 27853 de *P. aeruginosa*, foi observada maior sensibilidade desta, quando comparada as demais, ao tratamento apenas em HHP temperatura ambiente e sua completa inativação a 200 MPa a 25 ℃. O biofilme formado pela cepa ATCC *P. aeruginosa* em fragmentos de catéteres demonstrou-se altamente resistente a todas as exposições realizadas, sendo erradicado completamente apenas sob ≥ 3,67 mM, 300 MPa a 50 ℃ em 10 min.

Como outra técnica alternativa a esterilização de biomateriais, desafiamos cepas de *S. aureus* e *P. aeruginosa* com nanopartículas de prata biogênicas (AgNPs) associadas ou não com HHP. Neste tratamento, os resultados demonstraram redução de ≥ 70% das bacterias presentes nas cepas quando expostas a 200 μM de AgNPs sob pressão de 300 MPa a 25 ℃ por 15 minutos. Modificações superficiais e estruturais significativas nas cepas e nos biofilmes foram

observadas após o tratamento em análises de microscopia eletrônica de transmissão (MET) e microscopia de força atômica (AFM). A massiva inativação de diferentes cepas bacterianas garantida por este método ressalta sua possível aplicação na esterilização rotineira de biomateriais e implantes sem perda de integridade. Este tipo de técnica de esterilização moderada é favorável a eliminação de biofilme multi-espécies de materiais sensíveis à alta temperatura. Dada a importância da integridade destes biomaterias para a biomedicina e profissionais de saúde, a análise das propriedades superficiais do material exposto a estas condições pode ser uma perspectiva para futuros estudos.

Abstract

Antibiotic resistance acquiring the capability of several bacterial species is gaining attention day by day due to increase in incident rate and related consequences. *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* are potential opportunistic pathogen concerning Hospital-Acquired Infections (HAIs). All bacterial species concerning this work obtained different resistant profiles against various antimicrobial agents, however, the characteristic feature of biofilm formation of these bacteria, making them difficult to eliminate. For instance, biofilm produce from these bacteria correspondingly acts as a protective barrier against several external stress conditions. Biomaterials or implants contaminated with these bacteria often need harsh or extreme treatment condition for sterilization, which can ultimately compromise the properties of the biomaterial. In this concern, the use of an alternative technique coupled with High Hydrostatic Pressure (HHP) can be a very favourable approach for an adequate sterilization procedure. In this study, two different strains of gram-positive (*S. aureus*), and gram-negative (*P. aeruginosa* and *K. pneumoniae*) bacteria were subjected to HHP with or without combining to a disinfectant Glutaraldehyde (GA) at the moderate temperature (50℃) for 10 min. After treatment of both phytoplankton cells and biofilm grown on carrier material (lens and catheter fragments), we observed complete inactivation/eradication of both *S. aureus* and *K. pneumoniae* strains and one strain of *P. aeruginosa* NM 31 in the range of 0.01- 0.5 mM of GA, 300 MPa of high pressure at 50 ℃ for 10 min but showed negligible reduction in bacterial growth when exposed to these conditions individually. In the case of *P. aeruginosa* strain ATCC 27853, exhibited more sensitivity to only HHP at room temperature and completely inactivated at 200 MPa at 25℃. The biofilm of *P. aeruginosa* ATCC on carrier material (catheter fragments) strain demonstrated as high resistance to all such exposures and enabled to eradicate completely under ≥ 3.67 mM, 300 MPa at 50 ℃ in 10 min. We tested both strains of *S. aureus* and *P. aeruginosa* with another antibacterial agent i.e. Biogenic Silver Nanoparticles (AgNPs) uniquely and with HHP, aiming to reduce or minimize the utilization of AgNPs in high concentrations, comparing to conventional use. The results indicated ≥ 70% magnitude of bacterial reduction using 200 µM of AgNPs, 300 MPa pressure at 25℃ for 15 min of exposure. Transmission electron microscopy (TEM) and Atomic force microscopy (AFM) revealed significant surface and structural modifications before and after subsequent treatments equally in cells and biofilm. Achievement of such massive inactivation suggestive to the application of this methodology for routine biomaterial and implants sterilization with

negligible or no damage. This kind of moderate sterilization technique is approachable for multi-species biofilm obliteration for the materials sensitive to high temperature. Surface properties analysis of material expose with such conditions can be a perspective for future studies to ensure its importance for biomedical and health-related practitioners.

LIST OF PAPERS

I. **Synergism between high hydrostatic pressure and glutaraldehyde for the inactivation of Staphylococcus aureus at moderate temperature.**
Marriam Yamin[1], Ancelmo R. Souza[1], Bianca G. Castelucci[2], Juliana G. Mattoso[1] and. Carlos F. S. Bonafe[1,*]
Manuscript published in *Applied Microbiology and Biotechnology.*
https://doi.org/10.1007/s00253-018-9270-4

II. **AFM based surface analysis of High Hydrostatic Pressure Sensitive and Resistant *Pseudomonas aeruginosa* strains.**
Marriam Yamin[1] and Carlos Francisco Sampaio Bonafe[1,*]
Manuscript to be submitted to *Langmuir.*

III. **AFM-IR spectroscopic analysis of *Klebsiella pneumoniae* inactivation induced by high pressure, glutaraldehyde and moderate temperature.**
Marriam Yamin[1] and Carlos Francisco Sampaio Bonafe[1,*]
Manuscript to be submitted to *systematic and applied microbiology.*

IV. **High hydrostatic pressure mediated enhanced antibacterial activity of biogenic Silver nanoparticles (AgNPs) against *Staphylococcus aureus* and *Pseudomonas aeruginosa.***
Marriam Yamin[1] Luiz Alberto Bandeira Ferreira[2], Marcelo Bispo de Jesus[2] and Carlos Francisco Sampaio Bonafe[1,*]
Manuscript to be submitted to *Nanobiotechnology.*

COLLABORATIVE WORK

V. **Fabrication of layered Al-silicate magadiites for the removal of reactive dyes from textile effluents**
Khalid Ahmed[a], Asif Jamal Khan[b], Cleo T.G.V.M.T. Pires[a], Marriam Yamin[c], Fozia Rehman[a,d],*, Abdur Rahima,[d], Jinxi Song[b], Claudio Airoldi[a]
Article published in *Desalination and water treatment*.
doi: 10.5004/dwt.2018.21801

VI. **Entrapment of amphotericin B in plasma bead for a safe and sustained in vivo delivery**
Munazza Fatima, Zeyaul Islam, Marriam Yamin, Ejaj Ahmad, Mehboob Hoque
Manuscript submitted to *Journal of Pharmaceutical and biomedical analysis*

VII. **Porcine Parvovirus VP1/VP2 on a Time Series Epitope Mapping: exploring the effects of high hydrostatic pressure on the immune recognition of antigens.**
Ancelmo Rabelo de Souza[a], Marriam Yamin[a], Danielle Gava[c], Janice Reis Ciacci Zanell[a], Maria Sílvia Viccari Gatti[a], Carlos Francisco Sampaio Bonafe[a], Daniel Ferreira de Lima Neto[a,b]*
Manuscript submitted to *viral immunology*

PROCEEDINGS OF CONFERENCES/ WORKSHOPS:

a). *Papers:* No paper published in any conference.

b). *poster and oral presentations*

i. High hydrostatic pressure effect associated with disinfectant to inactivate pathogenic bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*), presented at VII Protemics workshop, arranged by the Brazilian Biosciences National Laboratory LNBio, at the Brazilian Center for Research in Energy and Materials (CNPEM), on November 8th and 9th, 2016, in SP, Brazil.

ii. High hydrostatic pressure effect associated with disinfectant to inactivate pathogenic bacteria (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) was exhibited in E-Poster Participation at "4th World Congress and Expo on Applied Microbiology" held during September 19-21, 2016 in Las Vegas, USA.

iii. Efeito da alta pressão hidrostática associado ao desinfectantes para a inativação de bactérias patogênicas (*Klebsiella pneumoniae* e *Pseudomonas aeruginosa*) was orally presented in Mostra Científica do 25º Congresso Nacional de Pós-Graduandos, held in 10 and 11 jully, 2016, in Universidade Federal de Minas Gerais, Belo Horizante, Minas Gerais, Brazil.

iv. Attended "Integrated analysis of shotgun proteomic data with PatternLab for proteomics 4.0" course, held by the Brazilian Biosciences National Laboratory (LNBio) at the Brazilian Center for Research in Energy and Materials (CNPEM), on November 10th and 11th, 2016.

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CHAPTER: 1

INTRODUCTION

Hospital-acquired infections: challenges of disease and risk factors

All the infections established in a patient after/during hospitalization stay of two days (48 hours) at a hospital, which did not exist before, are termed as Hospital acquired infections (HAIs). These infections have been believed to be contributing highly to the growing medical

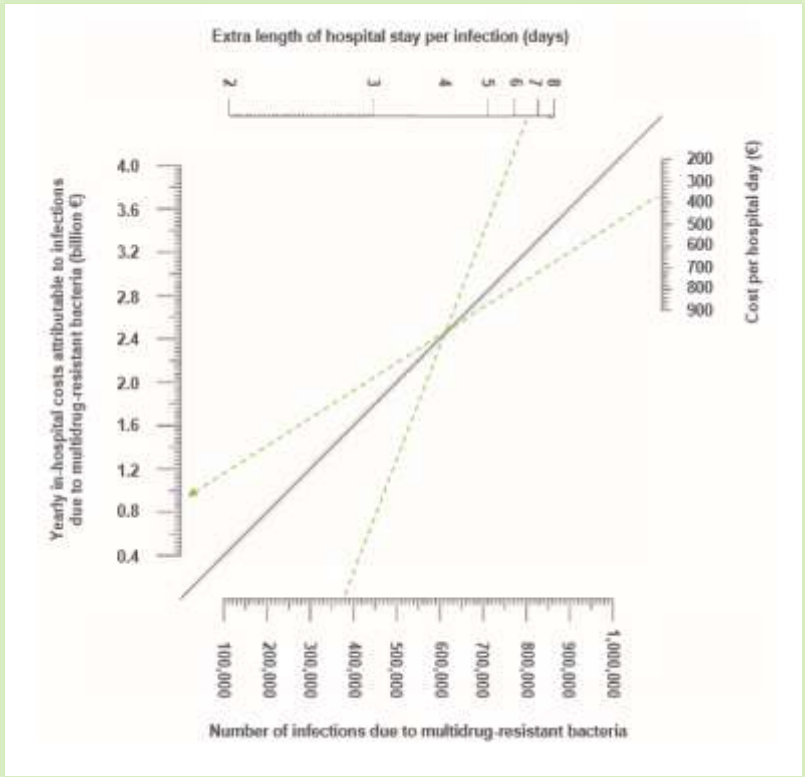


Figure 1.1. Nomogram: This nomogram can be used to calculate yearly in-hospital costs attributable to infections due to multi-drug resistant bacteria with various values for the total number of infections, the average extra length of hospital stays per infection and the average cost per day of hospitalization. Source: (ECDC et al. 2007).

expenses, time of hospitalization, high mortality rate (Figure 1). Some policies have been developed recently, to alleviate the consequences of these infections (Kelly and Monson 2012). In this context, the reuse of medical devices in hospitals raises additional difficulties for sterilization, such as the presence of biofilm, that may require more drastic conditions for efficient treatment (Ntsama-Essomba et al. 1997; Rutala and Weber 2016).

''Biofilms'': A protective barrier against external stress

Biofilm formation is crucial for chronic and refractory infections that organize organisms, cement cells to surface and possesses extracellular polymeric matrix made by exopolysaccharide, nucleic acid and proteins. Biofilm are extremely resistant to both innate and specific host immune system. On the other hand, due to exopolysaccharide thick matrix and decreased metabolic rate, biofilm-grown bacteria are less susceptible to phagocytic macrophages and resistant to antibiotic that attack on dividing cells (Bendouah et al. 2006) or may develop a specific-biocide resistance phenotype, owing the heterogeneous nature of biofilm showing multiple resistant mechanism with in a community (Mah and O'Toole 2001).

Conventional sterilization methods

All invasive procedures involve contact by a medical device or surgical instrument with patients' sterile tissue or mucous membrane. Therefore, the high or low-level sterilization is mandatory depending on use of critical (items that contact sterile tissue, such as surgical instrument) semi-critical (items that contact mucous membranes, such as endoscopes) or noncritical (items that contact only intact skin, such as stethoscopes) objects. Cleaning prior to disinfection and sterilization (Figure 1.2). Use of cutting-edge technologies (e.g. hydrogen per oxide mist) and its practice must to updated to the health-care practitioners and awareness with environmental factors responsible for infections (e.g. endoscopes) (Rutala and Weber 2016). Several other conventional methods of sterilization, such as steam autoclaving (Baier et al. 1982), gamma radiation, 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).

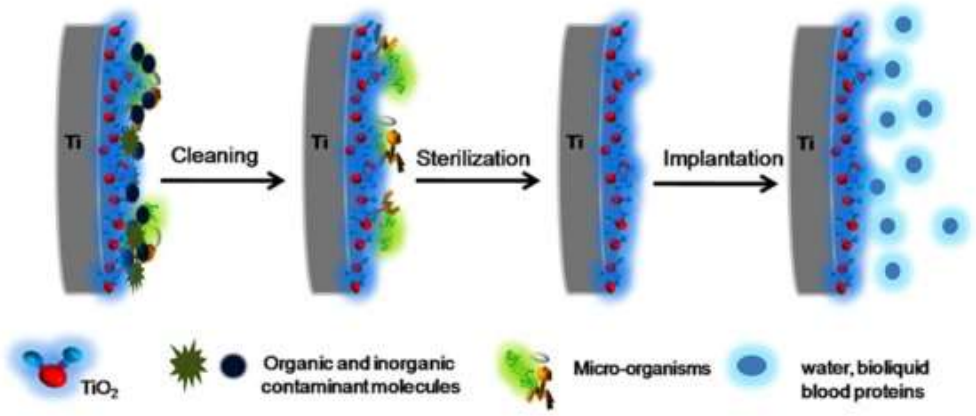


Figure 1.2. Illustration of events at the Ti surface during cleaning, sterilization, and implantation. Source: (Park et al. 2012).

1. Glutaraldehyde

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). Figure 1.3. explains in what manner Glutaraldehyde polymerizes and then interact with amino acids in proteins (left) or in peptidoglycan (right). As a result, the proteins are alkylated and cross-linked to other proteins, subsequently, inactivates bacteria. The amino acids in peptidoglycan are also alkylated and cross-linked, which prevents them from participating in other chemical reactions such as those involved in peptidoglycan synthesis. This 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; Reddy et al. 2015). GA also leaves residues on material surfaces that can cause the calcification of implants treated with this agent (Kim et al. 1999; Yang et al. 2017). A 2% buffered solution of glutaraldehyde solution is recommended for 10 minutes by FDA for hospital and laboratory equipment effective disinfection but may require 12 hr. exposure to destroy all spores, virus and bacterial inactivation (Rutala and Weber 2004).

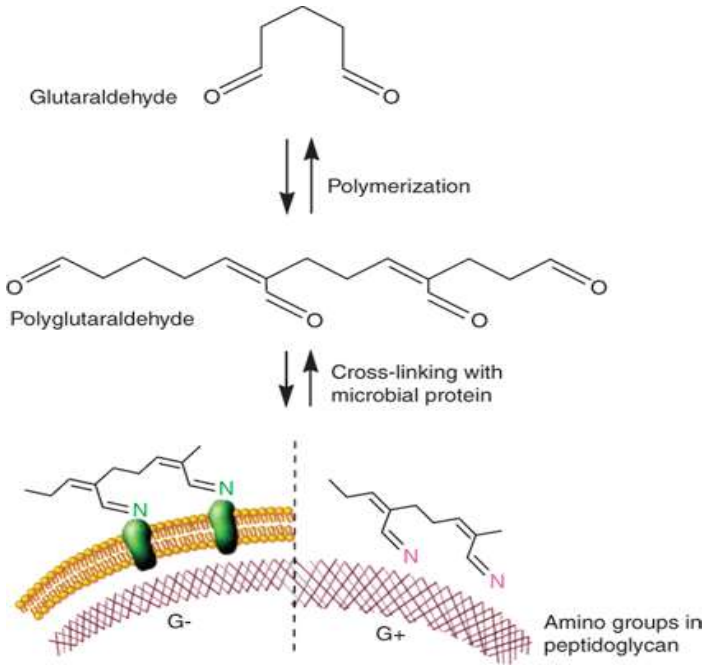


Figure 1.3. Effect of Glutaraldehyde. Source: Prescott's microbiology, 8th edition, 2008.

2. Biogenic silver nano particles (AgNPs)

Particle size less than 100 nanometres generally called nanoparticles (Durán et al. 2015). which deals with matter that ranges from one half the diameter of DNA is the nanotechnology (Dingman 2008). Biogenic silver nanoparticles (AgNPs) are another agent that is gaining attention progressively due to its antimicrobial activity, although its mechanism of action is not well understood but there is the indication of cell wall damage, inhibition of 30S ribosomal subunit, attachment with cell membrane, formation of free radicles, intercalation in nitrogenous bases of DNA by adhering on cell surface against multi-drug resistant (MDR) microorganisms (Durán et al. 2016). That's why we incorporated in this project to check its antibacterial activity with and without exposure to HHP. Baring the idea to minimize the use of silver nanoparticle in higher concentration by making combination with HHP as up to some range it (AgNP) possesses toxicity to human cells, by causing damage to mitochondria, DNA and increased production of reactive oxygen species (ROS) in a dose-dependent manner by reducing ATP content (P. V. AshaRani et al. 2009).

Mechanism of AgNPs antibacterial activity

The antibacterial activity of AgNPs working in several different manners, which has been extensively studied by various researchers along the last two decades. AgNPs may serve as a vehicle to deliver Ag^+ more effectively (being less susceptible to binding and reduced bioavailability by common natural ligands) to the bacteria cytoplasm and membrane, whose proton motive force would decrease the local pH (as low as pH 3.0) and enhance Ag^+ release (Figure 1.4. A). On the other hand, proposed a number of mechanisms of antibacterial action of silver nanoparticles: 1—electrostatic attraction, 2—production of free radicals, changes in permeability, disturbance of respiration, leakage of intracellular content, 3—modulation of phosphotyrosine profiles of proteins, involved in the cell cycle progression and in the synthesis of capsular polysaccharides, 4—interaction with SH-groups; inhibition of protein synthesis and function, 5—interaction with phosphorus-containing molecules (DNA) (Figure 1.4. B) (Durán et al. 2016) .

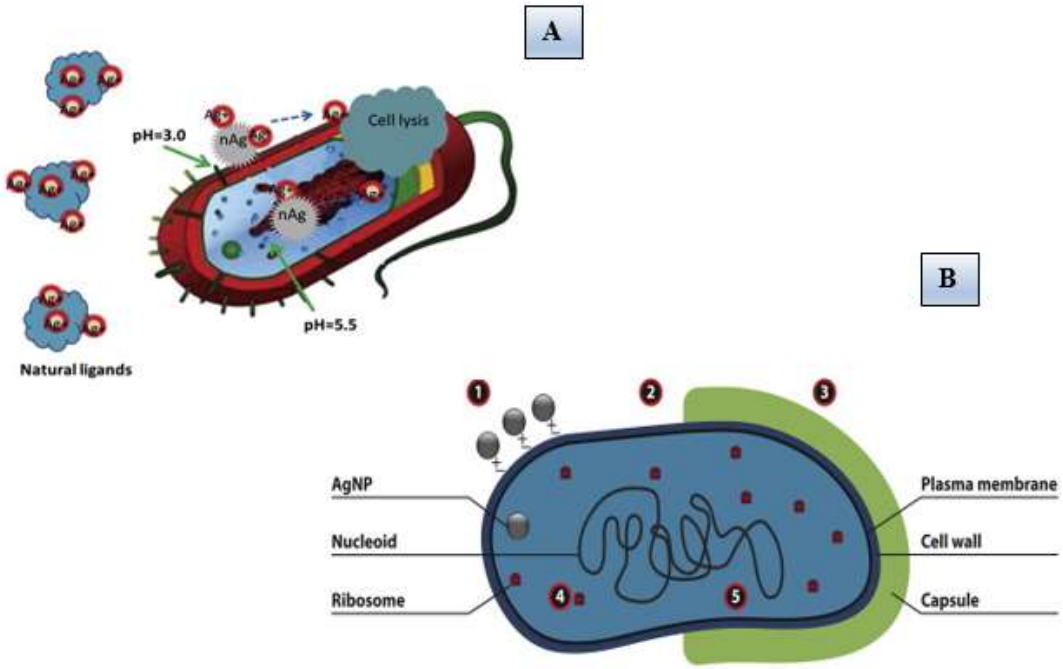


Figure 1.4. **(A)** Schematic representation of AgNPs, Ag^+, and cell interactions. **(B)** Proposed mechanism of antibacterial action of silver ion (Durán et al. 2016).

Staphylococcus aureus

Staphylococcus aureus is a gram-positive bacterium that inhabits human body, thus contributing to the normal human bacterial flora. It is also considered as a virulent pathogen that escapes detection by the host tissue by secreting various surface proteins that acts as biofilms and special capsules around it. In addition to these surface proteins, *Staphylococcus aureus* also secretes chemotaxis inhibitory proteins and leucocidins among many others which leads to infections in the host body (Kelly and Monson 2012). Figure 1.4. showed that *S. aureus* surface protein G (SasG), *S. aureus* surface protein C (SasC), *Staphylococcus aureus* protein A (Spa), as well as the cell surface extracellular matrix binding protein (Embp) and extracellular adherence protein (Eap), release of extracellular DNA based lysis controlled by autolysin are factors for biofilm formation (Zapotoczna et al. 2016). *S. aureus* is a pathogen that can form biofilm similarly on implants and medical devices. Central to biofilm formation is a very tight interaction between microbial surface proteins called adhesions and components of the extracellular matrix of the host. Thus, these adhesins can attach to their target with exceptionally resilient mechanostability, virtually independent of peptide side chains (Milles et al. 2018).

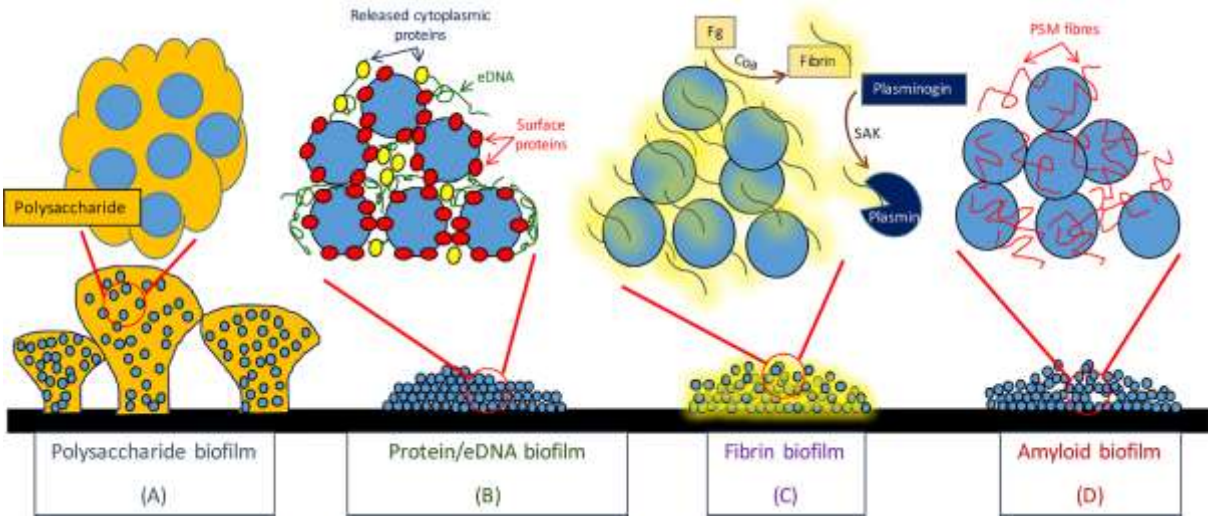


Figure 1.4. Major mechanisms of biofilm expressed by *S. aureus*. Source: (Zapotoczna et al. 2016).

Pseudomonas aeruginosa

Pseudomonas aeruginosa inhabits both biotic and abiotic environments, by virtue of its versatile metabolic activity. ***P aeruginosa*** is a leading cause of opportunistic infection in the eye (contact lenses), wounds, urinary tract, and burns (Figure 1.5). In a special situation it colonizes the respiratory tract of persons with cystic fibrosis by formation of a biofilm. Being capable of secreting biofilms and due to antibiotic resistance, this bacteria is a potential cause of various acute and chronic infections in human body (Balasubramanian et al. 2013). Specially in hospital settings, *P. aeruginosa* is responsible for biofilm formation on indwelling catheter causing catheter-associated urinary tract infections (CAUTIs) by utilizing urea (urine constituent) and propagate by the proclamation of extracellular DNA as support system (Cole et al. 2014).

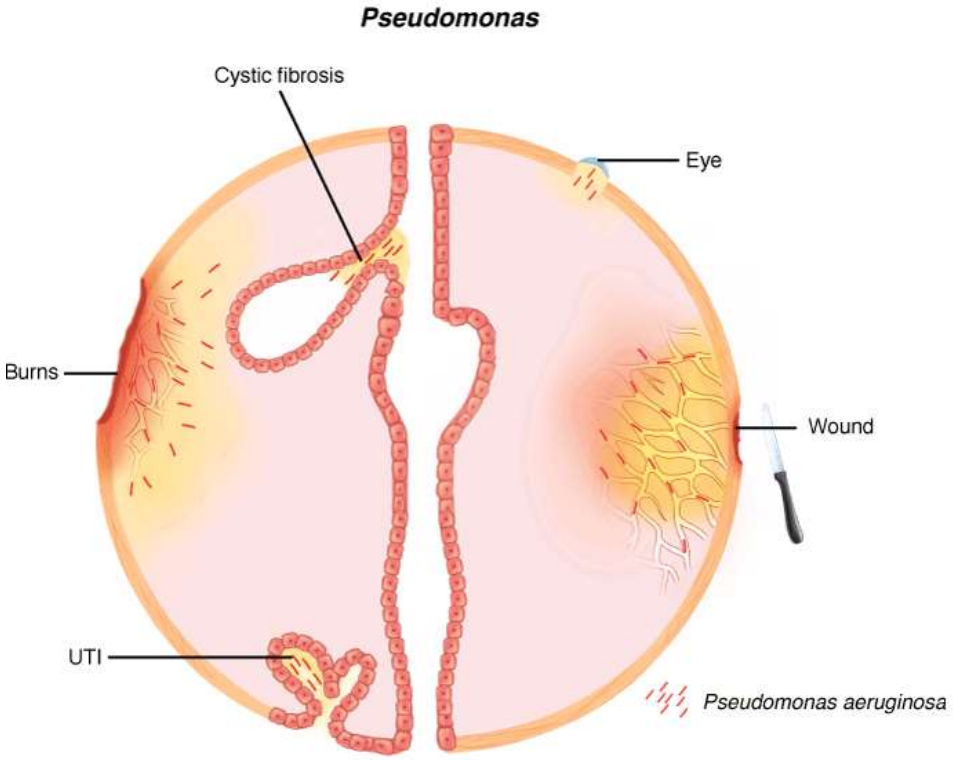


Figure 1.5. Pseudomonas disease overview. Source: Ryan KJ, Ray CG: Sherri's medical microbiology, 5th edition.

Klebsiella pneumoniae

Klebsiella pneumoniae is a widespread pervasive pathogen found in clinical and even nonclinical setups, which is believed to be associated with a wide range of infections related to urinary tract, respiratory system, and surgical wounds in individuals with suppressed immune system (Figure 1.4) (Janda 2015) . Their biofilm formation capability aids them in carrying out the pathogenic processes which causes infections in patients with weak immunity systems even in microgravity environment which is a stressful condition (Wang et al. 2016). An encapsulated bacterium a significant cause of hospital-acquired pneumonia. These virulence characteristics include capsule formation, iron scavenging systems, biofilm formation, allantoin metabolism, and serum resistance (McIver and Janda 2008; Shon et al. 2013).

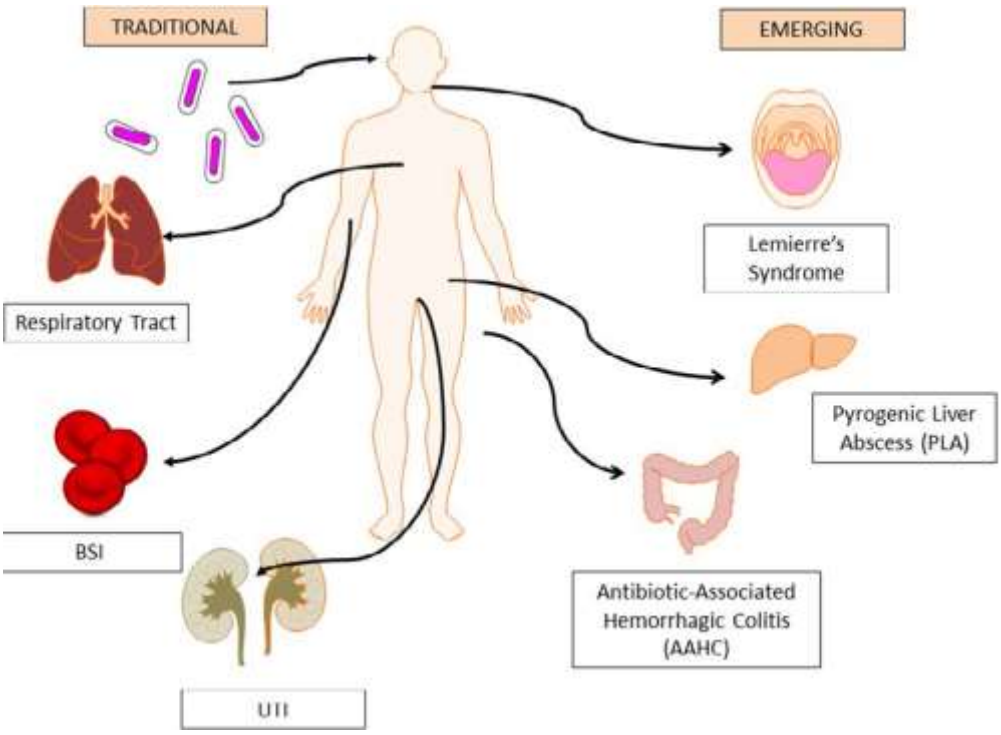


Figure 1.6. Diagrammatic representation of traditional and emerging diseases associated with the genus *Klebsiella*. Source: (Janda 2015).

Emerging Antibiotic resistance strains

Antibiotic resistance is a growing issue, which is considered to driven by the use of antibiotics which exert main selective pressure in increasing resistance. Consequently, a wide flora of commensal bacteria that exist on skin and other systems including digestive and excretory tract, have surfaced as pathogens (Alekshun and Levy 2004; Goossens et al. 2005), become opportunistic. Methicillin- resistant *Staphylococcus aureus* (MRSA) which is a notorious antibiotic resistant bacterium, can be found ubiquitously. In 2002, soon after the aforementioned microbe acquired five-gene plasmid cassette, vancomycin-resistant *Staphylococcus aureus* (VRSA) received attention. Almost 20,000 people in U.S are believed to die every year because of MRSA infections, which is more than mortality rate caused by HIV/AIDS (Walsh and Fischbach 2009). In addition, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* developed resistance against all the clinically available drugs by virtue of their double cell membrane and its complexity (ECDC et al. 2007). Besides, it also acquires resistance to last line antimicrobial agents e.g. Polymixins A (Chávez-Jacobo et al. 2018; Xu et al. 2018).

High hydrostatic pressure technology (HHP) and its Working principles

The application of high hydrostatic pressure (HHP) treatments to preserve food was first described only 23 years later. In 1899, Hite tried to find alternative preservation methods for heat sensitive food and was able to demonstrate that HHP treatments at approximately 700 MPa can significantly increase the shelf-life of milk with less detrimental effects on sensorial properties than heat treatments (Hite B.H 1899). Pressure is defined as the force per unit area applied on a surface in a direction perpendicular to this surface: mathematically:

$$P = F / A$$

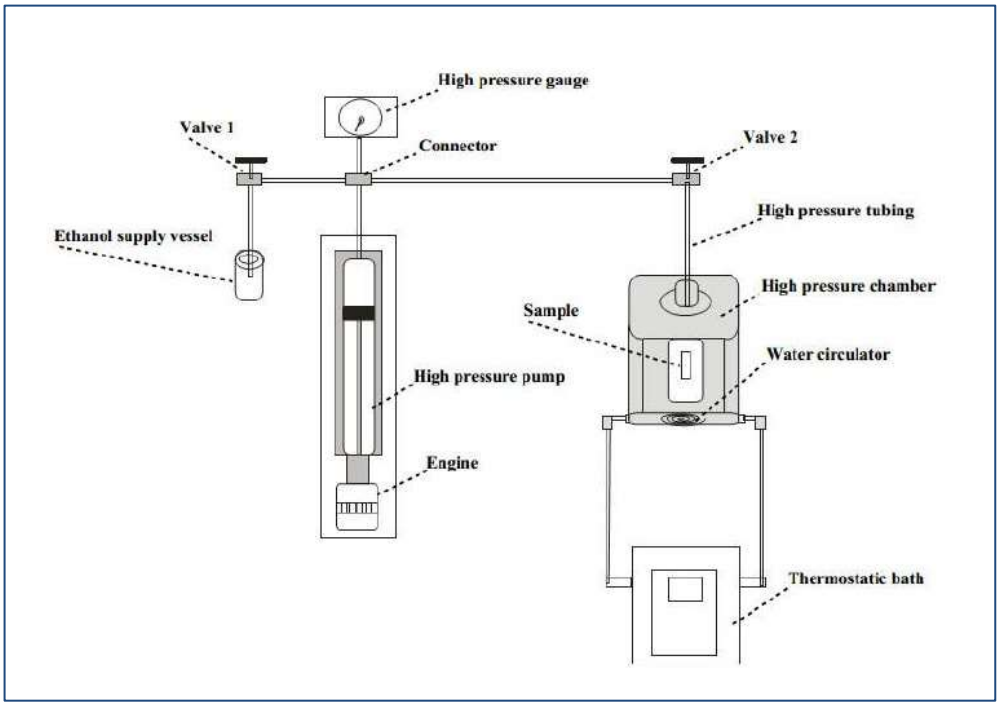
In which P is the pressure, F is the normal force applied to the surface and A is the area of the surface. The official pressure unit is the Pascal (Pa) (1 Pa=1 N/1 m^2=10^{-5} bar). The Newton representing a small force and 1 m^2 corresponding to a large surface, the Pascal unit is a very small pressure unit. Consequently, the Mega Pascal (MPa) [1MPa=10^6 Pa] is the pressure unit commonly used in high pressure studies. The conversion from MPa to other pressure units is given in the Table 1. Below, adapted from (Rivalain et al. 2010). This widespread approach reflected in the wealth of the equipment, which now ranges from laboratory and industrial Scale

HHP generators to systems that are able to invade the HHP biosphere, such as submersibles used to sample piezophilic organisms in the deep sea. While laboratory scale generators have vessels with sample volume as low as 8 mL and a maximum achievable pressure of approximately 800 MPa (Aertsen et al. 2009). Pressure is a thermodynamic parameter whose unique effects on biological systems are increasingly being studied in a growing number of scientific fields. As such, the effects of high pressure are currently being investigated at different levels, ranging from proteins, enzymes and viruses to microorganisms, mammalian cells and tissues. From past years (figure 1.6.), the use of HHP in biological and biotechnological applications is seeking more attention e.g. vaccine preparation, protein disaggregation and modulation of food to check its functionality and processing (Aertsen et al. 2009). At the same time, sterilization of bones grafts and implants by high pressure prior to transplantation gave another hope to biomedical practitioners to render proper solution to contamination consequences preserving biomechanical properties of tissues (van de Sande et al. 2017), blood and its derivatives (Yang et al. 2016). As HHP do not break covalent bond but weak ionic bonds so, the organoleptic properties are therefore not modified and stay quite close to fresh like product (Demazeau and Rivalain 2011). The effect of high pressure for bacterial inactivation (*E. coli* and *S. aureus*) was first studied in 1895 by Royer H. cited in (Rivalain et al. 2010). High pressure exerts many effects on living organisms, affecting not only cell structural organization but also its metabolic processes, which makes it difficult to pinpoint pressure effects in cell growth and viability (Bartlett 2002) and its damaging effect on bacteria and fungus, elevate with the increment in high pressure (Park et al. 2003). Illustrative figures 1.5. A, B & C are representing instrumental design of the HHP system originally of our laboratory of Protein Thermodynamics situated in Department of Biochemistry and tissue biology, State university of Campinas, that was utilized to this study.

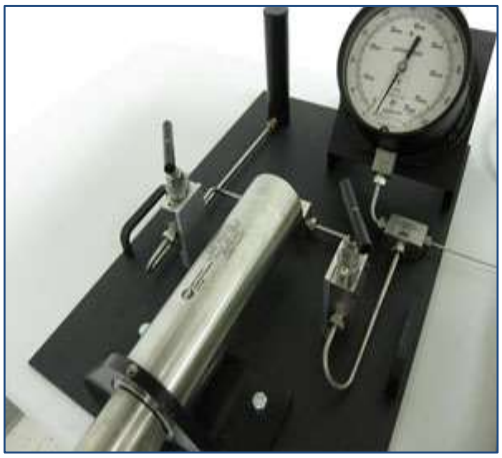
Table 1.1. Conversion of the different units used for pressure. Source: (Rivalain et al. 2010).

	Atmosphere	Bar	kg/cm^2	MPa	P.S.I. ($pounds/inch^2$)
Atmosphere	1	0.987	0.968	9.901	0.068
Bar	1.013	1	0.981	10.000	0.069
kg/cm^2	1.033	1.021	1	10.228	0.070
MPa	0.101	0.1	0.098	1	0.00689
P.S.I.	14.696	14.504	14.223	145.038	1

A



B



C

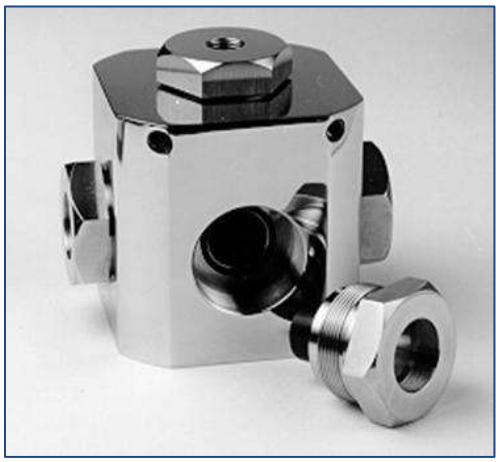


Figure 1.7. (A) high pressure engine, (B) high pressure chamber for sample loading and, (C) Schematic Representation of High Hydrostatic Pressure instrument.

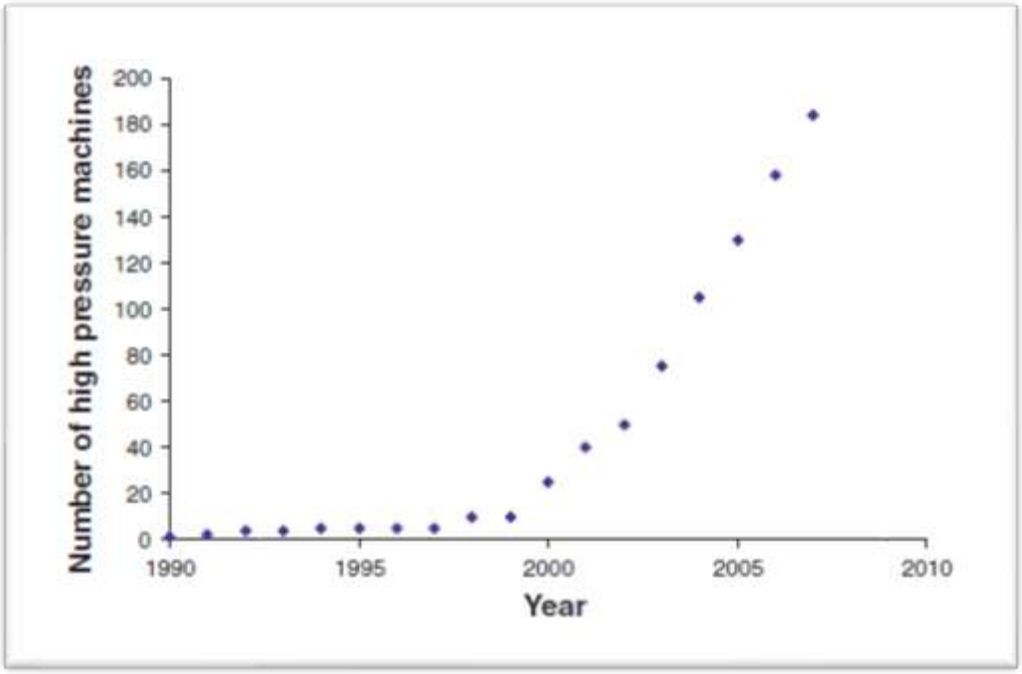


Figure 1.8. Development and increase in number of high-pressure machines around the world in the last 20 years. Source reviewed (Bermúdez-Aguirre and Barbosa-Cánovas 2011).

High hydrostatic pressure (HHP) effect and applications

The synergism is the process when any factor appears to facilitate other factor to achieve the goal. The synergism of HHP is being study in various systems e.g. with temperature, CO_2, nitric oxide, and water for food preservation (Pyatkovskyy et al. 2018), in biomaterial sterilization (Gollwitzer et al. 2009), inanimate objects (clothes, mobile phone and usb) (Calvo and Casas 2018) was reported recently headed for sterilized by HHP to avoid biohazards occurred due to bacterial contamination. Before selecting sterilization methods, the nature of material and evaluation of sterilization technique's effect is very sensitive issue to be keeping in mind, highlighting modification concerning the biomechanical, physiological and structural properties of material of interest to be sterilized. Park et al. (2012) studied the surface properties and immune response as a result of different cleaning and sterilization methods were implied on implants High hydrostatic Pressure inactivation of biological agents is expected to be applicable to sterilization of fragile biopharmaceuticals, or medical compounds. The enhanced immunogenicity of some pressure-killed bacteria and viruses could be applied for making new vaccines. Finally, storage at sub-zero temperatures without freezing is another potential

application of HP for cells, animal tissues, blood cells, organs for transplant, and so forth (Masson et al. 2001). HHP has potential to initiate various biotechnological developments for example change in its effect on biomolecules such as proteins, carbohydrates and fatty acids using isostatic pressure where the pressure value remains contact in all direction in liquid either water or alcohol (Rivalain et al. 2010). High pressure effect was also evaluated in mammalian cells by applying high hydrostatic pressure on different cell lines where around 100 MPa was observed in mammalian cell which activated intrinsic and extrinsic pathways apoptotic signalling during high pressure induced cell death (Takano et al. 1997; Agar et al. 2006; Yamaguchi et al. 2008), for such reason, pressure selection decision is very important to keep in mind prior to biomaterial treatment.

Mechanical stresses and cellular deformation was also observed in *Saccharomyces cerevisiae* under lower and higher level of pressure and presented by the help of simulation and modelling (Hartmann et al. 2006). In general, low temperature and high pressure have related effects on biological membranes, since both largely affect its fluidity (Royer 1995). With increasing pressure, lipid bilayers lose fluidity and became rapidly impermeable to water and other molecules, while protein–lipid interactions essential to the optimal function of the membrane are weakened (Winter and Jeworrek 2009). In general, all pressure effects arise from a single influence, which corresponds to the volume reduction of the biological system, favouring the acquisition of more compact structural forms. Besides the structural alterations in biomolecules, pressure also disturbs the equilibrium of (bio) chemical reactions (Mota et al. 2013).

High hydrostatic pressure interactions in biological systems

For various reasons, pressure appears as an important tool for the investigation of biological systems (Winter and Dzwolak 2005).

- ***The change in the volume and thermal energy in biological system induced by atmospheric pressure affected by temperature, so high pressure can be appropriate tool for such studies.***
- ***Non-covalent interactions play an important role in the stabilization of biological systems. Due to the low energy developed by pressure, high pressure alteration of weak bonds (in particular weak bonds characterized by a negative ΔV value) can play a crucial role in the investigation of the mechanisms of this stabilization. Pressure affects also chemical equilibria and reaction rates but all these parameters are governed by the Le-Chatelier's rule leading to the stabilization of the state corresponding to the smallest volume.***
- ***Due to the existence of hydrogen bonds, the specific pressure temperature diagram of water (the most common solvent in biological systems) favours the liquid state until −20 °C if the pressure value is high enough (−20 °C, 200 MPa). Consequently, experiments at sub-zero temperature in liquid phase are possible.***
- ***The change in volume ΔV can also open doors to execute new methods at ambient pressure.***

High pressure targets specific effect: Biomolecules

1. **Pressure effects on proteins**

Denaturation of proteins is induced by different factors: (i) heat, (ii) chemicals, and (iii) pressure. Temperature and/or chemicals lead to protein denaturation and often irreversibly unfold the complete protein because of covalent bond breaking and/or aggregation of the molecule. Indeed, works of Zhang et al. demonstrated, for example, that pressure denatured ribonuclease A preserved some partial secondary structure contrary to the heat unfolded one. The preservation of some β-like structures was also observed for pressure denatured staphylococcal nuclease (Zhang et al. 1995; Winter and Dzwolak 2005). High pressure can maintain some parts of the molecule unchanged due to the fact that only weak bonds are affected (and only weak bonds characterized by a negative ΔV value). Consequently, the denaturation mechanisms induced by pressure are different from the ones observed using temperature or

chemicals (Rivalain et al. 2010). HHP also responsible for causing conformational changes in proteins and ribosomes which has been considered as limiting factors for both microbial growth and survival under high pressure (Gayán et al. 2017).

Among the weak interactions stabilizing the protein conformation, hydrophobic interactions are the ones characterized by the most negative ΔV value and therefore the most pressure sensitive. These interactions play a major role in the stabilization of the tertiary structure and in protein–protein interactions. Columbic interactions are not favoured by pressure and hydrophobic interactions are destabilized by pressure (Table 2).

Table 1.2. Susceptibility to high pressure of chemical interactions. Adapted source: (Rivalain et al. 2010) originally from Federighi et al. 1995.

Type of interaction	$\Delta V_{dissociation}$ (ml mol^{-1})	Pressure effect
Covalent	+10	Stabilization
Ionic	−10	Destabilization
Hydrogen	+3 to −1	Stabilization or low destabilization
Hydrophobic	<0 (−10 to −20)	Destabilization

High hydrostatic pressure is a unique tool to study hydration, as increases in water binding usually lead to decreases in volume. Pressure changes can favour the formation or disassembly of amyloids depending on the volume changes associated with protein folding and misfolding/ aggregation. The packing and formation of cavities will also contribute to changes in volume, and therefore, to sensitivity to pressure. Therefore, the formation of water-excluding cavities is predicted to be an important event in folding and aggregation landscapes (Silva and Foguel 2009).

2. High pressure effect on lipids and bio-membranes

Lipids are the most sensitive biomolecular system to high pressure specially in presence of water e.g. phospholipids (Winter and Dzwolak 2005). These lipids displays a large structural polymorphism subject to different factors: their molecular structure, water content, pH, ionic strength, temperature and pressure (Rivalain et al. 2010). The basic structural element of biological membranes consists of a lamellar phospholipids bilayer matrix (Winter and Dzwolak 2005). When saturated phospholipids are placed into water, two phase transitions take place: a

gel-to-gel pretransition (Lβ′–Pβ′) and a gel-to-liquid- crystalline (Pβ′–Lα) main transition. The compression of the phospholipidic bilayer is anisotropic. Under high pressure conditions, the acyl chains straighten which result in a lateral shrinking and an increase in thickness. This phenomenon is also accompanied by a phase transition from the liquid–crystalline to the gel phase (Winter and Jeworrek 2009). On the other hand, barophilic organism also exists and they have membranes that are more fluid, and this is partly due to an increase of the unsaturated to saturated lipid ratio. Because of the sensitivity of lipids against pressure, these biological components are often considered as the main target in the pressure inactivation of micro-organisms (Winter and Jeworrek 2009).

3. High pressure effects on nucleic acids

Due to the stabilizing effect of high pressure on hydrogen bonds and in particular DNA hydrogen bonds, the duplex to single strand transition temperature (melting temperature T_M) is increased by pressure (Rivalain et al. 2010). The changes of RNA induced by pressure were not visible in the case of cells in the stationary phase. The degradation of DNA isolated from pressure treated *E. coli* strains from the exponential as well as from the stationary phase of growth was not observed (Malinowska-Pańczyk et al. 2011). Though the HHP cause DNA condensation and inhibit septum formation, however, cells could grow continuously (T. Sato et al. 2002). Past research on the physiology and molecular biology of deep-sea barophilic bacteria has identified pressure-regulated operons and shown that microbial growth is influenced by the relationship between temperature and pressure in the deep-sea environment e.g. *Pseudomonas* strains (Horikoshi 1998).

High pressure resistance

There are many bacteria that acquired resistance against high pressure. Research presented on the isolation of pressure-resistant mutants, high-pressure regulation of gene expression, the role of membrane lipids and proteins in determining growth ability at high pressure, pressure effects on DNA replication and topology as well as on cell division, and the role of extrinsic factors in modulating enzyme activity at high pressure (Bartlett 2002). Different bacterial species examined under extreme higher-pressure ranges and observed attainment of pressure resistance only in *E. coli* strains and mechanistically concluded that, HHP resistance was not essentially connected to de-repression of the heat shock genes and was not related to the phenomenon of persistence and its resistance stability maintained >80 generations (Vanlint et al. 2012).

Objectives of the study

The aims of the study are to investigate inactivation profile and surface level structural changes on S. *aureus, P. aeruginosa* and *K. pneumonia* and their biofilm, with and without treatment with the various physical; temperature and chemical; Glutaraldehyde and Silver-Nanoparticles (AgNPs) agents under high hydrostatic pressure (HHP) aiming to safe biomaterial sterilization.

Specific objectives

- To find the temperature resistance profile of all bacterial strains of *S. aureus, K. pneumoniae* and *P. aeruginosa* for 10 min of exposure time.
- To Investigate the inactivation of one or more strains of *S. aureus, K. pneumoniae* and *P. aeruginosa* by disinfectant as glutaraldehyde at different concentrations for 10 min of exposure time.
- Inactivation kinetics analysis of above-mentioned bacteria by HHP for different time of exposure.
- To investigate the combined effect of disinfectant; Glutaraldehyde and HHP on the inactivation of these bacteria.
- Investigate the biofilm formation by the above-mentioned bacterial strains and the effect of HHP and/or disinfectant in the respective contaminated carrier materials (lens and catheter fragments).
- Investigation of the effect of different physical and chemical conditions in combination with disinfectants and HHP, at different temperatures.
- Investigate the antibacterial effect of biogenic silver nanoparticles (AgNPs) with and without a combination of HHP against *S. aureus* and *P. aeruginosa* strains.
- Transmission electron microscopy (TEM) analysis of treated and untreated S. *aureus* and *P. aeruginosa* strains.
- Atomic force microscopy (AFM) of *P. aeruginosa* (ATCC 27853 & NM 31) and *K. pneumoniae* (ATCC BAA 1705 & ATCC 4352) strains to observe morphological and structural modifications after HHP treatment.

CHAPTER: 2

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

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Running head: Synergism in *S. aureus* inactivation

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Acknowledgments

The authors thank Prof. Dr. Paulo P. Joazeiro and Adriane C. S. Sprogis for their support during the electron microscopic analysis and for helpful discussions and Stephen Hyslop for editing the English of the manuscript.

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 ^{o}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 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 ^{o}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 4,000 *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 ^{o}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, United Kingdom) 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 ºC, 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 ºC). 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 ºC 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 ºC, 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 ºC, 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 ºC 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 (Alagumaruthanayagam 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 7,000 g. The pellets was 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 ºC 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 (46,460x). 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

Fig. 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 Figure S1); this finding may reflect a significant phenotypic change in these experimental conditions.

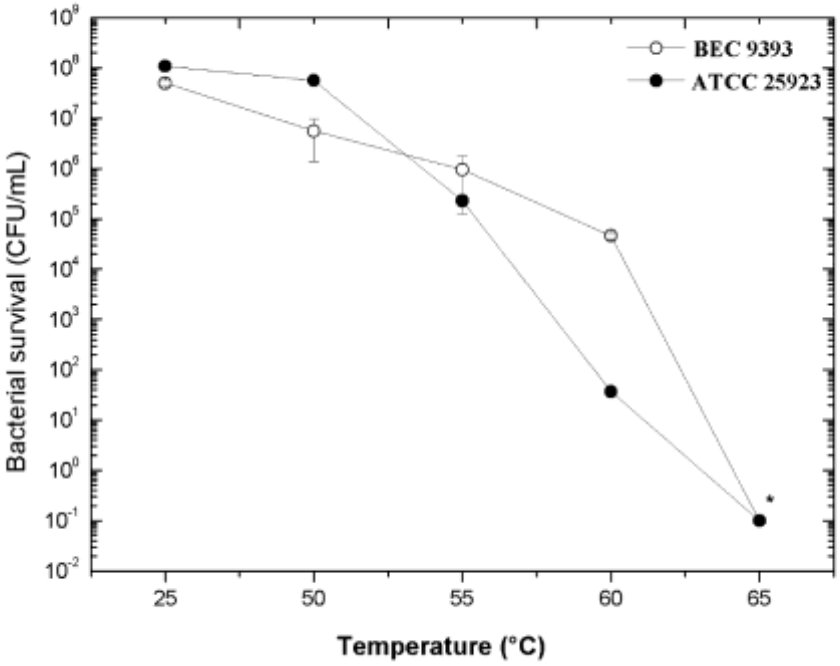

Fig. 1 Survival curves of *S. aureus* strains ATCC 25923 (*closed symbols*) and BEC 9393 (*open symbols*) after exposure to different temperatures for 10 min in absence of GA. Asterisk: no bacteria detected. The error bars represent standard deviations (n=3).

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 was about 100 times lower than those currently used for disinfection, which may reach up to 2% (212 mM). Fig. 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 ^{o}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 ^{o}C, even in the absence of GA (Fig. 1).

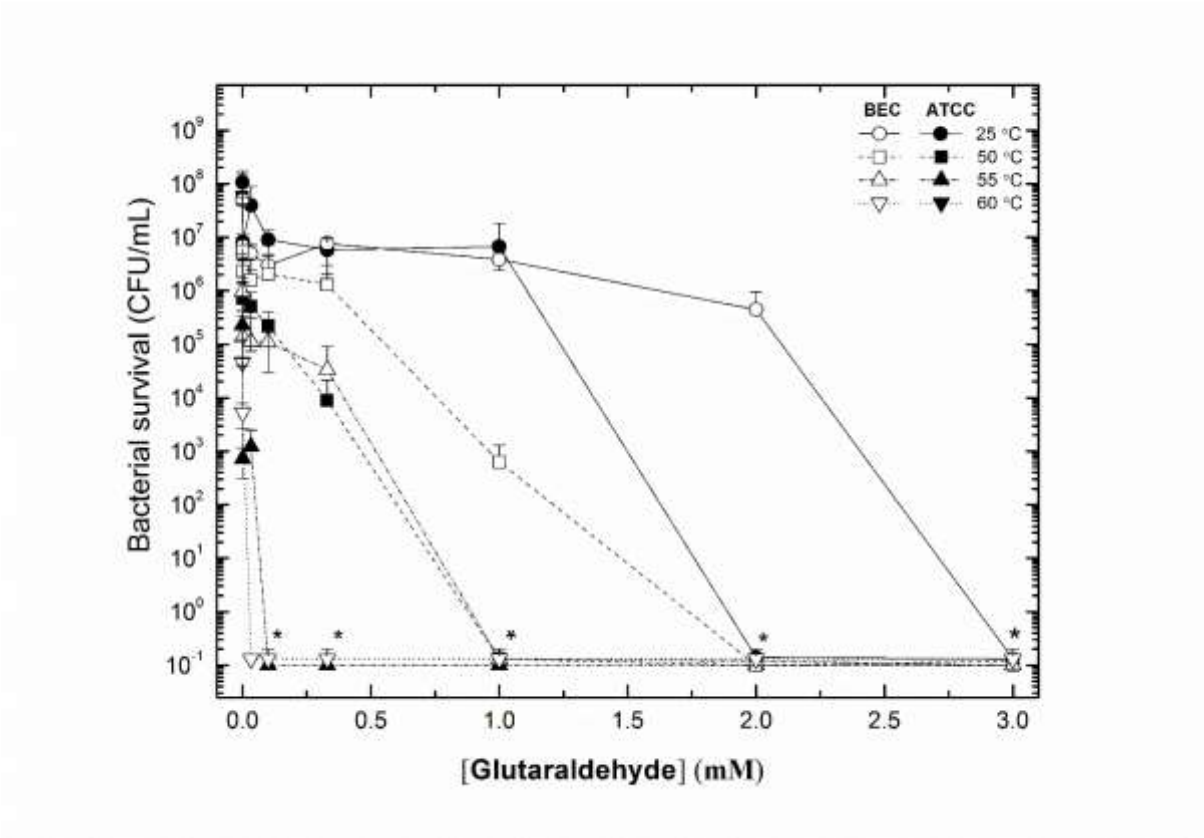

Fig. 2 Effect of GA on the inactivation of *S. aureus* strains ATCC 25923 and BEC 9393 at different temperatures (10 min exposure, pH 8.0). Asterisk: no bacteria detected. The error bars represent standard deviations (n=3).

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 ^{o}C and, was not affected by increasing the length of treatment to 60 min. At moderate temperature (50 ^{o}C), HHP caused inactivation in both strains that was 4-5 orders of magnitude greater than at 25 ^{o}C (Fig. 3). At 25 ^{o}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 ^{o}C and 300 MPa, 0.16 mM GA totally inactivated both strains, whereas, when tested separately, these conditions caused little or no inactivation.

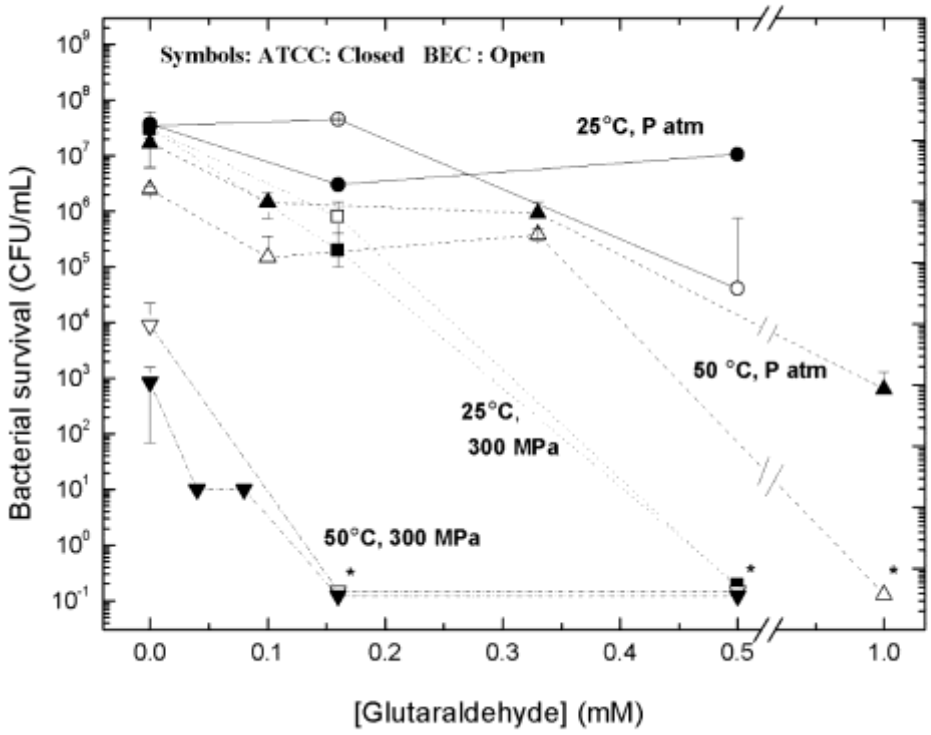

Fig. 3 Combined effect of HHP, GA and moderate temperature (50 ºC) on the inactivation of *S. aureus* strains ATCC 25923 and BEC 9393 after 10 min incubation. Asterisk: no bacteria detected. The error bars represent standard deviations (n=3). P_{atm}: atmospheric pressure.

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 ºC 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 ºC and HHP, and the respective untreated control. Total inactivation was observed in catheter with the same conditions of lenses.

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, P_{atm}	50 °C, 300 MPa (up to 24 h)		55 °C, P_{atm} (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.

BS: bacterial suspension. P_{atm}: atmospheric pressure.

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.

*: appearance of bacterial growth after 48 h.

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. N.D.: Experiments Not Done.

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 (Figure 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

Fig. 4a-d shows the morphological alterations induced by HHP in synergism with GA at room temperature. TEM 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 ºC 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.

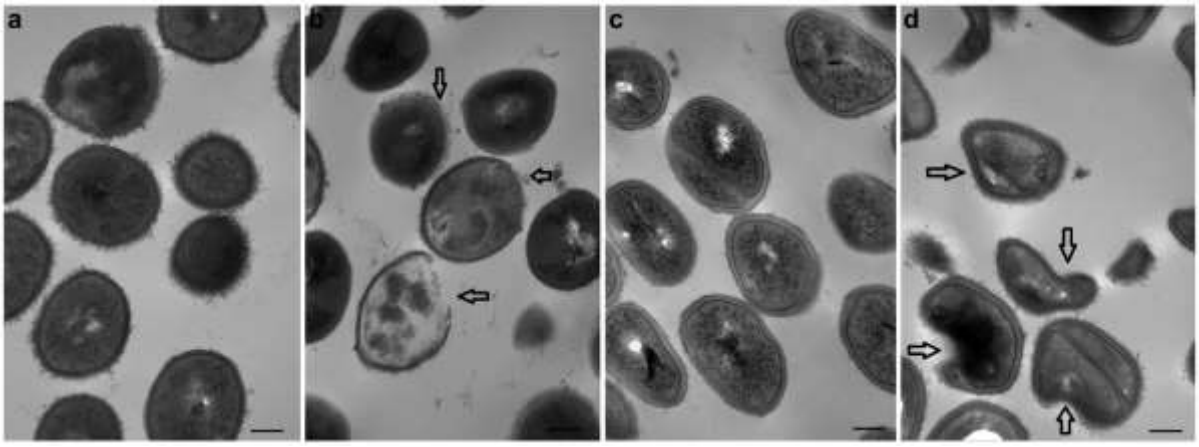

Fig. 4 TEM images of *S. aureus* ATCC 25923 (control (**a**) and pressurized (**b**) samples) and BEC 9393 (control (**c**) and pressurized (**d**) samples). The pressurization conditions for both strains were 300 MPa HHP at 25 ºC for 10 min in the presence of 0.5 mM GA. The arrows indicate morphological 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 ^{o}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. 5a-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. 5d-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 ($p<0.05$).

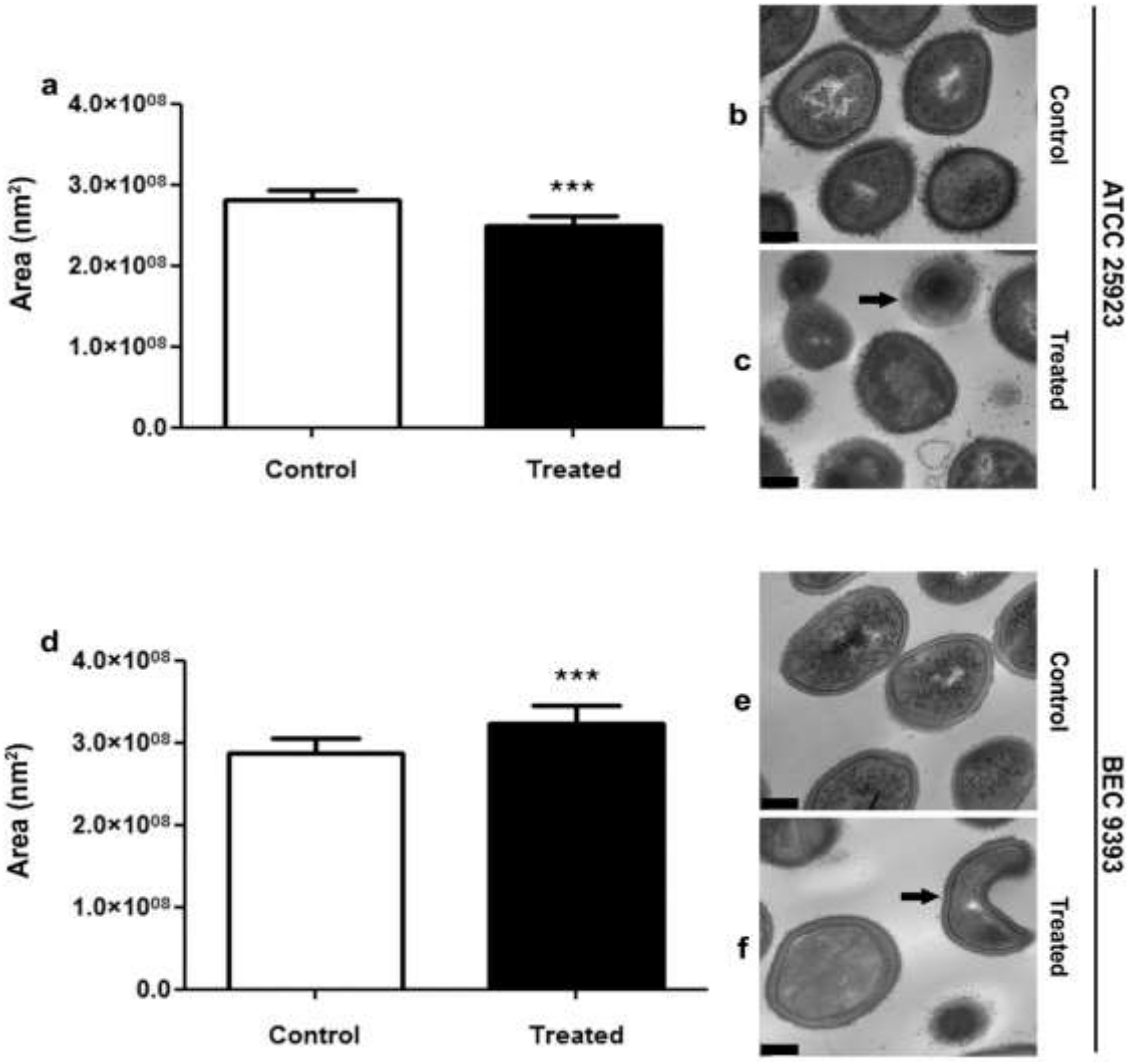

Fig. 5 Morphometric analysis of bacterial TEM images based on the change in bacterial surface area (nm^2) without (control) and with treatment with 0.5 mM GA and 300 MPa HHP and 25 ºC for 10 min of *S. aureus* ATCC 25923 (**a)** and BEC 9393 strain (d). The control and treated images are shown respectively 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.

Discussions

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 bacteria 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. 4S) 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 bio-materials. 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).

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. 5a-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 ^{o}C for 2 min or 75 ^{o}C for 1 min (Kennedy et al. 2005). Our temperature experiments showed marked bacterial inactivation between 55 ^{o}C and 60 ^{o}C, so we investigated the possible potentiation of HHP at a lower temperature (50 ^{o}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 ^{o}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 ^{o}C), or pH 4.0 or pH 9.0, and was less efficient at subzero temperature (-15 ^{o}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 ^{o}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 ^{o}C), as also reported by Gorman et al. (Gorman et al. 1980). The action of GA was very sensitive to an increase in temperature from 50 ^{o}C to 60 ^{o}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 ^{o}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 ^{o}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 crosslinking 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 crosslinking 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.

Competing interests' statement

The authors declare no competing interests.

Ethical statement

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

Funding information

This study was supported by Coordination for the Improvement of Higher Education Personnel (CAPES, grant no. PROEX0232084) and the São Paulo State Research Foundation (FAPESP).

CHAPTER: 3

Sensitivity susceptibility of *Pseudomonas aeruginosa* strains to high hydrostatics pressure and glutaraldehyde inactivation: correlation of morphological changes with AFM studies

To be submitted to *Langmuir*

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- **ACKNOWLEDGMENTS**

The authors thank Prof. Dr. Paulo P. Joazeiro, Ancelmo R. de Souza, Bianca G. Castelucci and Adriane C. S. Sprogis for their support during the electron microscopic analysis and for helpful discussions, Stephen Hyslop for editing the English of the manuscript, Carlos A. R. Costa and Evandro M. Lanzoni for their assistance during Atomic force microscopic analysis in Brazilian Nanotechnology National Laboratory (LNNano) at Centro Nacional de Pesquisa em Energia e Materias (CNPEM). This study was supported by The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, grant no. PROEX0232084).

ABSTRACT

In the hospital environment, the bacterium *Pseudomonas aeruginosa* is considered an important opportunistic pathogen, which is highly resistant, often causing infections in immunocompromised patients, as well as responsible for post-surgical infections, partly due to the inadequate sterilization and contamination of surgical equipment and biopharmaceutical solutions. There is a great concern for efficient sterilization of these materials, so the use of a technique called as high hydrostatic pressure (HHP) to inactivate microorganisms could be very effective, especially for the sterilization of temperature sensitive materials. Although the mechanisms of inactivation of microorganisms involved are not yet well established, yet there exists an indication of significant damage in the cell wall, ribosomes, cellular content and plasma membrane. In this study, we aimed to use high hydrostatic pressure treatment separately and in combination with disinfectant (glutaraldehyde) in low concentration and moderate temperature to check the synergistic effect for the sterilization of bacteria *Pseudomonas aeruginosa* (strains ATCC 27853 & NM 31). With subsequent treatments the observations detailed the sensitivity of ATCC 27853 strain only with 250 MPa HHP at 25℃ while NM 31 kept resistant. The synergistic combination treatment completely inactivates both bacterial strains on 0.01 mM GA, 150-300 MPa HHP at 50℃ in 10 min of exposure suggesting high efficacy of this method. High-resolution Transmission electron and Atomic Force Microscopy observations further supplemented the high impact of subsequent treatments for bacterial planktonic cell as well as biofilm. This technique draws attention to the biotechnological aspects of bacterial classification and certainly quality control measures including safe sterilization of the medical and biopharmaceutical manipulations.

KEY WORDS:

Pseudomonas aeruginosa, High hydrostatic pressure-sensitivity, Atomic force microscopy, transmission electron microscopy, synergistic effect, Biofilm.

▪ INTRODUCTION

Pseudomonas aeruginosa is a gram-negative pathogen that has become an important cause of infections, especially in patients with compromised defence mechanisms. It is frequently related to nosocomial infections such as pneumonia, urinary tract infections (UTIs) and bacteraemia. The biofilm formed by the bacteria allow them to adhere to any surface, living or non-living and thus *Pseudomona*s infections can occur in any part of the body. Further, the adaptive and genetic changes of the micro-organisms within the biofilm make them resistant to all known antimicrobial agents making the *Pseudomonas* infections complicated and life threatening (Sharma et al. 2014). In medical and surgical materials, several bacteria make biofilms. This work is based on inactivation of some pathogenic bacteria *Pseudomonas aeruginosa* that are responsible for making biofilm in medically important materials and biopharmaceutical solutions by forming thick exopolysaccharide (EPS) matrix, which helps them to escape from external stress environment and shocks i.e. antibiotics, phagocytosis and majorly host immune system by acquiring resistance genetically. These environmental bacteria can form biofilms on a variety of living and non-living surfaces such as the mucous plugs of the CF lung, contaminated catheters, and contact lenses (Toole et al. 2000; Høiby et al. 2010). The first stage of biofilm formation is attachment with the surface followed by micro-colony formation, maturation to form mushroom shaped structures, extension and propagation. Once the cells attach onto the surface, the micro-colonies pledge differentiating into a complex structure with overruling water channels (Müsken et al. 2010). There are some risk factors involved in *P. aeruginosa* infections for example, the use of antimicrobial drugs and the number of days of antibiotic therapy before positive blood culture in neonates intensive care unit (NICUs), exposure to particular health-care personnel, transfusion to blood products and intravenous injections (Jefferies et al. 2012). They are–also responsible for ventilator and, bronchitis-associated pneumonia in adults (Fujitani et al. 2011).

P. aeruginosa multi-drug resistance independently predicted higher hospital costs with a more than 70% increase per admission compared with non-resistant strains (Morales et al. 2012). Clinical isolate exhibits increased resistance to antibiotics making it almost impossible to treat *Pseudomonas* infection e.g. resistance to ciprofloxacin (last-line antibacterial drug) (Chávez-Jacobo et al. 2018). Ultimately, this bacterium fetching reason for high epidemiological events. i.e. a quarter of hospital-acquired infections occurred due to prevalence of Extensively-Drug Resistant *P. aeruginosa* (XDR-PA) and higher mortality (Bendouah et al. 2006; Palavutitotai et al. 2018). Therefore, proper sterilization procedures of the temperature

sensitive medical instruments and biopharmaceutical materials are of great importance for the inactivation of pathogenic bacteria to minimize the risk factors associated with occurrence of infections (De Souza et al. 2013).

High hydrostatic pressure (HHP) is alternative technique of non-thermal sterilization and exploration of its working in different systems is being carried out extensively since last two decades. The application of high hydrostatic pressure (HHP) in medical and pharmaceutical industries has also been researched in the preservation of biopharmaceuticals, blood products, cells and organs for transplantation, being a promising technique in the near future (Masson et al. 2001; Gollwitzer et al. 2009; Rivalain et al. 2010). Various wild type or mutant strains of bacteria showed survival against HHP (Hauben et al. 1997; Vanlint et al. 2011; Hazael et al. 2014). Previously done physiological and molecular biology research of deep-sea barophilic bacteria evident occurrence of pressure resistant operon responsible for resistance to HHP (Horikoshi 1998).

Another factor of consideration is Glutaraldehyde (GA) disinfection for inactivation of pressure-resistant strains. Glutaraldehyde is a convincing disinfectant that is normally used to sanitize and fix tissues of organs and inanimate entities, medical and surgical materials, but it has some toxic effects similar to it deposition on the surfaces, it may cause eye, lung and respiratory tract irritation and spontaneous abortion. It causes blood coagulation. All of these features should be taken into account once represent a serious risk in health settings (Takigawa and Endo 2006).

Atomic Force Microscopy is a versatile image analysis technique, which provides significant images besides measuring various surface properties of organism on nanoscale level, for example: morphology, topography, molecular interactions over membranes, damages as a result of stress conditions (Eaton et al. 2008) or chemical treatments (Camesano et al. 2000). In addition, this can observe the molecular determinants (Razatos et al. 1998), and force that a bacterium possesses to adhere on any surface either animate or inanimate (Milles et al. 2018), from single cell to bacteria in community i.e. in biofilm (Wright et al. 2010). We studied two strains of *Pseudomonas aeruginosa* ATCC 27853 and NM 31 (non-mucoid strain) to check their sensitivity and resistance, with an aim to achieve total inactivation by High hydrostatic pressure followed by determination of structural changes on bacterial cell surface by virtue of AFM.

▪ EXPERIMENTAL SECTION

Bacterial strains, inoculum preparation and quantification

For this study, strains of *Pseudomonas aeruginosa* ATCC® 27853™ and NM 31 were collected from department of Biotechnology, Institute of Biology, and Hospital das Clínicas (HC), UNICAMP, respectively. Bacterial isolates were cultured in BHI (Brain heart infusion) broth to prepare stocks by incubating overnight at 37 ℃, next day these cultures were added with glycerol in 1:10 ratio and stored at -80℃ in bio freezer for forthcoming experiments. Inoculum for each experiment was prepared by adding stock bacterial suspension in 5 mL of tryptic soy broth (TSB; Difco-BD) at 37 ℃ 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 4,000 *g* for 15 min; the resulting supernatant was discarded. Pellet bacterial cells were suspended in 0.9% (w/v) saline solution to achieve an estimated concentration of 10^9 cells/mL, which corresponds to an OD_{660} of 1.5 (Beckman DU640, Beckman Instruments, CA, USA), to be used in the experiments.

Quantification of bacterial viability 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 18-22 h incubation at 37 ºC in a biosafety incubator. All graphs were plotted on origin8 pro software by using mean and standard deviation of at least 3 independent experiments.

Temperature kinetics profiling

Survival profile of *P. aeruginosa* strains against different temperatures was analysed using 16-18 hr stationary phase bacterial cultures in TSB medium from above mentioned stock cultures. Bacterial suspension was exposed to different ranges of temperatures categorized as moderate i.e. ranges from 40 ℃ to 65 ℃ for 10 min in water bath and controls were kept untreated at room temperature. Both temperature exposed and unexposed samples were then serially diluted in 0.9% saline solution followed by plating on TSB plates and incubation at 37 ℃ for 24h. After 24 hr of incubation bacterial colonies were counted as colony forming unit (CFU/mL).

Effect of different GA concentrations at room temperature

The methodology for GA analysis was adopted to preserve the approach to minimize the use of higher concentrations of GA to reduce its toxic effects. The bacterial suspension of 1.4 OD_{660} that corresponds to $1.27x10^9$ cells/ mL was used to find survival kinetics against different concentrations of GA at room temperature (25 ℃). For this purpose, GA (J.T. Baker®) solution was prepared in sterilized distilled water from 0.001 to 29.4 mM final concentration from 25% stock solution. Before each treatment, GA solution was added with PBS of pH= 8.0 to activate for 2 min and then bacterial suspension was added for 10 min to check its antibacterial activity (Mcdonnel and Russell 2005; De Souza et al. 2013; Sehmi et al. 2016). After 10 min, 100 µL of this mixture was added to 900 µL of 3% glycine (Sigma®) solution to neutralize the effect of GA, as GA possess time dependent activity (Cheung and Brown 1982). The untreated and treated neutralized mixture was then serially diluted in 0.9% saline solution and plated on TSB plated for 24 h at 37 ℃.

Exposure to moderate temperature with GA

Antibacterial activity kinetics of *P. aeruginosa* strains was studied in this work by incubating bacterial suspension to six selected GA concentrations (0.001, 0.010, 0.100, 0.900, 1.000 and 2.000 mM) with and without combination with moderate temperature i.e. 55 ℃ for 10 min of exposure time using water bath. Afterwards, neutralization with 3% glycine was carried out as mentioned in earlier GA inhibitory assay. Subsequently samples were diluted in 0.9% saline, plated on TSB plates and incubated for 24 h at 37 ℃. Quantification was made using CFU/mL criteria.

High hydrostatic pressure (HHP) system

The HHP equipment (HIP model 37-5.75-60; Erie, PA, USA) connected with water bath (Marconi) supplied with controlled temperature, as well as the experimental method used in this study have been described earlier (Silva et al. 1989; Santos et al. 2004; Bispo et al. 2007; De Souza et al. 2013). The pressure and temperature were continuously monitored during assay, whereas, the sample used were sealed in pressure shock proof polyethylene bags.

The bacteria were treated under high pressure for different time of exposure in the suspension prepared with 0.9% saline because it exhibited negligible effect on bacterial surrounding medium.

P. aeruginosa exposure to HHP under different time ranges

Both above mentioned strains of *P. aeruginosa* (ATCC 27853 & NM 31) in suspension of 1.4 OD_{660} turbidity 2.70x 10^9 cells CFU/mL, were subjected to 250 and 300 MPa HHP for 5, 15, 30, and 60 minutes time of exposure in high pressure chamber at room temperature i.e. 25 ℃ (De Souza et al. 2013).

P. aeruginosa ATCC 27853 sensitivity testing to HHP

Sensitivity test of ATCC 27853 strain of *P. aeruginosa* was carried out by exposing bacterial suspension to different ranges of HHP (50, 100, 150, 200, 250 and 350 MPa) (Dommerich et al. 2012; Yang et al. 2016) for constant time i.e. 10 min, and controls were remained untreated. Later on, both treated and untreated samples were serially diluted in saline and plated on TSB plates followed by incubation for 24 h at 37 ℃.

Combined HHP, temperature and GA treatments

After exclusive temperature, GA and HHP survival sketching of both strains of *P. aeruginosa* (De Souza et al. 2013), selected values of all these factors were critically perceived to acquire complete inactivation and synergistic effect was observed in relation to temperature, GA and HHP.

In vitro Biofilm assays with catheter fragments

In previous studies, *P. aeruginosa* ATCC 27853 strain was reported as strong biofilm producer (Joe J. Harrison and Ceri 2005; Piasecki et al. 2013). So we utilized this ATCC 27853 strain to perform *in vitro* biofilm assay on carrier material with certain modification from Cole et al. (2014). Carrier material utilized was catheter (Jiangsu Jichun Medical Devices Co. Ltd., Jiangsu Province, China) fragments of 1 cm length. For *in vitro* biofilm formation, carrier materials were incubated with the ATCC 27853 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 cautiously 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 treatment with cell suspensions. The treated carrier materials were again incubated in fresh TSB for 24 h at 37 °C, through 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 treatment, 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.

Transmission electron microscopy

For transmission electron microscopy (TEM), both ATCC & NM strains of *P. aeruginosa* were treated with 300 MPa for 10 min at 25 °C. Treated and untreated 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 7,000 g. The pellets were then washed and the samples were prepared as previously described (Durães-Carvalho et al. 2012).

Bacterial preparations & immobilization for AFM

For mapping bacterial cell morphology and to evaluate their mechanical properties with atomic force microscopy. Park NX10, Park Systems Inc. Santa Clara, CA, USA and Nanosurf AG, Gräubernstrasse, Liestal, Switzerland was used. Both strains of *P. aeruginosa* in suspensions were tested in the presence of milli-Q water instead of saline to avoid salt crystal interference while capturing images. These suspensions of 0.4-0.5 OD_{600} were subjected to 300 MPA at 25 °C for 10 min in high pressure chamber, subsequent to removal from HHP chamber, 5 µL of exposed and unexposed samples were deposited on the fresh cleaved mica surface and allowed to dry for 10 min. Biofilm was formed using ATCC 27853 strain, inoculate overnight in BHI broth for 24 h at 37°C, next day thick exopolysaccharide biofilm material was exposed to 3.67 mM of GA, 300 MPa at 50°C for 10 min. The untreated and treated biofilm material were then reinoculated to fresh BHI broth for 24 h incubation at 37°C. Material from these tubes were separated by help of loop, fixed over the surface on mica for AFM analysis. After the exposures (or following the exposure), ATCC 27853 treated and untreated biofilm material was fixed over mica surface by putting with the help of loop and allowed to dry for 10 min prior to imaging. Both bacterial suspension and biofilm fixed mica surfaces were analyzed on tapping mode. Each image took almost 20-25 min to appear completely on computer screen. The tip model was PPP-FMR from Nanosensors with nominal resonance 75kHz and nominal force constant 2.8 N/m (Xie et al. 2016). The bacterial plantonic cells (ATCC 27853 & NM 31) images were initially measured in 15 µm and finally with 5 µm resolution and ATCC 27853 biofilm was observed in 20 µm, capturing the fields that contained large number cells in that

confined area to the isolated cell to study surface topology of single cell. To treat AFM images offline, Gwyddion 2.50 software was used, distance across cells verses height was measured in the treated and untreated biofilm surface.

Antibiotic susceptibility testing

The antibiotic susceptibility of the *P. aeruginosa* 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 overnight (12-14 h) at 37 ℃, 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 ℃, 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 ^{o}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 (Alagumaruthanayagam et al. 2009).

▪ RESULTS AND DISCUSSION

To analyse the effect of combination of HHP with other factors, chemical, Gluataraldehyde and physical, temperature, the survival profiling against each factor was performed. Starting from the very first physical and an abiotic factor i.e. temperature, the results obtained showed a preserved survival profile of both strains from 25 to 55 ℃, after this range drastic decrease in growth of both strains was prominently recorded at 60 and 65 ℃, that leads to total bacterial inactivation (Figure 1). The untreated controls for both strains were noted at room temperature (25 ℃). Various studies already mentioned *P. aeruginosa* growth conditions in relation to different temperature where, *P. aeruginosa* PAO1 grows well at 37℃ (Chan et al. 2016). Contrarily, the growth starts reducing with the increase in temperature. However, in observation of this recent result, *P. aeruginosa*, both NM 31 and ATCC 27853 strains showed

stable growth in the range of 40-55 ℃, bearing characteristic resistance to elevated temperature (Figure 1).

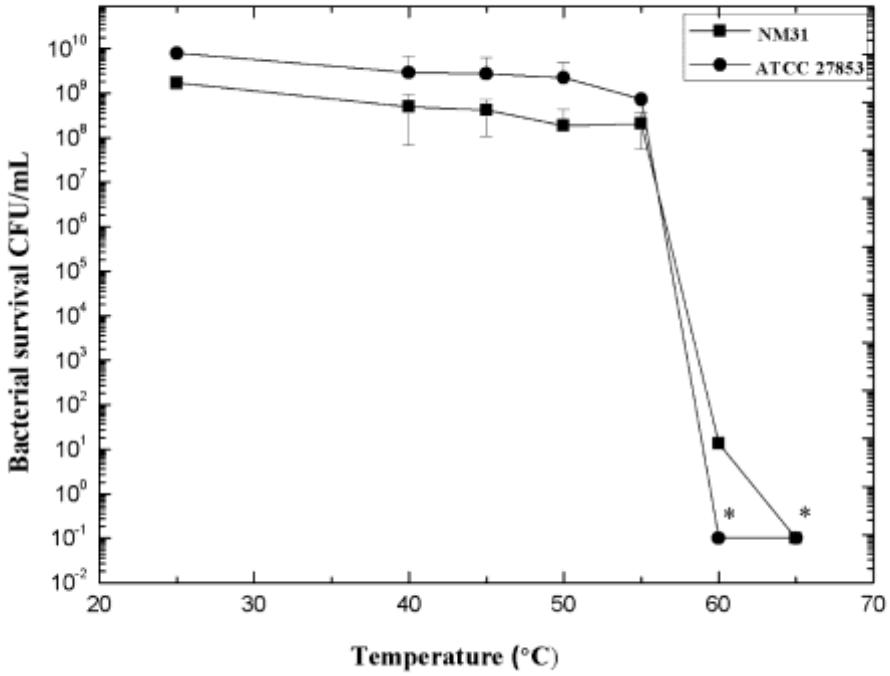

Figure 1. Effect of temperature on survival of *Pseudomonas aeruginosa* strains. Error bars corresponds to mean and standard deviation, Where n= 3. And total inactivation of bacterial is indicated with (⁎) asterisks.

Glutaraldehyde (GA), being an efficient disinfectant has very fast antibacterial activity i.e. 1-10 min for different microorganism like bacteria (vegetative cell and spores), viruses and fungi through 2% alkaline aqueous solution (Gorman et al. 1980). However, it is being widely used as cross-linking agent when it comes to improvement in biomaterials prior to transplantation though possession of cytotoxicity as well (Reddy et al. 2015). GA has time dependent activity against microorganisms and 3% glycine is normally used to neutralize its effects (Cheung and Brown 1982). In this study, GA was evaluated for its bactericidal activity, the resulting total inactivation of both strains of *P. aeruginosa* was achieved with 3.67 mM concentration of GA at 25 ℃ in 10 min, with the reduction of ≥8 log magnitude in bacterial

growth (Figure 2). In comparison with (Gorman et al. 1980), the results are relatively similar which comprises that up to 4 mM concentration of GA inactivate *P. aeruginosa* and other bacterial species.

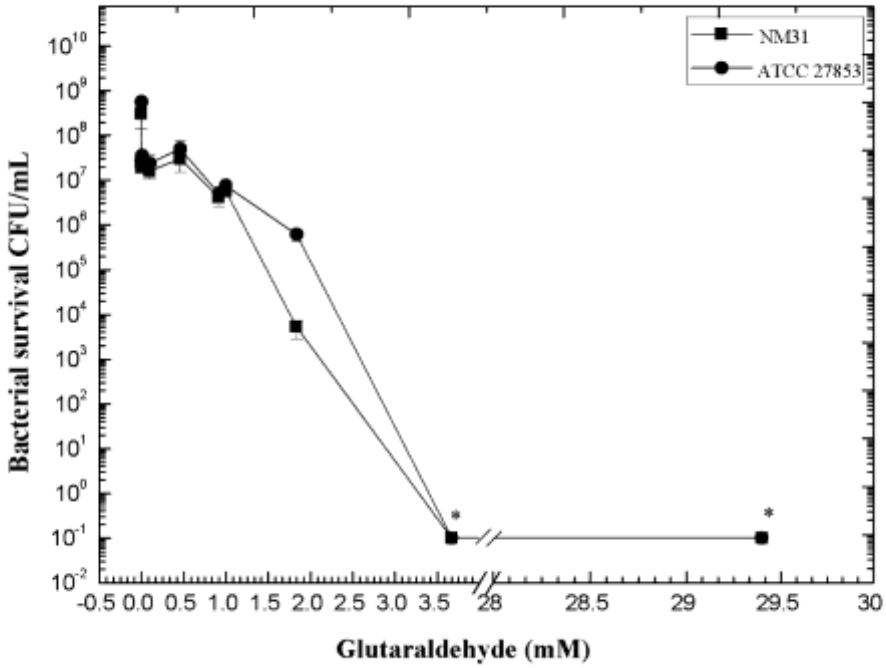

Figure 2. Survival curves of *Pseudomonas aeruginosa* strains ATCC 27853 and NM 31 under exposure of different concentration of disinfectant Glutaraldehyde at room temperature 25 ℃. Error bars corresponds to mean and standard deviation, Where n= 3. And total inactivation of bacterial is indicated with (⁕) asterisks.

In order to verify the combination of moderate temperature and GA, both strains of *P. aeruginosa* were subjected to 55 ℃ in the presence of different concentrations of alkaline GA solution, consequently, the total inactivation of ≥ 8 logs CFU/mL was noted in lesser concentration i.e. 2mM of GA in comparison to total elimination observed at room temperature (25 ℃) (Figure 3). The temperature more than ≥ 54 ℃ was evaluated previously (Sierra and Boucher 1971), in synergism with lower concentrations of GA, which results in high-density inactivation of bacterial spores. Such studies were conducted to overcome the hazards directly or indirectly ended up with environmental fate, (Leung 2001) which demonstrated the toxic effects of GA to marine life after its disposal. Although the current study demonstrates its

novelty as GA was never investigated before in combination with moderate temperature and HHP for bacterial inactivation. In another literature (Blenkinsopp et al. 1992), low-strength electric current was applied in the presence of lower concentration (lower than normally required without combination with low-density current) of GA to eliminate *P. aeruginosa* biofilm developed over stainless steel. After analysing GA and temperature effect we select 50℃ to further treat in the combination of HHP to gain complete inactivation on maximum moderate conditions of treatment.

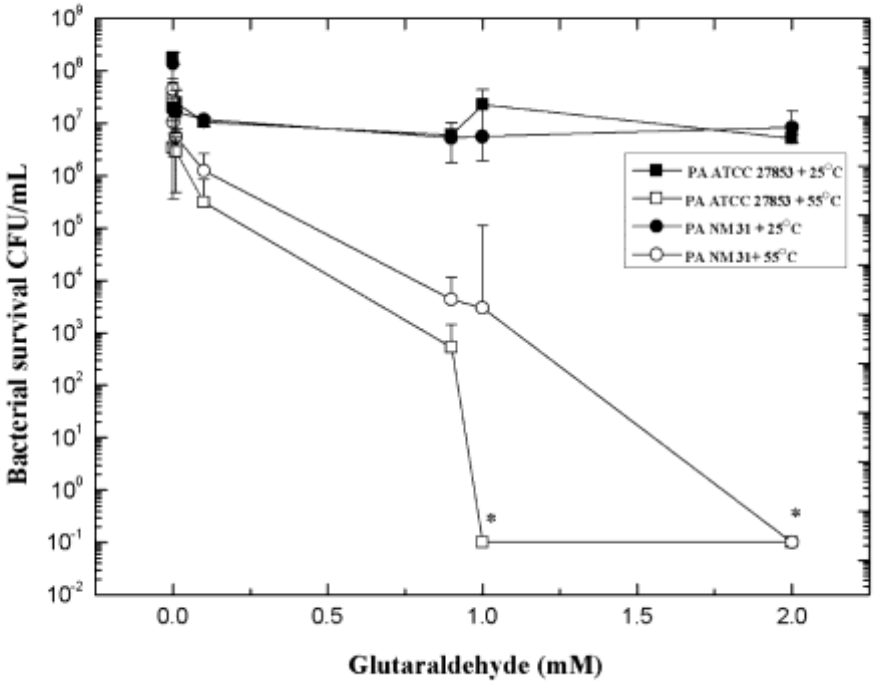

Figure 3. Evaluation of GA effect in combination with moderate temperature (55 ℃) for *P. aeruginosa* strains for 10 min. Error bars corresponds to mean and standard deviation, Where, n= 3 individual experiments and total inactivation of bacterial is denoted with (⁎) asterisks.

Different bacteria respond variably towards varying stress conditions, acquiring resistance or showing sensitivity. High hydrostatic pressure stress sensitivity was examined using both recruited strains and applied 250 and 300 MPa pressure at 25 ℃ for different time of exposure, after the treatment, serial dilution and plating, 24 h incubation results showed ATCC 27853 strain completely sensitive to 250 MPa even with 5 min exposure and onwards,

which is a significant property of this strain. whereas NM 31 strain remained prominently resistant to both 250 and 300 MPa from 5- 60 min exposures and showed a neglected reduction of one log magnitude in bacterial growth (Figure 4). A past study (Govers and Aertsen 2015) suggested dominant inactivation of *E. coli* bacteria from lower to elevated level of high hydrostatic pressure by the help of time-lapse fluorescence microscopy revealing increased protein aggregation and change in cellular dynamics after 15 min of contact time. Related level of HHP was analysed previously by Durães-Carvalho et al. (2012) using 250 and 350 MPa at 25 ℃ and up to 30 min of exposure, total inactivation of *A. hydrophila* strain AH 191 was highly distinguished. Discussing the difference from the past reviews about HHP sensitivity among gram-negative bacteria, (Pilavtepe-Çelik et al. 2008) concluded that gram negative bacteria are more sensitive to HHP with the reason of having less cell wall content but the resistance of NM 31 being gram-negative bacteria, from current work completely differ with this image (Figure 4).

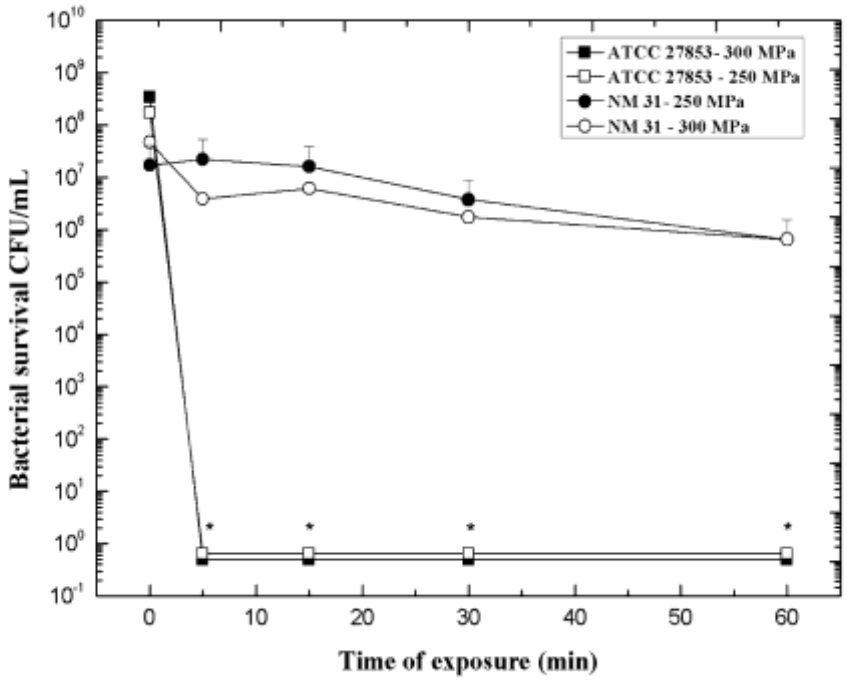

Figure 4. Assessment of HHP sensitivity of both *P. aeruginosa* strains ATCC 27853 and NM 31 to 250 & 300 MPa at room temperature (25 ℃) for different time of exposure. Errors bars corresponds to mean and standard deviation, where n= 3 individual experiments and total inactivation of bacterial is denoted with (⁎) asterisks.

Sensitivity of ATCC 27853 strain to elevated pressures prompted testing at gradually lowered levels of high pressure. In this regard, above mentioned Pseudomonas strain was challenged with different ranges of HHP for 10 min, the results show a continued resistance of bacterial growth from 50-150 MPa pressure while after 200 MPa starts declining (4 log CFU/mL) significantly, although, pressure of 250 MPa and onwards showed overall inactivation of ≥ 8 log CFU/mL magnitude (Figure 5). Previous study also reported this type of inactivation of *Mycobacterium abscessus* at 250 MPa and higher pressure, showing drastic decrease in inactivation following total elimination of bacteria (De Souza et al. 2013). Application of 200- 250 MPa high-pressure was made to inactivate different pathogens (bacteria and viruses) in plasma preceding to transfusion, results from their study confirmed the inactivation of 7.5-8 log reduction of *E. coli* and *B. subtilis* conserving porcine parvo virus and *S. aureus* resistant closer to 0 ℃ (Yang et al. 2016).

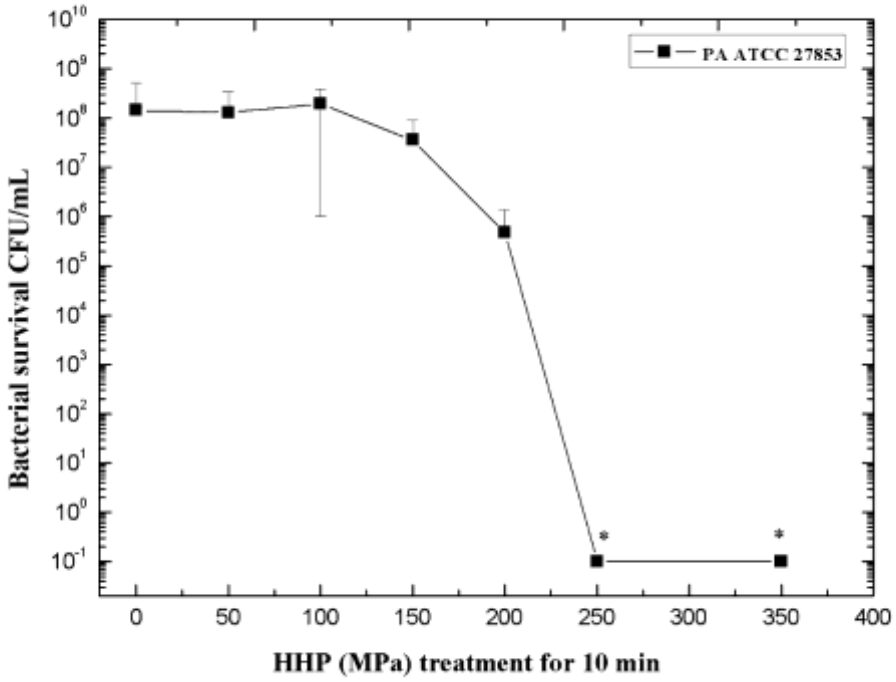

Figure 5. Sensitivity of *P. aeruginosa* ATCC 27853 strain at different values of High hydrostatic pressure (MPa) for 10 mins time of exposure at 25 ℃. Errors bars corresponds to mean and standard deviation, where n= 3 individual experiments and total inactivation of bacterial is denoted with (*) asterisks.

Previously assessed HHP, GA and temperature kinetic curves of both *P. aeruginosa* strains of our study were employed to hand-picked the values that exhibit 75% bacterial growth, furthermore, moderate for biomaterial treatment to associate all three factors together aiming overall inactivation. Consequently, strain NM 31 when experienced treatment with 0.01 mM GA, and 300 MPa pressure at 50 ℃ for 10 min suffered reduction of ≥8 log CFU/mL magnitude in bacterial growth, while when these conditions were implemented individually, demonstrated no difference in decrease in growth. Moreover, treatment of NM strain with 300 MPa at 50℃ showed significant difference and presented 5 log CFU/mL reduction in 10 min. Although other combinations of abovementioned stress conditions did not yield substantial difference (Figure 6).

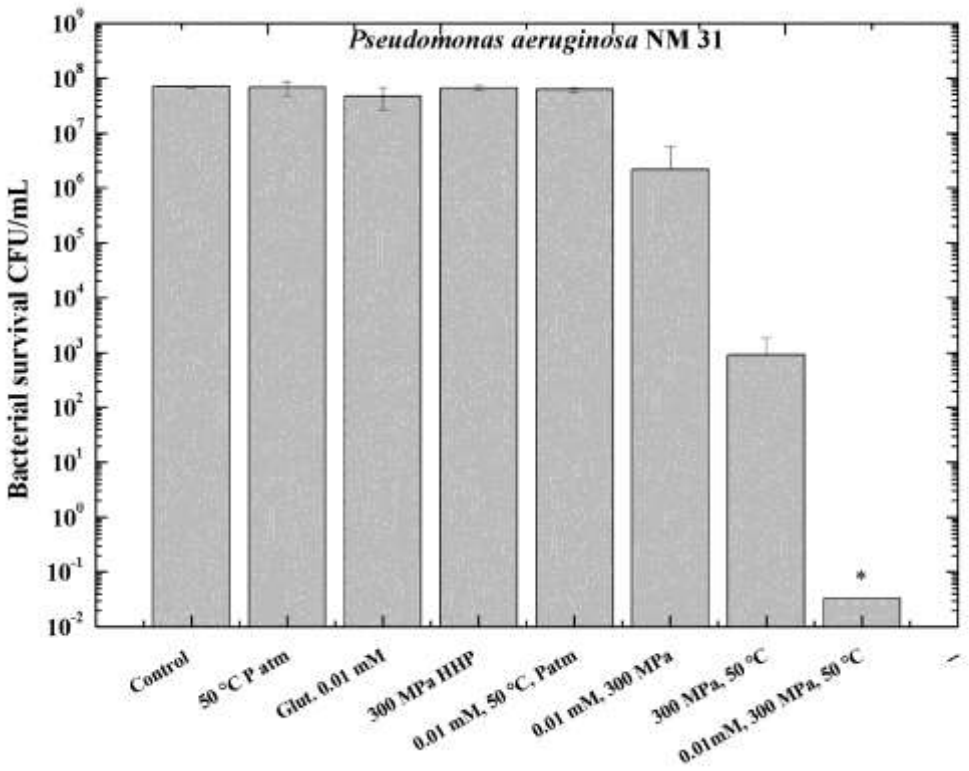

Figure 6. Representative synergistic effect of 0.01 mM GA, and 300 MPa high pressure at moderate temperature 50 ℃ on bacterial inactivation with all controls of GA, temperature and HHP exclusive and in combinations. Errors bars demonstrating the mean and standard deviation of n= 3 individual experiments and asterisks (⁎) showing total inactivation.

In case of ATCC 27853 strain, we selected 150 MPa pressure value when we consider to combine with lower GA concentration i.e. 0.01 mM at 50℃, as this strain was already sensitive to ≤ 250 MPa, we observed its (ATCC strain) sensitivity to various combined factors simultaneously. The resultant inactivation measured was ≥ 8 log CFU/mL magnitude in bacterial survival. The ATCC strain comparatively demonstrated the 4-5 log CFU/mL reduction when 150 MPa was combined in 0.01 mM GA and 50℃ separately (Figure 7), we used this pressure as ATCC 27853 strain is already sensitive to pressure > 200 MPa. In past several studies, researchers implemented HHP in various combinations for example mild heat, Carbon dioxide (CO_2), nitric acid etc. Synergism of mild heat was assessed in many schemes in order to potentialize the bactericidal activity, in a recently reported study (de Carvalho et al. 2018) developed a methodology by collecting essential oils (Nano-emulsions of peppermint), pulse electric field (PEF), mild heat and HHP to inactivate *E. coli*, the treatment of 15 min exposure to this method results in 5- $\log^{10}$ reduction in bacterial inactivation cycles. This kind of cooperative interaction was gauged earlier in another work (Moody et al. 2014) consuming HHP, pulsed electric fields and ultrasound against *E. coli* aimed to food preservation, they concluded that the best treatment that show 7 log decrease in bacterial survival was HHP (300-600 MPa for 1 min). Food model based HHP conducted study is important as biomaterials are sharing same sensitivity to high temperature exposures concerning bacterial inactivation. (Patterson et al. 1995) Tested different vegetative pathogens in phosphate-buffer saline solution under different ranges of HHP 275-700 MPa for 15 min and observed *Yersinia enterocolitica* as most sensitive at 275 MPa, while rest of pathogens (*S. typhimurium, L. monocytogenes, S. aureus and E. coli*) needed elevated pressure treatment for 15 min. Additive effect of HHP, pulse electric field (PEF) and sonication was screened at 400 MPa for 100 sec resulted in reduction of 8 log CFU of *Listeria innocua* in water (Pyatkovskyy et al. 2018).

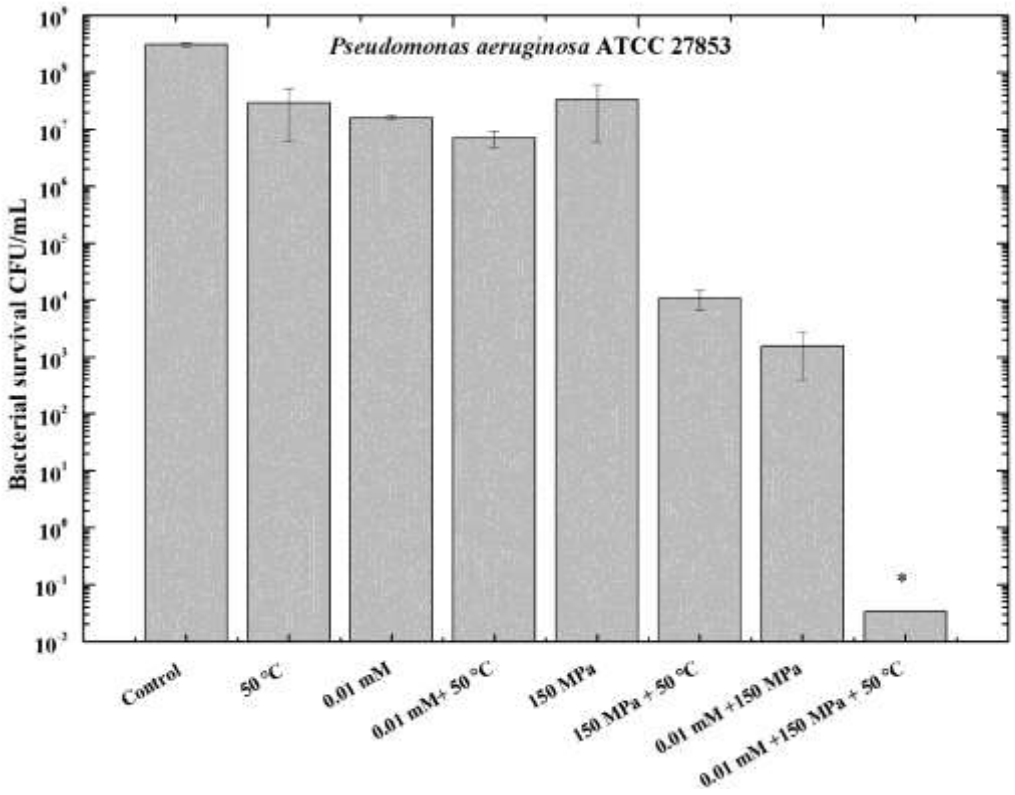

Figure 7. Illustrative synergistic effect of 0.01 mM GA, and 300 MPa high pressure at moderate temperature 50 °C on bacterial inactivation with all controls of GA, temperature and HHP exclusive and in combinations. Errors bars demonstrating the mean and standard deviation of n= 3 individual experiments and asterisks (*) showing total inactivation.

To identify structural and morphological changes among both strains, we performed transmission electron microscopy (TEM) before and after treatment with 300 MPa for 10 min, the observation from analysis depicted morphological damages inside cell cytoplasm and ribosomes. Moreover, it caused noticeable destruction of cell wall and membrane in ATCC (HHP sensitive) strain. Contrarily, NM 31 strain reserved its integrity overall before and after treatment with HHP, proving its resistance property (Figure 8). *Mycobacterium* strains (De Souza et al. 2013) and *Aeromonas* AH 191 (Durães-Carvalho et al. 2012) were studied under transmission electron microscopy after HHP exposure, that revealed distinctive damage to cell membrane and leakage of cell cytoplasmic content to surrounding, suggestive of sensitivity to HHP. This correlation of HHP treatment with cellular damage was described using *S. epidermidis* illustrating significant change in cell shape and structure after 10 min exposure with 540 MPa (Dommerich et al. 2012).

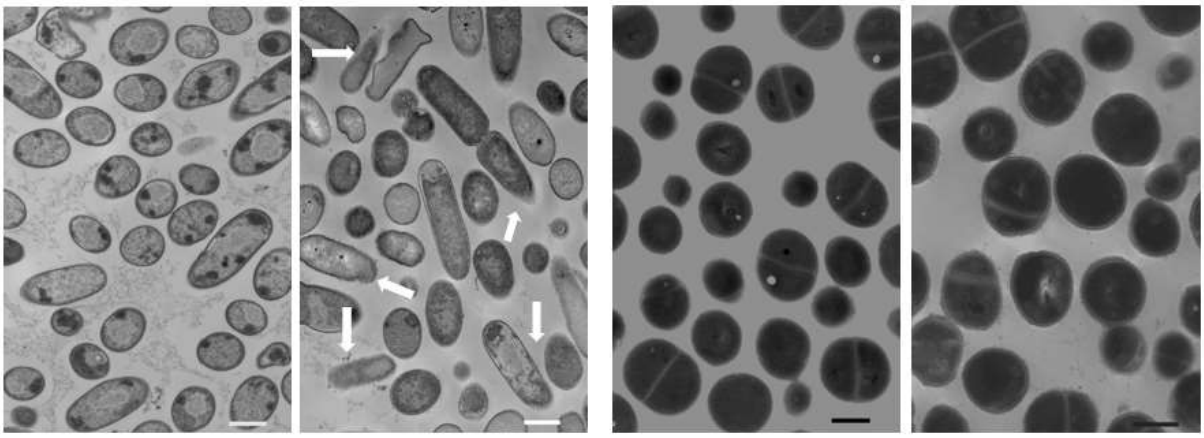

Figure 8. Transmission electron microscopic observations of *P. aeruginosa* strains incubated under 300 MPa, at room temperature for 10 min; ATCC 27853 untreated cells kept intact (A), while treated cells demonstrated prominent damages (B) indicated with arrow. NM 31 strain exhibited comprehensive resistance sharing among both control (C) and treated cells (D). The Scale *bars* indicating 2 μm.

Another type of microscopic technique, useful for three-dimensional image to study biological structures is called Atomic force microscopy, is exploited in this work. Figure 9 represents cell suspension of untreated and treated bacteria (ATCC 27853 and NM 31) with 300 MPa pressure at 25 ℃ for 10 min. The untreated bacterial cells in Milli-Q water displayed reproducible and intact surface structure, furthermore, the bacterial polar flagella appeared undamaged (Figure 9A and 9C), whereas, treated cells of ATCC strain showed significant irregular surface, damages and abnormalities as well as leakage of cytoplasmic content due to multiple cleavages (Figure 9B). NM 31 strain showed no damage on surface and structural properties for being resistant to HHP treatment. The centre of each cell was considered as a reference point to capture the whole image to give 3D impression. Changes in bacterial surface properties vary from species to specie, Xie et al. (2016) previously verified change is pattern of surface properties in *E. coli* and *B. subtilis* after high-pressure jet treatment. The importance of analysing bacterial adherence to different surfaces is gaining attention due to biomedical and pharmaceutical applications, for example, drug designing and antimicrobial surface attachment targets. Sahoo et al. (2016) frameworked single bacterial cell *of Xylella fastidiosa* adhesiveness using nanowires arrays to understand biofilm propagation on surface starting by single cell anchoring via atomic force microscopy. Through AFM imaging several surfaces can also be spotted to verify its properties and mode of action, in such intention examination of ultra-thin films and nanofilms of polyethylenimine and Hyaluronan/chitosan was performed respectively,

to find antibacterial effect against *S. aureus* and *P. aeruginosa* (Hernandez-Montelongo et al. 2016; Hernandez-Montelongo et al. 2017).

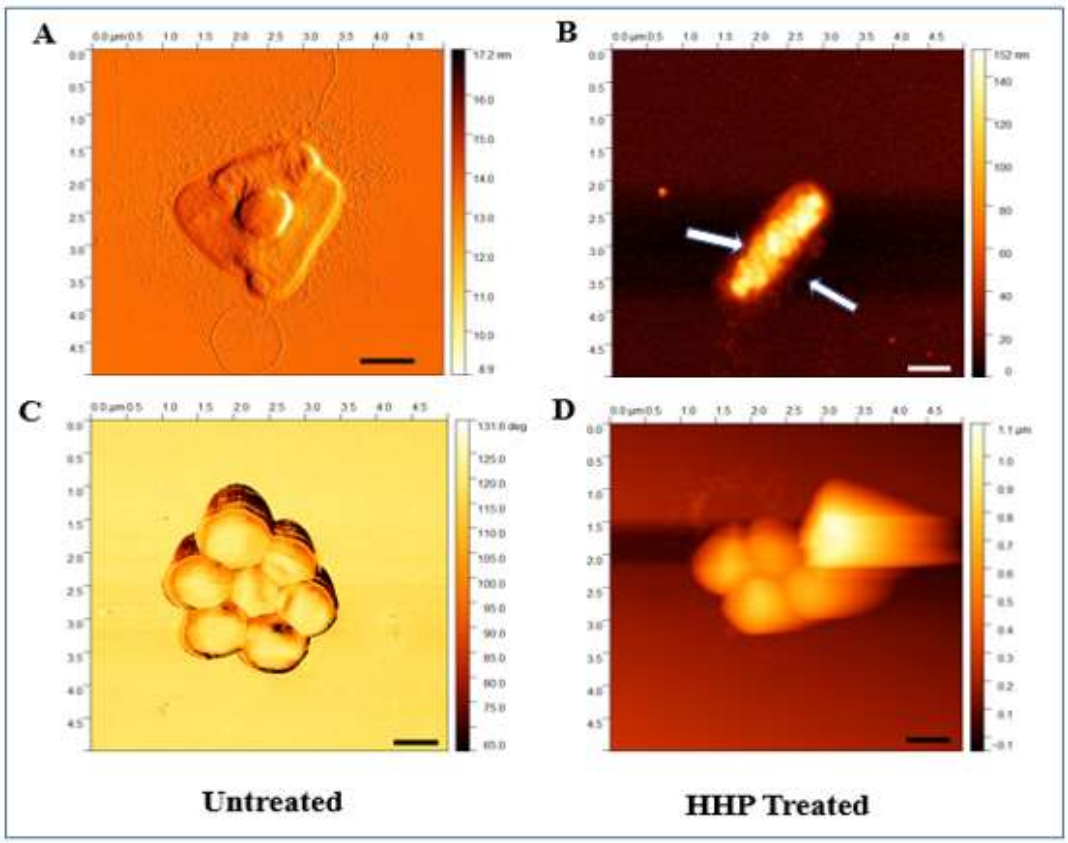

Figure 9. AFM investigation of *P. aeruginosa* strains with and without treatment under 300 MPa HHP at 25 ℃ for 10 min, showing (A) untreated (B) treated ATCC 27853 cells whereas (C) untreated and (D) treated NM 31 cells.

Biofilm formed by both strains of *P. aeruginosa* over the catheter surface was tested with different GA concentrations, 300 MPa at 50 ℃ for 10 min and reinoculated in fresh TSB liquid medium for 24 h. After 24-48 incubation, the recorded results exhibit that biofilm moulded by ATCC 27853 strain is much more resistance as compared to NM 31, later we associated these outcomes with bacterial suspension growth records and found that ATCC strain in biofilm is more resistant to external shocks since in cell suspension the total inactivation was achieved with less harsh condition i.e. 0.01 mM GA, 150 MPa at 50 ℃ for 10 min however, to inactivate biofilm embedded cells 3.67 mM GA, 300 MPa at 50 ℃ for 10 min was required. On the other hand, NM 31 strain remained sensitive at the same conditions that was critical to inactivate cells in suspension (Table 1, Appendix III). The inhibition of *P. aeruginosa* planktonic cells and

biofilm was evaluated earlier by epifluorescence microscopic method to find the effect of chlorine disinfectant and they determined that biofilms are more tolerant towards disinfectant than planktonic cells (Olszewska et al. 2016). Different cationic metals were assessed to eradicate *P. aeruginosa* ATCC 27853 planktonic cells and biofilm for 2 and 4 h exposure, resulting observations also again shows that biofilm thick material in 2-25 times more resistant to cationic metals effect in comparison with planktonic bacteria (Harrison et al. 2005).

Test with biofilm formed by ATCC 27853 strain was similarly analysed using AFM with and without treatment with 3.67 mM GA, 300 MPa HHP at 50 ℃ for 10 min in air. We selected these conditions as we attained inactivation of biofilm embedded cells in abovementioned conditions. Figure 10 expressed very well the changes in cells surrounded in biofilm before and after treatment, showing complete eradication of polysaccharide material in Figure 10B as compared to untreated sample. (Figure 10A) presents unbroken and compactly attached cells within biofilm. The cells that underwent combined GA, HHP and 50 ℃ treatments, display distinctive morphological changes, loss of agglomeration and detachment from biofilm material. Biofilm was measured using 20 µm resolution which is indicated with scale bar in both untreated and treated image. (Pelling et al. 2005) successfully visualized and characterized the bacterial cell and its extracellular components under AFM of *Myxococcus xanthus* in their native environment in fluid, highlighting the importance of AFM analysis capable of expressing ultrastructural and nanomechanical properties of bacteria. *Streptococcus mutans*, a main etiological agent of dental carries was observed in biofilm using AFM imaging to check their ability to acquired resistance against different antibiotics by identifying surface protein responsible for mutation (Cross et al. 2006).

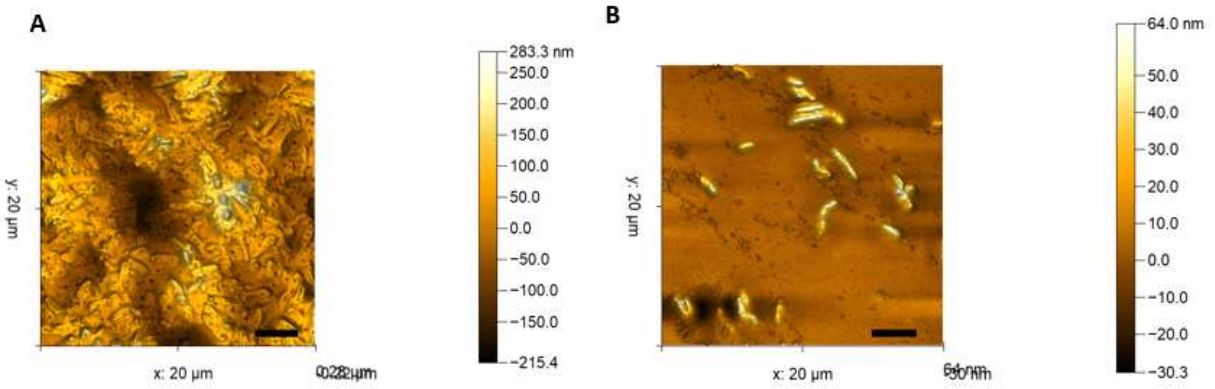

Figure 10. Topographical representation of *P. aeruginosa* ATCC 27853 biofilm, untreated (A) and treated (B) with 3.67 mM GA, 300 MPa high pressure at 50 ℃ for 10 min.

Antibiotic resistance for fifteen different antibiotics were profiled against both strains of *P. aeruginosa* ATCC 27853 and NM 31, the concluded results displayed high resistance of ATCC strain then NM towards gram negative specific antibiotics. NM 31 strain showed resistance only towards Ceftazidime and Aztreonam while staying susceptible and intermediate susceptible to all tested antibiotics (Appendix I-Table 2 or Figure 3S). The purpose to perform antibiogram for these strains was to check its susceptibility or resistance assuring these strains as threat to environment and to highlight the need of preventive measures against these bacterial strains meanwhile justifying and supplementing our work. *P. aeruginosa* is a common cause of hospital-acquired infection, therefore, the validation and review of epidemiological aspects is very important for disease management and control (Fujitani et al. 2011).

▪ CONCLUSIONS

On the basis of TEM and AFM analyses, we were able to visualize the surface and membrane level damages due to HHP synergistic combination effects. Material surface imaging can add valuable remarks to study this combination effects as well. *P. aeruginosa* inactivation with HHP and its combinations has broken the resistance standpoint and can open doors towards evaluating other bacterial species with such conditions. This preparation technique can be interesting for use in AFM analysis without limiting chemical interference for bacteria as well as for other variety of cells.

CHAPTER: 4

AFM analysis of *Klebsiella pneumoniae* inactivation induced by synergistic effect of high hydrostatic pressure

To be submitted to: Systematic and Applied Microbiology

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▪ ACKNOWLEDGMENTS

The authors thank Stephen Hyslop for editing the English of the manuscript and Carlos A. R. Costa and Evandro M. Lanzoni for their assistance during Atomic force microscopic analysis in National Laboratory of Nanotechnology (LNNano) at Centro Nacional de Pesquisa em Energia e Materias (CNPEM). This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, grant no. PROEX0232084).

ABTRACT

Klebsiella pneumoniae is an opportunistic pathogen, important cause of hospital-acquired infections due to its capability of attaining resistance to multiple antibiotic, some of them are classified as MDR-KP (Multi-drug resistance *Klebsiella pneumoniae*) and carbapenems-resistant Klebsiella. Concerning to high risk factor for infection and resistance to several biocides and disinfectants, emphasized other alternative technique to combat with their infections. High hydrostatic pressure is a cold sterilization technique, its impact varies from specie to specie among bacteria. In this study, we assessed two different strains of *Klebsiella pneumoniae* with HHP separate and/or in combination with glutaraldehyde at moderate temperature to find its susceptibility with these exposures. Our findings show that solely 300 MPa of HHP is able to give significant inactivation of only ATCC BAA 1705 strain up to 5 log CFU/mL in 10 min of exposure, but in combination with 0.01- 0.05 mM GA at 50 ℃ for 10 min, 300 MPa hydrostatic pressure enabled to completely eliminate all bacterial cells with indicative reduction of ≥8 log CFU/mL magnitude. The alteration in Klebsiella treated cell surface provides indication of loss of viability and integrity of cell structures was analyzed using AFM. High pressure effect accoupled with AFM analysis suggest its impact on single bacterial cell and directing its attention towards biotechnological aspects and their impact to study nanotechnological parameters.

Keywords

Klebsiella pneumoniae inactivation, high hydrostatic pressure, synergistic effect, AFM, surface and membrane damages.

▪ INTRODUCTION

The genus Klebsiella and its most significant species, *K. pneumoniae*, have long been familiar as an important human pathogen. This specie has a longstanding association with serious hospital-associated infectious progressions including blood stream infections (BSI), respiratory tract ailments such as pneumonia and urinary tract infections. The majority of these infectious processes in the past have been linked to long-term hospitalization and/or invasive medical procedures in general acute care hospitals (Janda 2015). *Klebsiella pneumoniae* including other *Enterobacteriaceae* members also acquired resistance to last-line antibiotics e.g. Polymyxin by horizontal gene transfer (Liu et al. 2016) and their ability to produce carbapenemase enzyme i.e. KPC strains, these properties are making difficult to treat their infections (Beirão et al. 2011; Band et al. 2018), accounting almost 70% of infections prevalent in hospital setting (Batchoun et al. 2009; Cerqueira et al. 2017). With this concern, the prophylactic measures to control these infections are of great importance to search some alternative ways.

Glutaraldehyde (GA), is an efficient disinfectant and crosslinking agent, which has been utilizing routinely in hospital settings, but has some controversial background due to its toxicity, difficult handling and residues formation on material and tissue surfaces (Gorman et al. 1980; Leung 2001; Reddy et al. 2015).

High hydrostatic pressure is an emerging biotechnological technique that coupled with other chemical and physical factors, shows very promising effects when it concern to sterilization of biomaterials for example Lens, tissues and bone etc. or materials sensitive to high temperature treatment (De Souza et al. 2013). HHP effect has indication of different kind of damages to bacterial cell for example, loss of protein content, enzyme dysfunctionality, and membrane damage ultimately loss of cell viability (Klotz et al. 2010).

Atomic force microscopy in tapping mode is helpful for analyzing surface based damages on confined cell selected from the group of bacterial cells as a result of certain physical and chemical application (Camesano et al. 2000). It provides delicate details of images on nanoscale resolution using sharp cantilever tip and laser beam, indicating cell-surface interaction (Razatos et al. 1998). In this study, we are aiming to evaluate the surface and membrane level damages, while two *Klebsiella pneumoniae* strains (ATCC BAA 1705 &

ATCC 4352) experienced inactivation as matter of HHP synergistic effect in combination with GA at moderate 50 ℃ by using AFM.

- ***EXPERIMENTAL SECTIONS***

Bacterial strains preparation and quantification

For this study strains of *Klebsiella pneumoniae* ATCC BAA 1705 and ATCC 4352 were granted by Prof. Marcello Lancelotti from department of Biotechnology. Bacterial isolates were cultured in BHI (Brain heart infusion) broth to prepared stocks by incubating overnight at 37 ℃ and next day these cultures were added with glycerol in 1:10 ratio and stored at -80 ℃ in bio freezer for upcoming experiments. Inoculum for each experiment was prepared by adding stock bacterial suspension in 5 mL of tryptic soy broth (TSB; Difco-BD) at 37℃ 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 4,000 *g* for 15 min; the resulting supernatant was discarded. Bacterial pellet cells were suspended in 0.9% (w/v) saline to achieve an estimated concentration of 10^9 cells/mL, which corresponds to an OD_{660} of 1.4 (Beckman DU640, Beckman Instruments, CA, USA), to be used in the experiments.

Quantification of bacterial viability 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 18-22 h incubation at 37 ºC in a biosafety incubator. All graphs were plotted on origin8 pro software by using mean and standard deviation of at least 3 independent experiments.

Temperature exposures

Survival profile of *K. pneumoniae* strains against different temperatures was analyzed using 16-18 h stationary phase bacterial cultures in TSB medium from above mentioned stock cultures. Bacterial suspension was exposed to different ranges of temperatures categorized as moderate i.e. from 40 to 60 ℃ for 10 min in water bath and controls was remained untreated at room temperature. Both temperature exposed and unexposed samples were then serially diluted in 0.9% saline solution followed by plating on TSB plates and incubation at 37 ℃ for 24 h. After 24 h of incubation bacterial colonies were counted as colony forming unit (CFU/mL).

Bacterial evaluations using glutaraldehyde and temperature

Due to bactericidal activity of GA, we have treated both strains of *K. pneumoniae* with its different concentrations. 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 suspensions of $1x10^9$ 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 qualified subsequently.

Afterwards GA profiling at room temperature both *Klebsiella pneumoniae* strains were studied by incubating bacterial suspension to four selected GA concentrations (0.001, 0.010, 0.100, and 1.000 mM) with and without combination with moderate temperature i.e. 55 ℃ for 10 min of exposure time using water bath. Afterwards the treatment, neutralization with 3% glycine was carried out as mentioned in earlier GA effect assay. Subsequently samples were diluted in 0.9% saline, plated on TSB plates and incubated for 24 h at 37 ℃. Quantification was carried out using CFU/mL standards.

Exposures to 300 MPa high hydrostatic pressure

To find susceptibility of both bacterial strains (ATCC BAA 1705 & ATCC 4352) of *Klebsiella*, 300 MPa pressure was exerted for different periods of time (5, 15, 30 and 60 min) at room temperature. The bacterial suspension of $5x10^9$ CFU/mL was prepared in 0.9% saline solution. The HHP equipment with water bath supply, as well as the experimental method used in this study have been described before (Silva et al. 1989; Durães-Carvalho et al. 2012; De Souza et al. 2013). The time required to elevate and remove the pressure till and from the 300 MPa limit is 1 min, were not included in time of exposure. A polyethylene bag (Polisilk®) filled with the sample was sealed at high temperature and placed in the high-pressure chamber. After the exposure samples were diluted and plated to incubate at 37 ℃ for 24 h for next day CFU/mL bacterial counts.

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

Bacterial suspension treatments with GA, HHP at moderate temperature

To find the correlation between isolated and combination treatments 0.01 and 0.05 mM GA concentrations for ATCC BAA 1705 and ATCC 4352 strains respectively, were used with and without 300 MPa pressure combination at 50 ℃. The bacterial suspension concentration was adjusted at 1.4 OD_{660} that corresponds to $5x10^9$ cells CFU/mL. All the treatments were applied for 10 min to measure comparisons. Quantification afterwards exposure was performed as above-mentioned pattern.

Atomic force microscopy (AFM)

To perform AFM analysis, $1x10^8$ CFU/mL concentrated bacterial suspension was prepared using sterilized Nano-pure water to avoid interference during imaging. Both *K. pneumoniae* strains were treated with their respective inactivation conditions i.e. ATCC BA 1705 (0.01 mM GA) and ATCC 4352 (0.05 mM GA) with 300 MPa at 50 ℃ for 10 min exposure. To analyze, 5 µL of bacterial suspension were deposited on free cleaved mica substrate. For mapping bacterial cell morphology and to evaluate their mechanical properties with atomic force microscopy. Park NX10, Park Systems Inc. Santa Clara, CA, USA and Nanosurf AG, Gräubernstrasse, Liestal, Switzerland was used. Each image took almost 20-25 min to appear completely on computer screen. The tip model was PPP-FMR from Nanosensors with nominal resonance 75kHz and nominal force constant 2.8 N/m (Xie et al. 2016). All the klebsiella cell were initially measured at 10 µm and finally on 4 µm, when confined to single cell observation. Gwyddion 2.50 software was used to treat AFM retrieved images.

Antibiotic susceptibility testing

The antibiotic susceptibility of the *K. pneumoniae* strains was evaluated using the Kirby-Bauer disc diffusion method (DDM). Initially brain heart infusion (BHI) broth (Neogen-Acumedia) was prepared and *S. aureus* were allowed to grow for overnight (12-14 h) at 37 ℃, 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 ℃, 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 ºC 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 ℃ 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 (Alagumaruthanayagam et al. 2009).

▪ RESULTS AND DISCUSSIONS

Effect of temperature on *K. pneumoniae* strains

Temperature resistance analysis was made using both strains of *K. pneumoniae* and the resultant findings showed significant reduction in growth with 55 ℃ and total inactivation of both strains were recorded at 60℃ (Figure 1). Temperature always works as control system in various applications, (Hennecke and Shanmugam 1979) reported that optimal temperature for *K. pneumoniae* is 37℃ and can resist at 39℃ to growth but with elevated temperature, decline in growth and propagation of various growth factors were noticed. In this study, we outlined temperature effect to select moderate temperature condition to be used in combination treatment as it plays deleterious effect to biomaterials and implants, when utilized in high ranges e.g. steam autoclaving (Park et al. 2012).

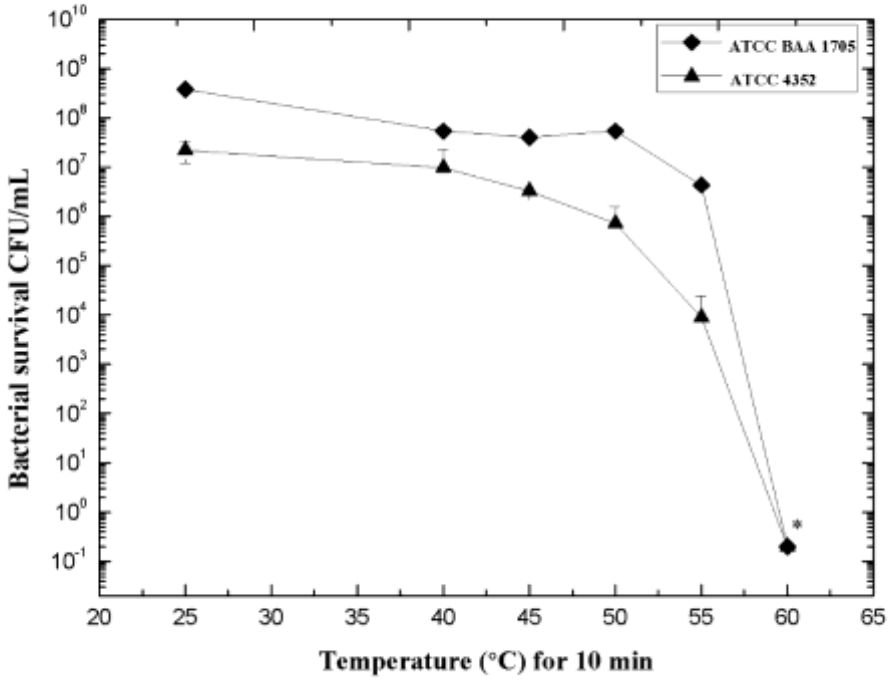

Figure 1. Measurement of survival curve of *Klebsiella pneumoniae* strains using different temperature ranges. (*) indicated complete inactivation. The data is represented using mean and standard deviation values of 3 individual experiments.

Glutaraldehyde effect with and without moderate temperature

The effect of Glutaraldehyde was well assessed in past studies (Gorman et al. 1980; Sehmi et al. 2016) showing its optimal efficacy for all forms or microorganisms from 0.5- 2% which is a higher concentration in terms of leaving traces and making cross-linkage with different proteins and carbohydrates ions, (Reddy et al. 2015) ultimately lessens the life span of biomaterial and implants while change their surface properties as well as its cytoxicity. In this concern *Klebsiella* strains were evaluated to bactericidal activity of GA with the different concentrations at room temperature i.e. 25℃ and with 55℃. The bactericidal activity at room temperature results in inactivation of both strains ATCC BAA 1705 and ATCC 4352 was found at 3.67 mM (Figure 2) giving ≥ 8 log magnitude reduction.

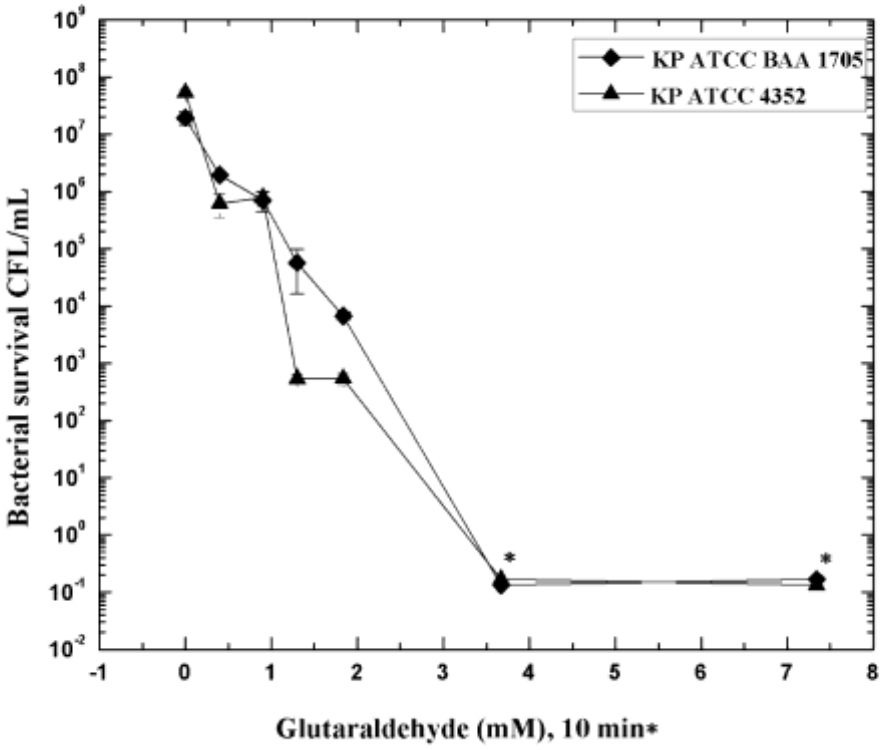

Figure 2. Glutaraldehyde effect using different concentrations at room temperature for 10 min against both strains of *K. pneumoniae*, (*) represents total inactivation while the data is plotted using mean and standard deviation values of 3 individual experiments.

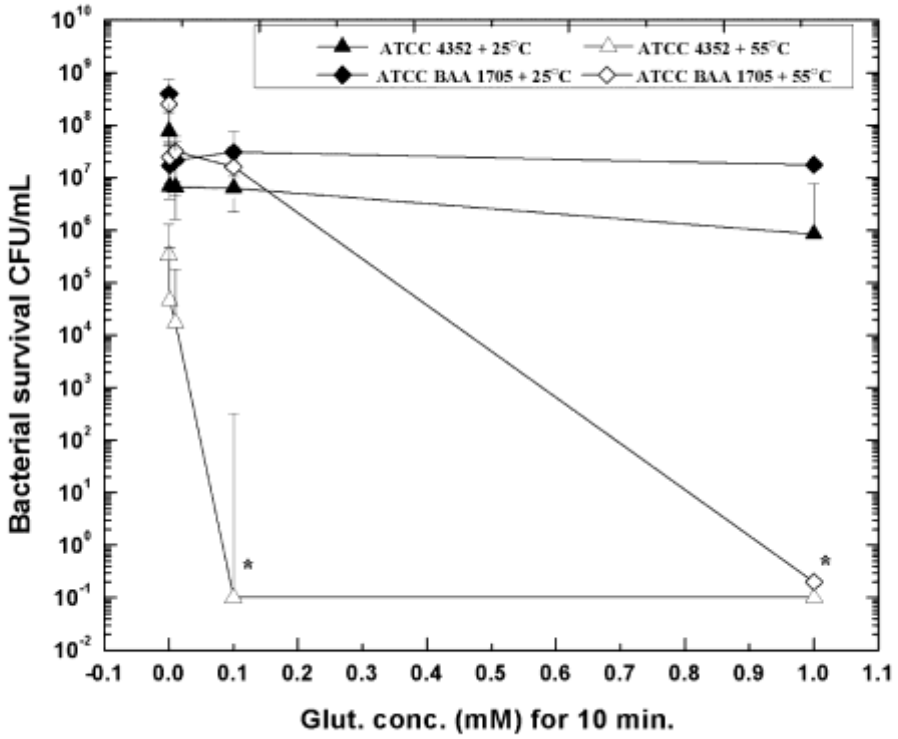

Figure 3. Effect of different concentrations of GA on room temperature and 55 ℃ for 10 min time of exposure. (⁕) showing total inactivation of bacteria while mean and standard deviation values of 3 individual experiment were used to plot graph.

The combination with 55℃ the bacterial strains completely reduced at 1.0 mM GA (Figure 3) representing ≥8 log CFU/mL reduction in bacterial growth. These indicative results demonstrate benefit of combination treatment and their correlation. This type of combination was studied earlier by Sierra and Boucher (1971), to inactivate bacterial spores by using GA with 54℃. After treatment GA exposure 3% glycine neutralization was also recommended (Cheung and Brown 1982) to remove its residues.

K. pneumoniae strains outcomes through HHP treatment

18 h cultures of *K. pneumoniae* was subjected to 300 MPa for different time of exposure after preparing with 0.9% sterilized saline, the bacterial cells concentration was adjusted at 1.4 OD_{660} that corresponds to $1x10^9$ CFU/mL. As a result of treatment ATCC 4352 strain display reduction of 5 log CFU/mL while, ATCC BAA 1705 strain kept resistant after all exposure and only showed fluctuation in growth, which can be considered as negligible effect for this strain (Figure 4). Phuvasate and Su (2015) tested two strains of *vibrio parahaemolyticus* with same range of high pressure and observed significant surface damages and alteration in quantity of protein content using SEM and SDS-PAGE, respectively. In Previously showed data, contaminated femur bone was disinfected prior to transplantation under 300 and 600 MPa, after treatment no bacterial growth was observed after sub-culturing of bone fragments (van de Sande et al. 2017).

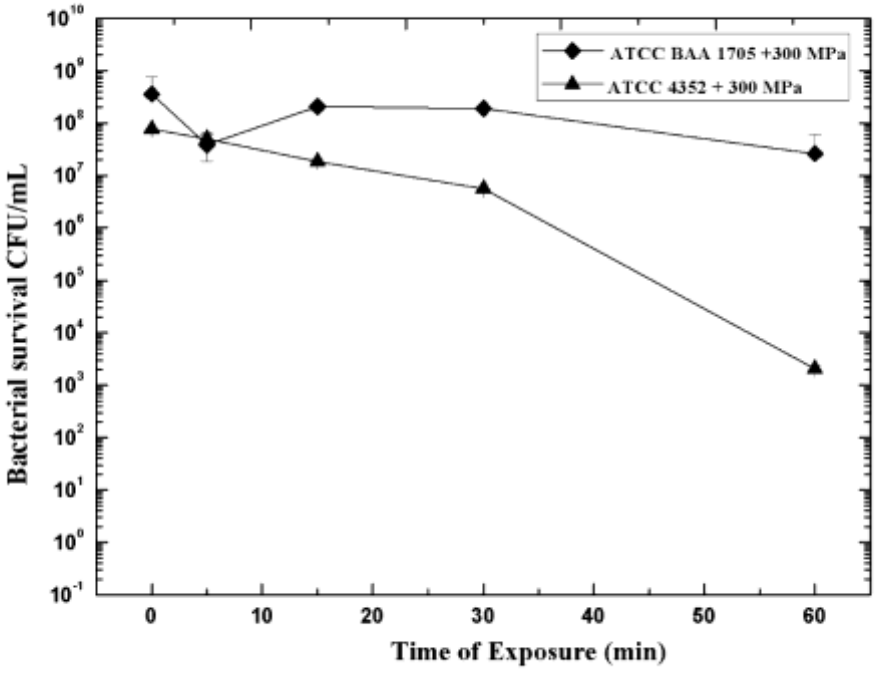

Figure 4. Effect of high hydrostatic pressure for different time of exposure at room temperature. Error bars indicting mean and standard deviation values of 3 individual test performed using 300 MPa to find *K. pneumoniae* survival profile.

Bacterial inactivation through HHP synergism

Figure 5 and 6 are representative of high hydrostatic pressure-induced inactivation of both *K. pneumoniae* strains, where ATCC BAA 1705 strain showed total inactivation with 0.01 mM GA and ATCC 4352 by 0.05 mM GA after subjecting to 300 MPa pressure at 50 ℃, for 10 min of exposure highlighted the advantage of this methodology to implement for routine sterilization procedures. HHP in combination with mild and low temperature was previously reported in both case conditions the antibacterial activity was noticed (Malinowska-Pańczyk et al. 2011; De Souza et al. 2013; Meng et al. 2016). Same combination we detected in case of ATCC BAA 1705 strain that inactivated completely in 300 MPa with 50℃ temperature treatment group (Figure 5).

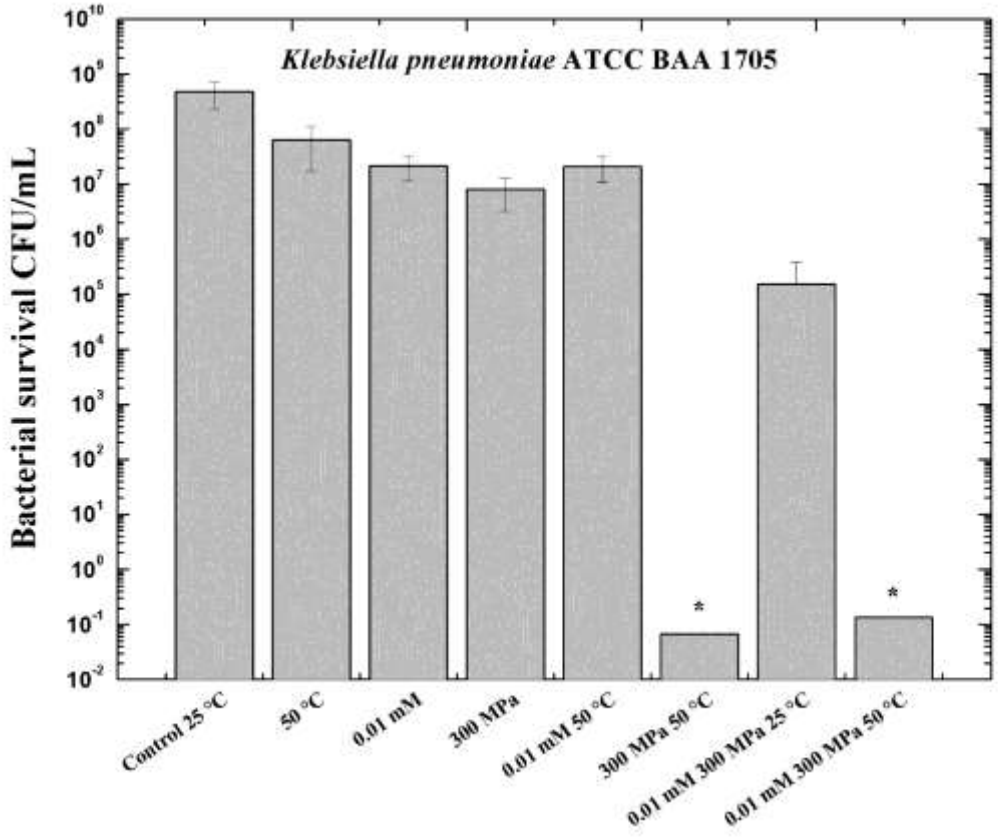

Figure 5. Bar diagram of combined assessment of 300 MPa HHP with 0.01 mM GA at 50 ℃ and individual group showing *K. pneumoniae* ATCC BAA 1705 survival. (⁕) indicating no growth observed on such condition. Error bars exhibiting mean and standard deviation values of 3 individual experiments.

In comparison, ATCC 4352 strain showed slight (about 4 log CFU/mL) reduction towards 300MPa and 50 ℃ temperature contact (Figure 6). Earlier experiments of tobacco mosaic virus with urea using 2.5kbar pressure at -19 ℃ revealed 18% of protein dissociation (Bonafe et al. 1998). In several arrangements HHP is capable of exerting its effect, aim to various applications similarly vaccine preparation , alterations in enzyme functionality and modulation of food and pharmaceuticals (Aertsen et al. 2009). *Klebsiella pneumoniae* is causative agent of blood stream infection so sterilization of blood products and factors are of great importance as (Yang et al. 2016) described HHP effectiveness by treating blood plasma and factors to noticeable inactivation of 7.5-8 log and 8.5 logs of bacterial and viral species respectively after treating between 200-250 MPa at 0 ℃ in ice-water bath. Mañas and Pagán (2005) reviewed the observation of Casadei et al. 2002 to discuss the mechanism around HHP resistance, stated that membranes with more fluidity exhibit more resistance towards high pressure due to unclear reasons.

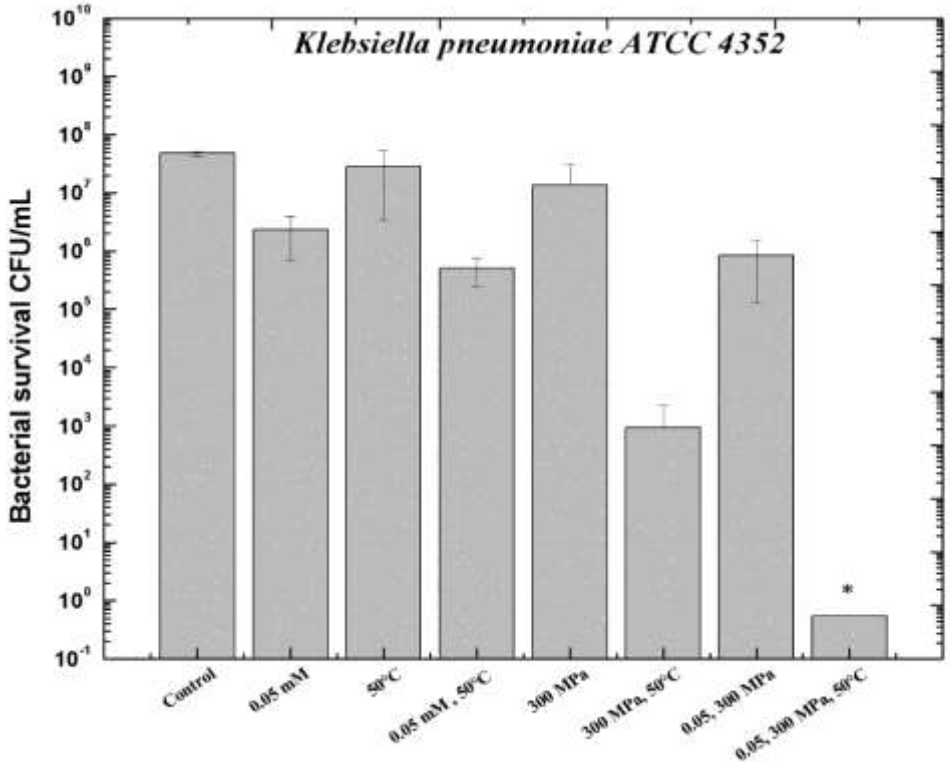

Figure 6. Representative illustration showing combination treatment of HHP, Ga and moderate temperature towards *K. pneumoniae* ATCC 4352 strain. Treatment condition sare mentioned below each group. (⁎) presence above the bar displaying complete bacterial elimination while error bars sketching mean and standard deviation of three individual experimentations.

AFM analysis of HHP altered *K. pneumoniae* cells

Surface structure analysis has benefit in both ways once to provide sensitive detail about substrate on which bacteria can be establish and second to evaluate topography of bacterial surface to study the change as a result of any treatment or external shock. In Figure 7. The observed topography of bacterial cell surface showing significant change upon HHP treatment in both strains as compared to their untreated bacteria cells. Figure 7 A and C showing untreated sample of both ATCC BAA and ATCC 4352 strains, respectively, where bacteria were kept preserve intact in their native state in. However, in Figure 7B, ATCC BAA 1705 strain suffered shrinking showing release of cell content and bursting. Contrarily, ATCC 4352 strain demonstrated membrane damage and cellular debris around while cell become swollen by known reason, Figure 7D. Boyd and Verran (2002) analyzed the change in surface roughness and topography effect on bacterial adhesion, and concluded that increase in surface roughness increases the bacterial adherence and retention. Razatos et al. (1998) extended the understanding of bacterial and biomaterial surface interaction by directly depositing *E. coli* growth on cantilever, showing the sensitivity of AFM analysis that is capable of analyze the establishment of single cell to the formation of thick biofilm structures.

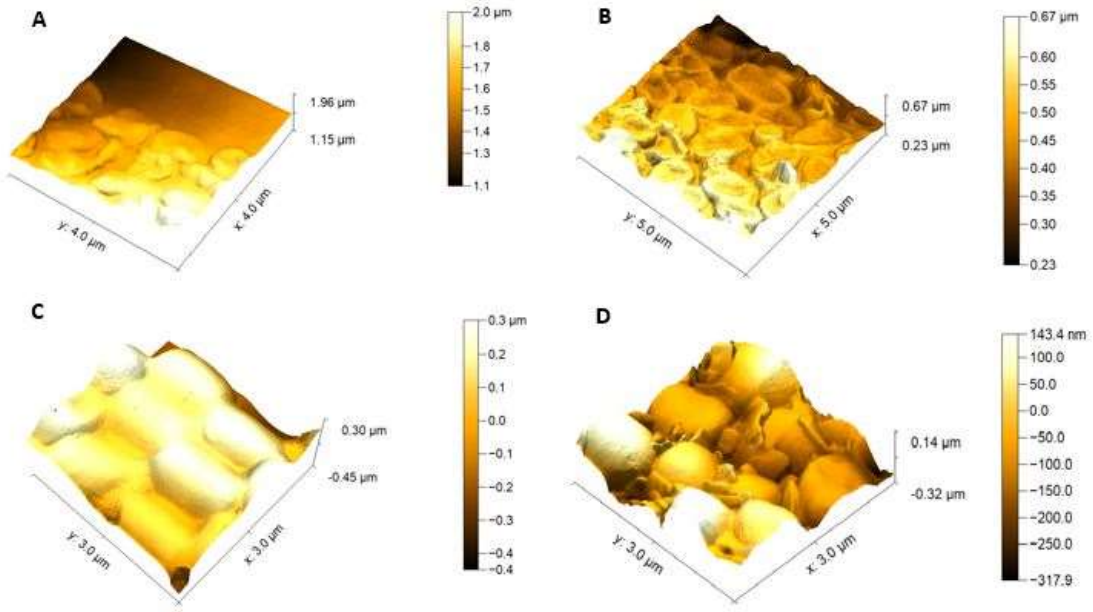

Figure 7. AFM results obtained by representative gram-negative bacteria *K. pneumoniae*. Images collected before and after treatment of both strains with 0.01/0.05 mM GA concentration with 300 MPa HHP association at 50 ℃. (A) ATCC BAA 1705 and (C) ATCC 4352 described, untreated bacterial cells, whereas, (B) ATCC BAA 1705 and (D) ATCC 4352 represents strain tested under above-mentioned conditions displaying difference in topography and surface structure as compared to untreated cells.

Antibiotic resistance profiling

Resistance to antibiotics is main global health risk factor. The results of disc diffusion antibiotics susceptibility showed high prevalence of resistance of ATCC BAA 1705, which is also standard strain recommended by CLSI manual for antibiotics testing. Moreover, ATCC 4352 strain displayed partial resistant and susceptible profile with for-mentioned tested antibiotics (Figure 4S).

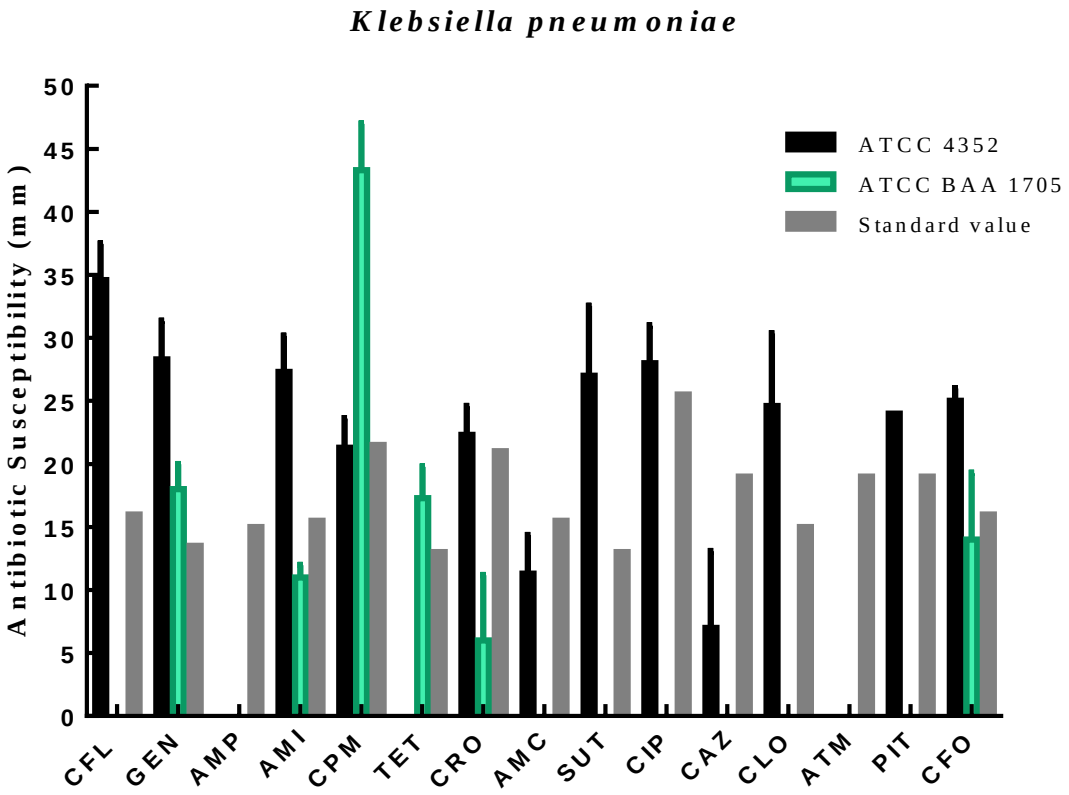

Figure 4S Antibiotic susceptibility testing of *K. pneumoniae* ATCC BAA 1705 & ATCC 4352 strains showing zone of inhibition (**mm**) against 15 antibiotics. Antibiotics symbols: **CFL** *(Cephalothin, 30 µg)*, **GEN** *Gentamycin, 10 µg*), **AMP** *(Ampicilin, 10 µg*), **AMI** *(Amikacin, 30 µg*), **CPM** *(Cefepime, 30 µg)*, **TET** *(Tetracyclin, 30 µg)*, **CRO** *(Ceftriaxone, 30 µg)*, **AMC** *(Amoxilin-clavulanic acid, 20/10 µg)*, **SUT** *(trimethoprim-sulfamethoxazole, 1.25/23.75 µg)*, **CIP** *(Ciprofloxacin, 5 µg)*, **CAZ** *(Ceftazidime, 30 µg)*, **CLO** *(Chloramphenicol, 30 µg)*, **ATM** *(Aztreonam, 30 µg)*, **PIT** *(Piperacilin- tazobactum, 100/10 µg) and* **CFO** *(cefoxitin, 30 µg).* Standard values adopted by CLSI manual, 2017.

▪ CONCLUSIONS

With the help of obtained results and observations, we assume that HHP combination with glutaraldehyde and moderate temperature can be interesting for biomaterial sterilization and AFM technique can equally benefit to explore HHP impact on biomaterials as well as bacterial surface. *Klebsiella pneumoniae* is a bacterium resistant to several antibiotics, so preventive measures and their influence on environments should be extensively studied for future perceptions.

CHAPTER: 5

High hydrostatic pressure mediated antibacterial activity of biogenic Silver nanoparticles (AgNPs) against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Manuscript to be submitted to *Nanobiotechnology*

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- **ACKNOWLEDGMENTS**

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, grant no. PROEX0232084) to provide financial support.

▪ ABSTRACT

The antimicrobial activity of biogenic silver nanoparticles (AgNPs) against *S. aureus (gram-positive) and P. aeruginosa (gram-negative)* was investigated as a model bacterial strain. For this purpose, 96-well plate method was used to evaluate using different concentrations (μM) of AgNPs with and without combination of high hydrostatic pressure (HHP). Exponential phase bacterial cultures of two different strains of *S. aureus* and *P. aeruginosa* in BHI medium was tested. After 15 min HHP exposure and subsequent incubation of 24 hr with AgNP the results were noted on the basis of appearance of turbidity at 600 wave length, the obtained results show that 200 μM/mL of silver nanoparticle displayed ≥ 70 % inhibition of bacterial growth in the presence of 150-300 MPa of HHP. Suggesting the use of HHP accoupled with silver nanoparticle can be interesting to minimize the use of high concentrations of AgNPs as these particles bares toxicity up to certain limit. This methodology can be used as model for testing effect of other nanoparticle in combination with HHP.

▪ KEYWORDS

Biogenic silver nanoparticles, *S. aureus* and *P. aeruginosa*, high hydrostatic pressure-application, disinfection, synergism.

▪ INTRODUCTION

One of the main causes of death and public health concern in the worldwide is different type of infection in health settings and there is a chance it will be increase in near future due increasing in population. The development of nanocarriers has provided a new hope in the fight against this terrible disease. Drugs have long been used to improve health and extend lives. One of the main fact that people developing the drug delivery carriers, sometime must face that the delivery systems may be harmful to healthy tissues. The side effects are often not good for health and gets fatal sometime. Nanocarrier systems with cell targeting ability offer site specificity and deliver cargos directly to infected sites with much less damage to healthy tissues, thus improving therapeutic efficacy (Jain 2010; Sasidharan et al. 2013).

Particle size less than 100 nanometres generally called nanoparticles (Silvera Batista et al. 2015), which deals with matter that ranges from one half the diameter of DNA is the nanotechnology (Dingman 2008). The size of nanoparticles are so small, even like bacteria would need a microscope to see them (Sekhon 2010). Nanoparticle can be used as an appropriate surface for molecular assembly and can also be composed of inorganic or polymeric materials. In some cases, the size and size distribution might be important specially when quantum-sized effects are used to control material properties. The surface of nanoparticles can be decorated with several molecules in order to reach their target more efficiently (Pauwels et al. 2008; Gary-Bobo et al. 2012).

One of the most important materials in the nanotechnology industry is silver nanoparticles. Recently, it received a great attention because of its distinctive physicochemical and biological properties. Silver nanoparticles are exceptionally small and has potential antibacterial effect and it can be used in many different products (Durán et al. 2015). Silver plays a vital role in antimicrobial, catalytic and biological systems among the other metals and the synthesis of silver nanoparticles as an antimicrobial agent has gained more importance against the increasing threat posed by antibiotic resistant microbes (Reddy et al. 2014).

Some studies reported that AgNP size, shape, surface charge, surface coating, solution chemistry and solubility affect AgNPs' toxicity (Zhang et al. 2016; Liu et al. 2018). However, the extent to which these factors affected toxicity directly by influencing particle-specific biological effects or indirectly by affecting silver ion release remains an open question. Discerning the relative importance of a particle-specific effect in the antibacterial activity of AgNPs requires careful quantification of the silver ion concentration contributed by the nanoparticle, as well as the role that complexing ligands present in the exposure media could

have on silver ion and particle bioavailability. One fundamental aspect that remains to be established concerns the identification of exactly which of the physical and chemical properties of Nano-Ag are responsible for the effective manner in which they deliver their antimicrobial activities.

Synthesis of silver nanoparticles is of much interest to the scientific community because of their wide range of applications. Chemical reduction is the most frequently applied method for the preparation of silver nanoparticles (AgNPs) as stable, colloidal dispersions in water or organic solvents. Commonly used reductants are borohydride, citrate, ascorbate, and elemental hydrogen. The reduction of silver ions (Ag^{+}) in aqueous solution generally yields colloidal silver with particle diameters of several nanometres (Sharma et al. 2009). These silver nanoparticles are being successfully used in the cancer diagnosis and treatment as well.(Ahmed et al. 2016). In addition, there is growing interest in utilization of Nano-Ag as a special class of biocidal agents, owing to the extraordinary antimicrobial properties of silver. like wound dressings containing sputtered nanocrystalline silver materials are currently used in clinical practice to suppress the microbial infection of burn wounds. There are also a number of silver composite materials that contain Nano-Ag as the active antimicrobial ingredient (Lok et al. 2007).

High hydrostatic pressure (HHP) is thermodynamic parameter, by virtue of its effect we can be able to control and manipulation the influence different other agents for biotechnological applications. It is capable to exerting its effect with various combination, this approach has been utilized in several previous studies (Silva et al. 1989; Bonafe et al. 1998; Rivalain et al. 2010; De Souza et al. 2013). In this study, we aimed to discover the inhibitory effect of silver nanoparticles (AgNPs) with and without combination with HHP to minimize the use of AgNPs, for this purpose two different strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were tested.

▪ EXPERIMENTAL SECTION

Synthesis of biogenic silver nanoparticles (AgNPs)

For this work, silver nanoparticle was synthesized using protocol of Durán et al. (2005) consuming *Fusarium oxysporum* to check its antibacterial activity with and without HHP induction.

Bacterial strains and inoculum preparation for AgNPs testing

Bacterial strains were pre-inoculated in BHI broth overnight at 37ºC, after 12 -13 hrs bacterial growth was re-inoculated in fresh BHI medium to achieve exponential phase cell after 6 hrs about $5x10^5$ CFU/mL or 0.4 O. D turbidity was measured in spectrophotometer. Second day bacterial suspension of 0.4 O. D from spectrophotometer, adjusted in BHI broth. 140 µL of two-fold diluted silver nanoparticles from the stock of concentration of 10mM. The antibacterial activity of bacteria was tested with 50, 100, 150, 200 and 250 µM/mL concentrations of biogenic AgNPs to find its inhibitory concentration (IC). 10µL of bacterial suspension was pipetted to each well of flat bottom 96-well microtiter plate. Incubated plate aerobically at 37 ºC for 24 hrs in dark. Next day absorbance of each well was measured cytation 5 reader (BioTek, USA) after 1 min sonication step by using 600 nm wavelength. Positive controls were run by inoculating bacteria in BHI broth medium and PBS pH= 7.2 and negative controls included BHI with AgNPs. After measuring turbidity at 600nm, 100 µL of each AgNPs tested bacterial suspension was collected in sterile conditions, serially diluted and spread on TSB plates for further growth estimation. (Raghupathi et al. 2011; Holtz et al. 2012).

AgNPs treatment with HHP

Above mentioned AgNP concentrations were tested with both *S. aureus* and *P. aeruginosa* strains cultures in BHI broth, sealed in polyethylene bags (Polisilk®) under 300 MPa high pressure for 15 min at room temperature. Afterwards sample were loaded in high pressure chamber. Next to treatment, the samples were added in respective wells and incubate 96-well plate for 24 h at 37°C. The procedure for HHP treatment was already described earlier (Durães-Carvalho et al. 2012; De Souza et al. 2013).

All results were expressed as average percentage of at least triplicate independent experiments. All data analysis was done using Graphpad Prism 6 software.

▪ RESULTS AND DISCUSSIONS

Antibacterial activity of silver nanoparticles with and without HHP exposure

Duran and collaborators previously tested *S. aureus* to discover antibacterial activity of biogenic silver nanoparticles by producing cotton clothes incorporated with silver nanoparticle and they observe significant antibacterial activity towards *S. aureus* (Durán et al. 2007). In this study, we used different concentration of silver nanoparticles with and without 300 MPa high pressure for 15 min at room temperature followed by 24 hr incubation of bacterial cells in the presence of silver nanoparticles to exert its effect. The resultant observations revealed that efficiency of antibacterial activity of AgNPs can be increased with the use of 300 MPa high pressure as shown in figure 1. *P. aeruginosa* strain ATCC 27853 is susceptible to AgNP equally without HHP combination for 15 min exposure however, NM 31 strain showed significant difference in reduction when exposed to AgNPs combined with 300 MPa pressure (Figure 1).

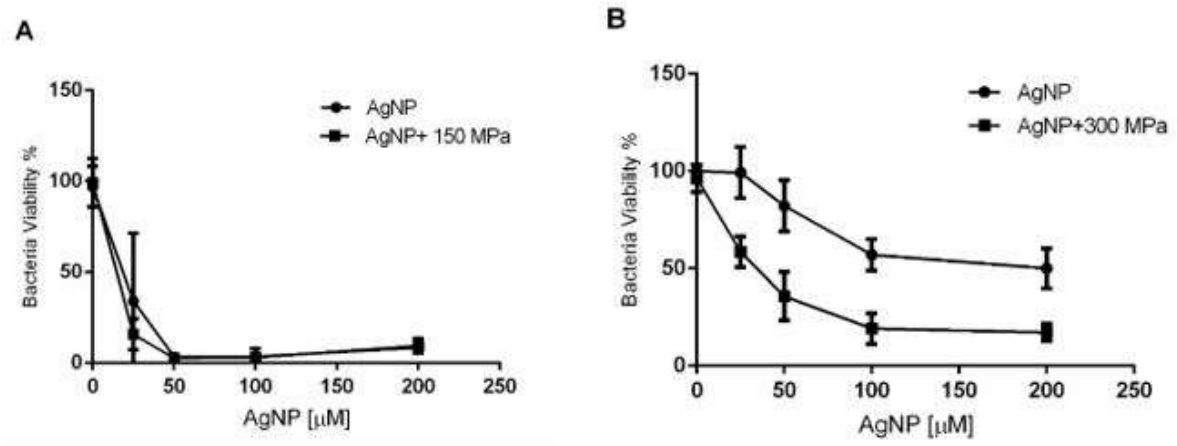

Figure 1. **Antibacterial activity of biogenic silver nanoparticles with and without HHP.** Curves showing percentage (%) decrease in bacterial viability of *Pseudomonas aeruginosa* strains in graph (A) ATCC 27853 and (B) NM 31. Error bars elucidating percentage of average values of experiments in triplicate for 15 min exposure time at room temperature.

HHP exceeded AgNPs effect almost from 50 % to 70 %, when combined with HHP for NM 31 strain. Figure 2. A and B showed the same isolated and combined effect of AgNPs and HHP activity, resulting in reduction of 70% and 80% bacterial viability for *S. aureus B*EC 9393 and ATCC 27853 respectively. Thus, 200 µM of AgNP appeared to be the Inhibitory concentration required to combine with HHP to achieve significant inactivation of both *S. aureus* and *P. aeruginosa* strain whereas, ATCC 27853 strain of *P. aeruginosa* Is showing its sensitivity even at lower concentrations of biogenic silver nanoparticle. We combined AgNPs with 150 MPa of pressure for *P. aeruginosa* ATCC strain as this strain is already sensitive to pressure less than 200 MPa (results from chapter 2 and 3 can be consulted in HHP effect section).

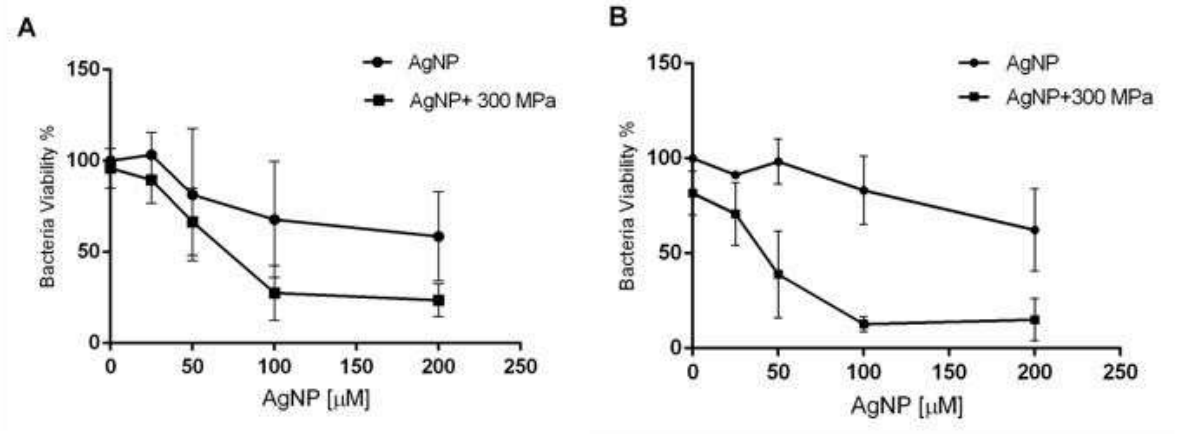

Figure 2. **Antibacterial activity of silver nanoparticles with and without HHP combination.** Illustrations showing effect on *Staphylococcus aureus* strains (A) BEC 9393 and (B) ATCC 25923. Curves showing percentage reduction in bacterial viability and error bars displaying average percentage of 3 individual experiments subjected for 15 min of exposure at room temperature.

Inhibitory concentration corresponds to give 50% reduction is called IC50, which is represented in Table 1. for each bacterial strain, expressing the decrease in AgNPs need when combined with 150 and 300 MPa for their respective conditions. The effect of *in situ* high-pressure small-angle X-ray scattering was first studied by using gold nanoparticles through mechanical annealing to improve in structural properties, suggesting HHP as thermodynamically helpful for various transformations (Wu et al. 2014). ZnO nanoparticles was previously utilized to check its antibacterial activity to reduce *P. aeruginosa* biofilm and virulence factors and found extensive inhibition without affecting cell propagation (Lee et al. 2014). Effect of silver

nanoparticles was evaluated against *E. coli*, the observation by TEM indicated the deposition of particles on bacterial membrane, causing high membrane permeability and ultimate cell death (Sondi and Salopek-Sondi 2004). Biosynthesized silver nanoparticles were tested earlier for *E. coli* and *P. aeruginosa*, showed that 4.7 and 2.7 µg/mL of nanoparticles inhibited 100% growth of both bacteria, respectively (Ramalingam et al. 2016). Since the results from our study evident inactivation of both *S. aureus* and *P. aeruginosa* strains on even lesser AgNPs concentration due to HHP combination effect.

Table 1. IC50 values of silver nanoparticle for bacterial strains.

IC50 (µM)	*P. aeruginosa* strains		*S. aureus* strains	
	ATCC 27853	**NM 31**	**ATCC 25923**	**BEC 9393**
AgNP	20.79	167.5	264.3	234.1
AgNP + 300 MPa	9.82 **150 MPa**	31.45	39.41	69.98

CHAPTER: 6

Effect of High hydrostatic pressure combination treatment for the inactivation of *Bacillus Subtilis* spores

- **INTRODUCTION**

High hydrostatic pressure (HPT) sterilization processes have several advantages in comparison with conventional (thermal) autoclaving, particularly, less detrimental effects on organoleptic and nutritional food quality and other materials. One major reason for this can be found in the lack of knowledge on basic mechanisms for the inactivation of pathogenic spore-forming organisms such as *Bacillus subtilis*. The aim of this study was to contribute to closing the knowledge gap regarding the HHP-mediated inactivation of spores from *B. subtilis* ATCC 6633, which primarily cause of environmental contaminant. Is a threat for several preparation either food or other biopharmaceutical solutions.

Morphological changes during sporulation putatively occur in a similar manner in *Bacillus* species and lead to a stepwise development of the extreme resistance of spores. Although sporulation is a continuous process and intermediate forms do not present discrete entities in which a sporulating cell remains, this developmental process is commonly divided into eight stages of sporulation. According to the favorable conditions the sporulated bacteria change in to vegetative cell for further propagation. Formation of highly resistant spores is a concern for the safety of low-acid foods as they are a perfect vehicle for food spoilage and/or human infection. For spore inactivation, the strategy usually applied in the food industry is the intensification of traditional preservation methods to sterilization levels, which is often accompanied by decreases of nutritional and sensory properties (Lopes et al. 2018). For this concern, alternative technique is being utilized in industries i.e. high pressure. Our approach in this study is to evaluated the effect of novel methodology that we observe inactivation of *Bacillus subtilis* spores. High hydrostatic pressure (HHP) is the most-widely adopted novel non-thermal technology for the commercial pasteurization of foods. However, HHP-induced

inactivation of bacterial spores remains a challenge due to spore resistance to the treatment limits of currently available industrial HHP units (i.e. ~650 MPa at 50 ℃). The germination of bacillus through HHP is common and HHP cause many transformations while making cells more susceptible to treatment (Sarker et al. 2013). Keeping the view of Bacillus resistance, we tried the methodology that we test with our bacterial species and their strains in form of planktonic cell and biofilm and achieved total inactivation. The methodology we developed in the combination of HHP, lower concentration of glutaraldehyde and moderate temperature (i.e. 50 ℃) for 10 min of exposure.

▪ EXPERIMENTAL SECTION

Media preparation for *Bacillus subtilis* sporulation

To sporulate bacteria, we inoculated *Bacillus subtilis* ATCC 6633 strain on Nutrient agar medium that contain metal using (glycerol contained stock stored at -80 ℃) to proliferate the sporulation and incubate for 24-48 h at 37 ℃. After incubation, bacterial growth was collected to make suspension of 0.4 OD_{660} that corresponds to $12x10^9$ CFU/mL for subsequent experiments. The confirmation of spore formation, we performed by spore staining with malachite green staining.

Bacterial spore 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 were treated in 0.1 M Tris-HCl, pH 8.0, with 5 different concentrations of GA in the range of 0.5- 14.7 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.

▪ RESULTS AND DISCUSSION

Figure 1. Showed the survival of *B. subtilis* ATCC 6633 spore under different concentrations of GA, 300 MPa at 50 ℃ for 10 min of exposure, the results admit the resistance of bacillus spore to such conditions though, there is significant reduction of 6-7 log CFU/mL magnitude of bacterial spore concentration, but we are unable to achieve total inactivation with our subsequent treatments. Inactivation spore and their germination is well known under high temperature and pressure (Reineke et al. 2013). But here in this study we did not aim to use extreme conditions to inactivate bacteria. Bacterial spores are more resistant forms, reflecting the approach to use more harsh conditions for total inactivation. Ahn et al. (2007) reported the high temperature and high-pressure mediated 7-8 log inactivation of different species of Bacillus under 0.1 and 700 MPa pressure at the range of 101-121 ℃ high temperature, these results expressing the demand of using very extreme conditions to achieve total spore inactivation.

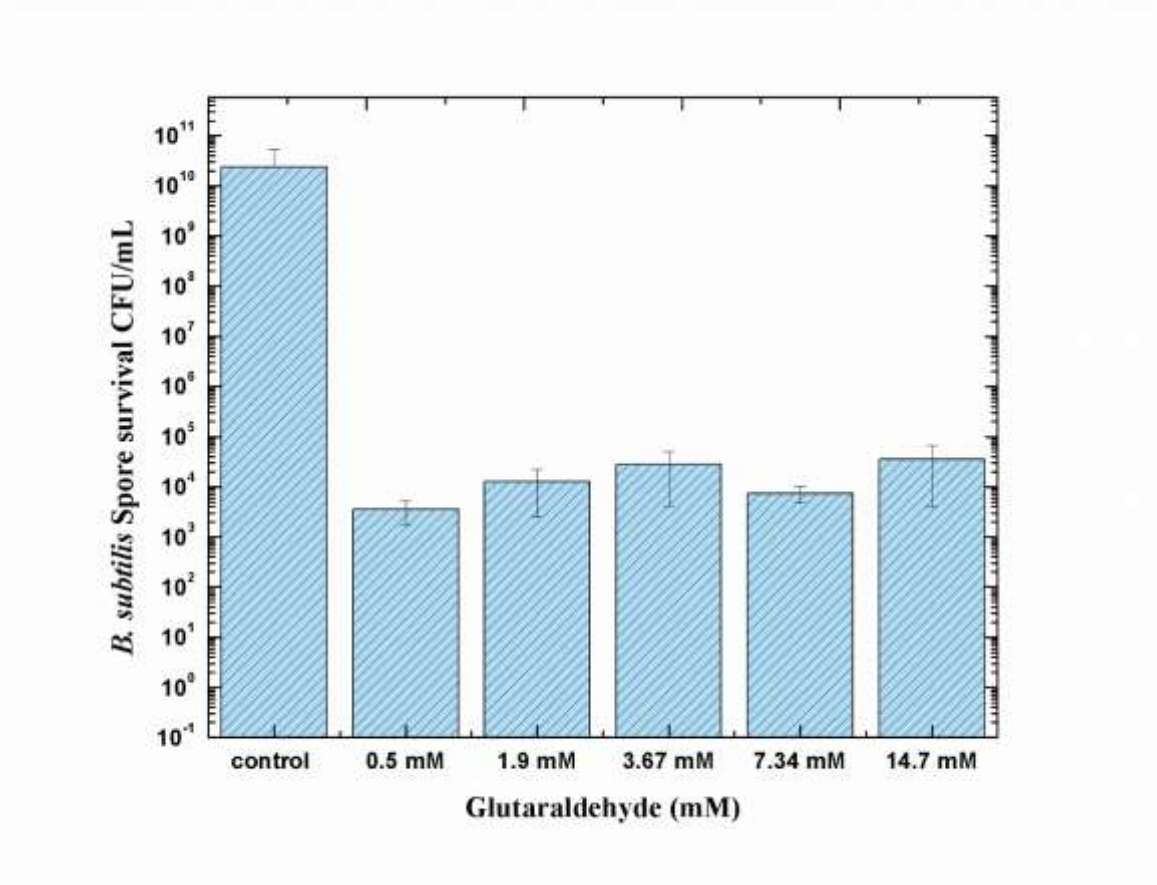

Figure 1. Survival of *Bacillus subtilis* with the treatment of different concentrations of GA, 300 MPa at 50℃ for 10 min of exposure. Error bar represents mean and standard deviation of three individual experiments.

MAIN CONCLUSIONS OF STUDY

This PhD project investigated the effect of synergism between moderate temperature, disinfectant; Glutaraldehyde (GA) and High hydrostatic pressure to inactivate three different bacteria *S. aureus, P. aeruginosa* and *K. pneumoniae* strains. The effects were compared using both planktonic and biofilm embedded bacterial cells to observed microbial survival profiles by using carrier materials. On the other hand, transmission electron microscopy and Atomic force microscopy revealed significant cell membrane damage and cytoplasmic content leakage appeared to be very promising tools for measuring effects of different treatment on bacterial cell. Overall results indicated that the novel mechanisms examined in this study could offer an opportunity for achieving moderate sterilization conditions compared to traditional heat autoclaving of temperature sensitive medical and pharmaceutical materials. Results from this thesis can be beneficial for designing validation studies on similar products and optimization of GA, temperature and high hydrostatic pressure can be helpful to modulate the inactivation profile of different bacteria and their biofilms aiming surgical and biomaterial sterilization. The quality control testing of novel method with *bacillus subtilis* spores also showed its significance as the obtained spore inactivation was at significant level but was not enough to achieve complete inactivation.

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Chapter 1

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APPENDIX I

Table 1. Antibiotic susceptibility profile of *Pseudmonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae* strains.

Bacterial strains / Antibiotics doses (µg)	*Pseudomonas aeruginosa*		*Staphylococcus aureus*		*Klebsiella pneumoniae*	
	ATCC 27853	NM 31	ATCC 25923	BEC 9393	ATCC BAA 1705	ATCC 4352
Cephalothin (30 µg)	R	S	S	R	R	S
Gentamycin (10 µg)	R	S	S	R	S	S
Amikacin (30 µg)	S	S	-	-	R	S
Ampicillin (10 µg)	R	I	R	R	R	R
Cefepime (30 µg)	S	S	-	-	S	SDD
Tetracyclin (30 µg)	R	S	S	R	S	R
Ceftriaxone (30 µg)	S	S	-	-	R	S
Amoxilin-clavulanic acid (20/10 µg)	R	S	S	R	R	R
Trimethoprim-sulfamethoxazole (1.25/23.75 µg)	R	S	S	R	R	S
Ciprofloxacin (5 µg)	S	S	S	R	R	I
Ceftazidime (30 µg)	S	R	-	-	R	R
Chloramphenicol (30 µg)	R	S	S	R	R	S
Aztreonam (30 µg)	S	R	-	-	R	R
Piperacilin-Tazobactum (100/10 µg)	S	S	-	-	R	S
Cephoxitin (30 µg)	R	S	S	R	I	S
Penicilin (10 units)	-	-	R	R	-	-
Oxacylin (1 µg)	-	-	R	R	-	-
Erythromycin (15 µg)	-	-	S	R	-	-
Rifampicin (5 µg)	-	-	S	S	-	-
Vancomycin (30 µg)	-	-	R	S	-	-
Clindamycin (2 µg)	-	-	R	S	-	-

Abbreviation: R= Resistant, I= Intermediate, S= Susceptible, and SDD= Susceptibility dose dependent.

APPENDIX II

SUPPLEMENTARY DATA (CHAPTER 2)

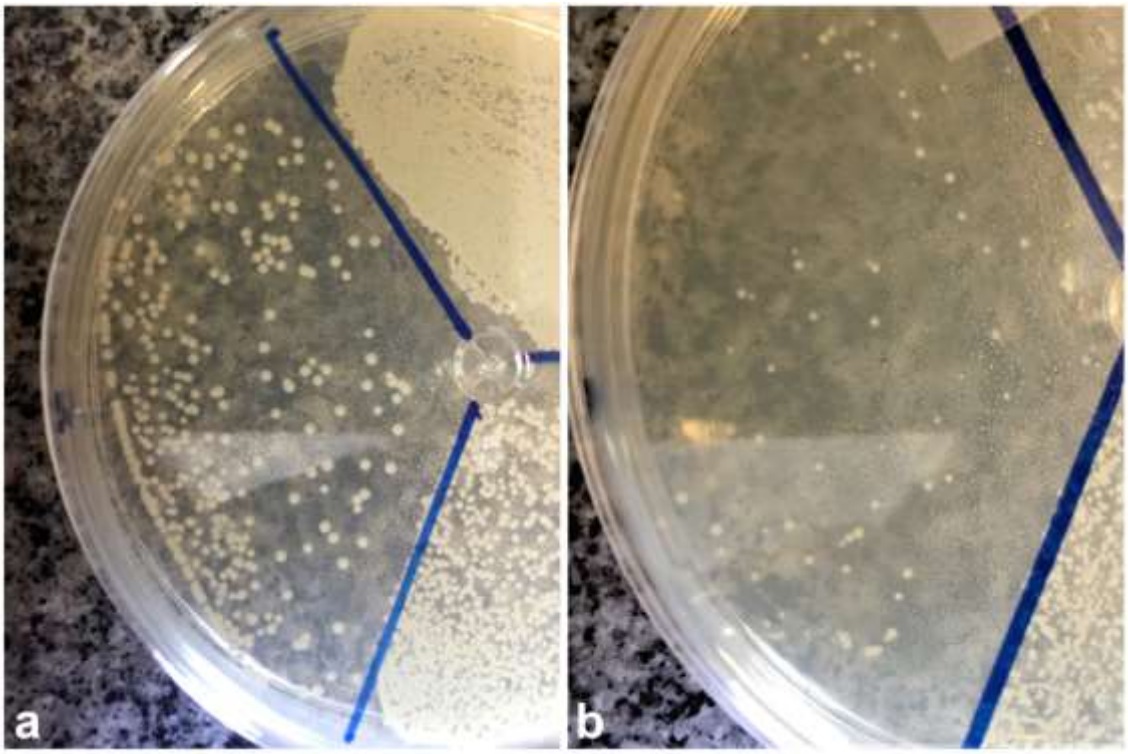

Figure 1S. *S. aureus* strain ATCC 25923 showing decrease in size of colonies. (**a**) control colonies not subjected to temperature treatment and (**b**) treated with 55 °C temperature for 10 min.

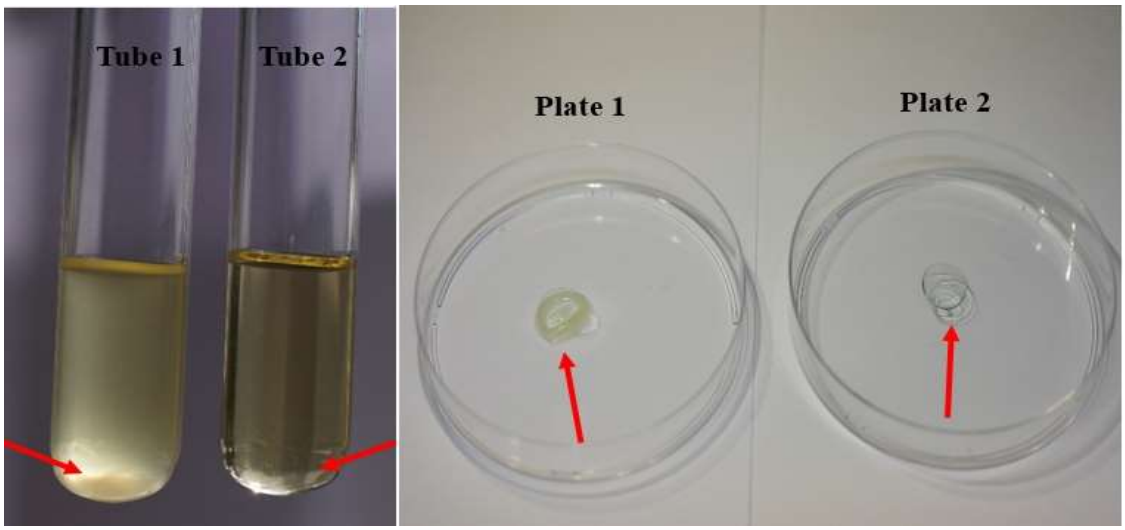

Figure 2S: Tube 1: positive control corresponding to lens contaminated with *S. aureus* strain ATCC 25923 without further treatment, and tube 2: lens contaminated with *S. aureus* strain ATCC 25923 and treated with 0.5 mM glutaraldehyde, 300 MPa HHP and 50 °C for 10 min. Plates 1 and 2 correspond to the lenses taken from tubes 1 and 2, respectively.

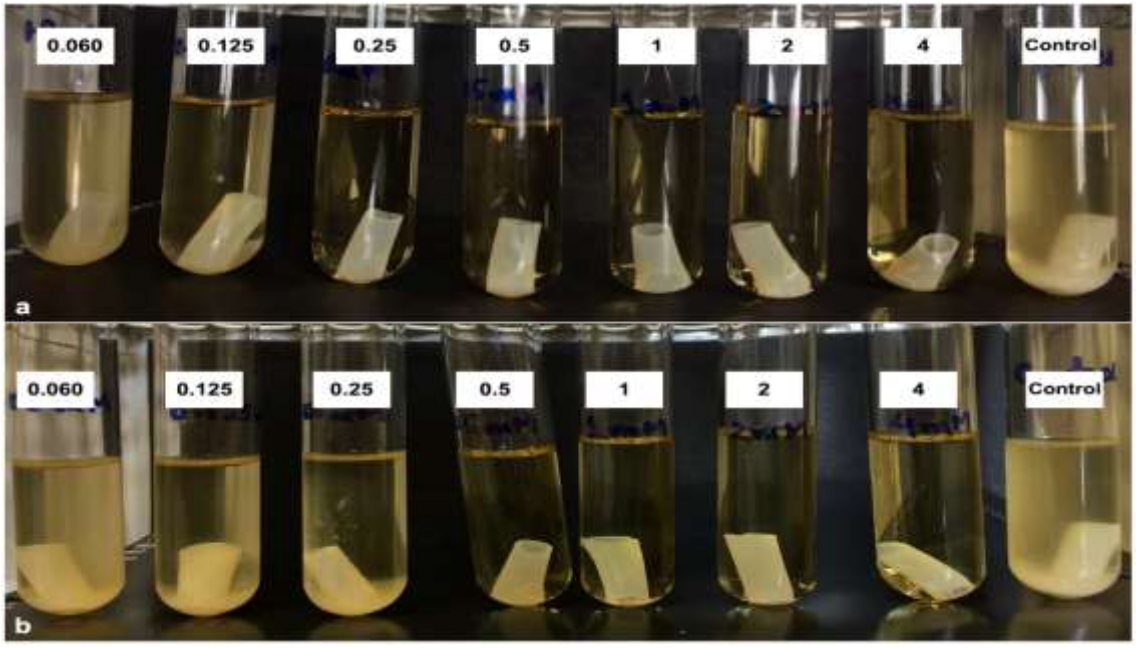

Figure 3S: Tubes containing catheter fragments previously inoculated with *S. aureus* strain ATCC 25923 and incubated for (**a**) 24 h and (**b**) 48 h at 37° C. From left to right: catheter fragments treated with glutaraldehyde (0.060-4 mM) in association with 300 MPa HHP at 50 °C for 10 min. The control is a catheter fragment only washed with sterile distilled water without above mentioned treatments.

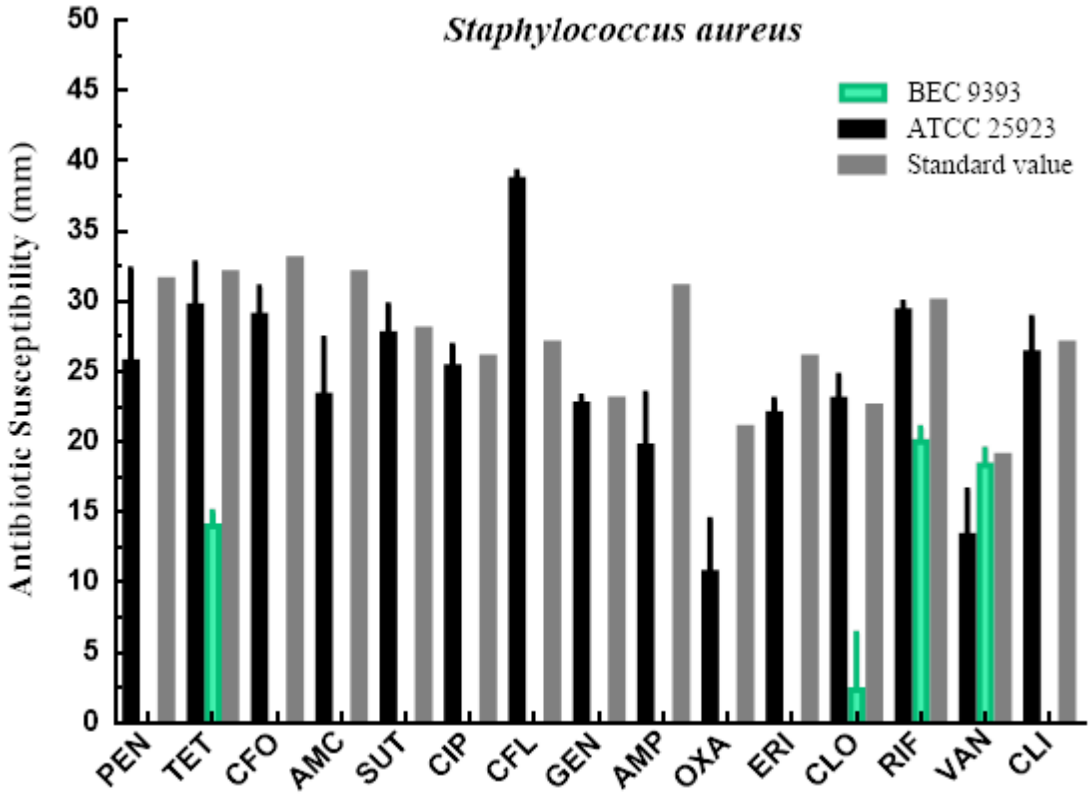

Figure. 4S: Antibiotic susceptibility testing of *S. aureus* ATCC 25923 & BEC 9393 strains showing zone of inhibition (**mm**) against 15 antibiotics. Antibiotics symbols: ***PEN*** *(Penicillin, 10 units)*, **TET** *(Tetracycline, 30 µg)*, **CFO** *(Cephoxitin, 30 µg)*, **AMC** *(Amoxilin-clavulanic acid, 20/10 µg)*, **SUT** *(Trimethoprim-sulfamethoxazole, 1.25/23.75 µg)*, **CIP** *(Ciprofloxacin, 5 µg)*, **CFL** *(Cephalothin, 30 µg)*, **GEN** *(Gentamycin, 10 µg)*, **AMP** *(Ampicillin, 10 µg)*, ***OXA*** *(Oxacylin, 1 µg)*, **ERI** *(Erythromycin, 15 µg)*, **CLO** *(Chloramphenicol, 30 µg)*, **RIF** *(Rifampicin, 5 µg)*, **VAN** *(Vancomycin, 30 µg)* and **CLI** *(Clindamycin, 2 µg)*. Standard values adopted by CLSI manual (J B. Patel, M P. Weinstein, G M. Eliopoulos, Sandra S. Richter and George M. Eliopoulos and S 2017).

APPENDIX III

Table 1. Comparison of the inactivation of *P. aeruginosa* strain ATCC 27853 present in biofilm in carrier material (catheter fragments) with the inactivation of a bacterial suspension of the same strain by different concentrations of GA at moderate temperatures and pressure of 300 MPa.

BIOFILM TREATMENT CONDITIONS

	ATCC 27853	**NM 31**
GA (mM)	50 °C, 300 MPa (up to 24-48 h)	50 °C, 300 MPa (up to 24-48 h)
14.70	-	-
7.34	-	-
3.67	-	-
2.0	+	-
1.0	+	-
0.5	+	-
0.16	+	+
0.08	+	+
0.04	+	+
0.01	+	+

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 with catheter with absence of growth at 24 h were monitored up to 48 h to check the sterilization.

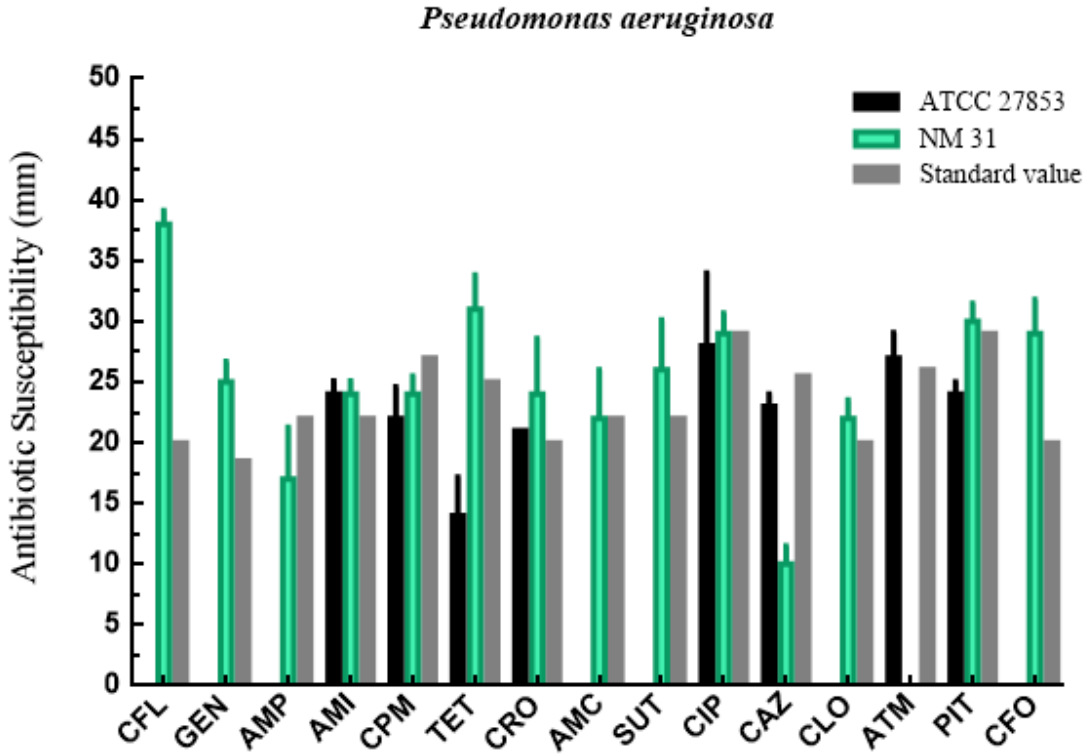

Figure 3S Antibiotic susceptibility testing of *P. aeruginosa* ATCC 27853 & NM 31 strains showing zone of inhibition (**mm**) against 15 antibiotics. Antibiotics symbols: **CFL** *(Cephalothin, 30 µg)*, **GEN** *Gentamycin, 10 µg*), **AMP** *(Ampicilin, 10 µg*), **AMI** *(Amikacin, 30 µg*), **CPM** *(Cefepime, 30 µg)*, **TET** *(Tetracyclin, 30 µg)*, **CRO** *(Ceftriaxone, 30 µg)*, **AMC** *(Amoxilin-clavulanic acid, 20/10 µg)*, **SUT** *(trimethoprim-sulfamethoxazole, 1.25/23.75 µg)*, **CIP** *(Ciprofloxacin, 5 µg)*, **CAZ** *(Ceftazidime, 30 µg)*, **CLO** *(Chloramphenicol, 30 µg)*, **ATM** *(Aztreonam, 30 µg)*, **PIT** *(Piperacilin- tazobactum, 100/10 µg) and* **CFO** *(cefoxitin, 30 µg).* Standard values adopted by CLSI manual, 2017.

Annexure

Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-018-9270-4

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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Received: 4 April 2018 / Revised: 4 July 2018 / Accepted: 24 July 2018

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

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-018-9270-4) contains supplementary material, which is available to authorized users.

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Published online: 08 August 2018

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

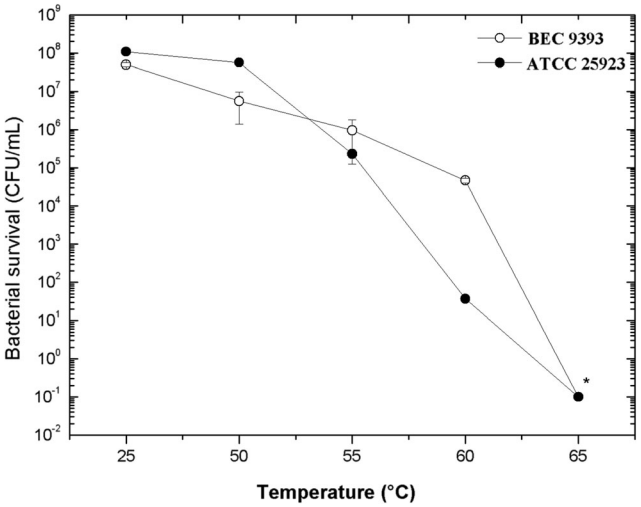

Fig. 1 Survival curves of *S. aureus* strains ATCC 25923 (*closed symbols*) and BEC 9393 (*open symbols*) after exposure to different temperatures for 10 min in the absence of GA. Asterisk: no bacteria detected. The error bars represent standard deviations ($n = 3$)

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

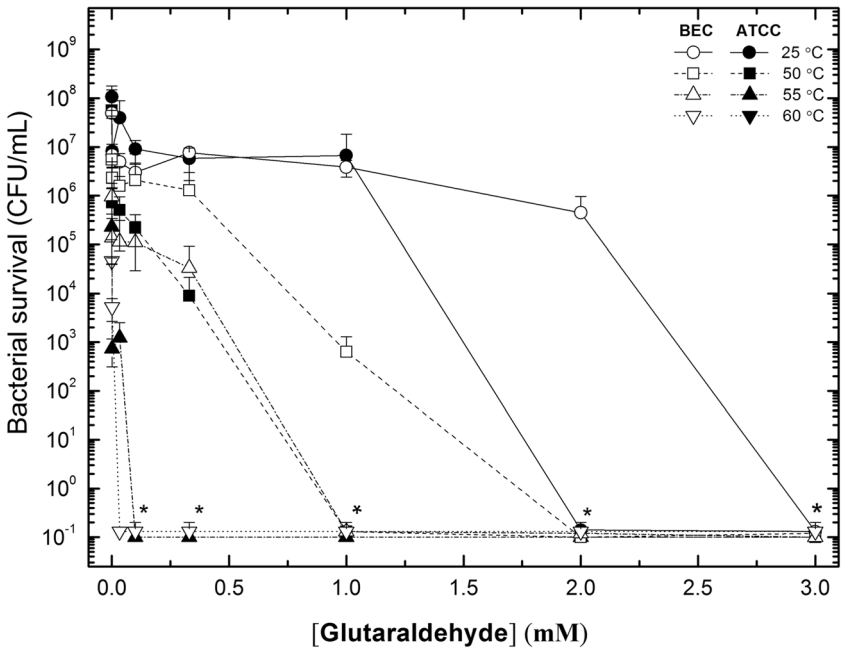

Fig. 2 Effect of GA on the inactivation of *S. aureus* strains ATCC 25923 and BEC 9393 at different temperatures (10-min exposure, pH 8.0). Asterisk: no bacteria detected. The error bars represent standard deviations (n = 3)

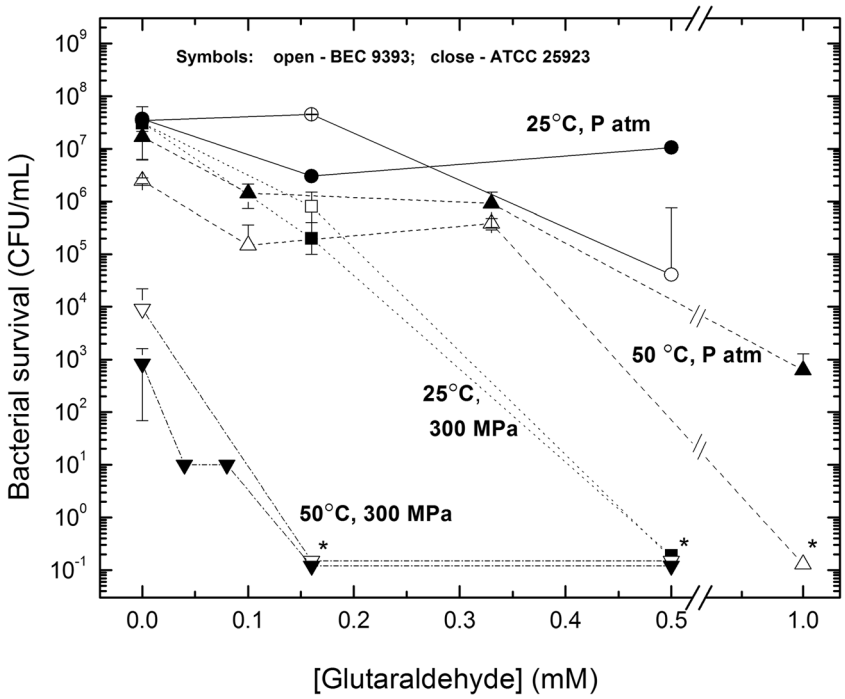

Fig. 3 Combined effect of HHP, GA, and moderate temperature (50 °C) on the inactivation of *S. aureus* strains ATCC 25923 and BEC 9393 after 10-min incubation. Asterisk: no bacteria detected. The error bars represent standard deviations ($n = 3$). P_{atm} atmospheric pressure

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

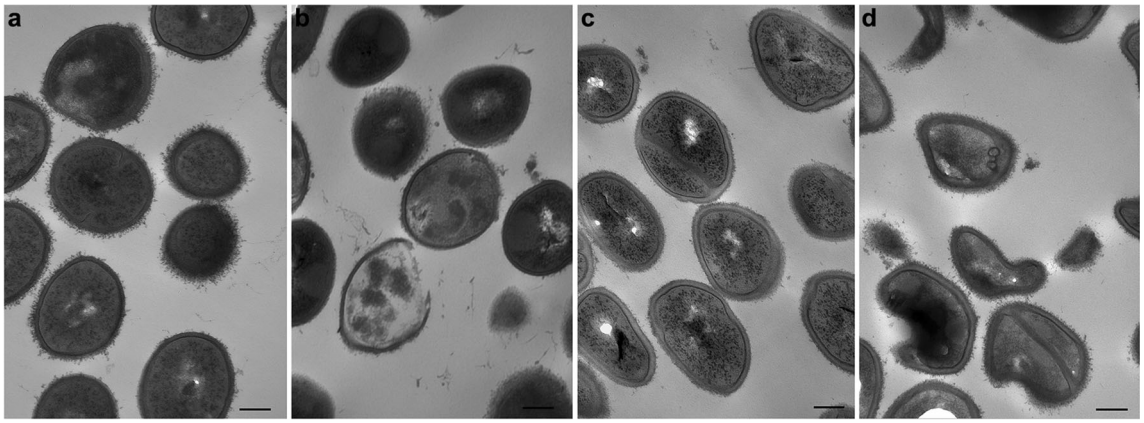

Fig. 4 TEM images of *S. aureus* ATCC 25923 (control (**a**) and pressurized (**b**) samples) and BEC 9393 (control (**c**) and pressurized (**d**) samples). The pressurization conditions for both strains were 300 MPa HHP at 25 °C for 10 min in the presence of 0.5 mM GA. The arrows indicate morphological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

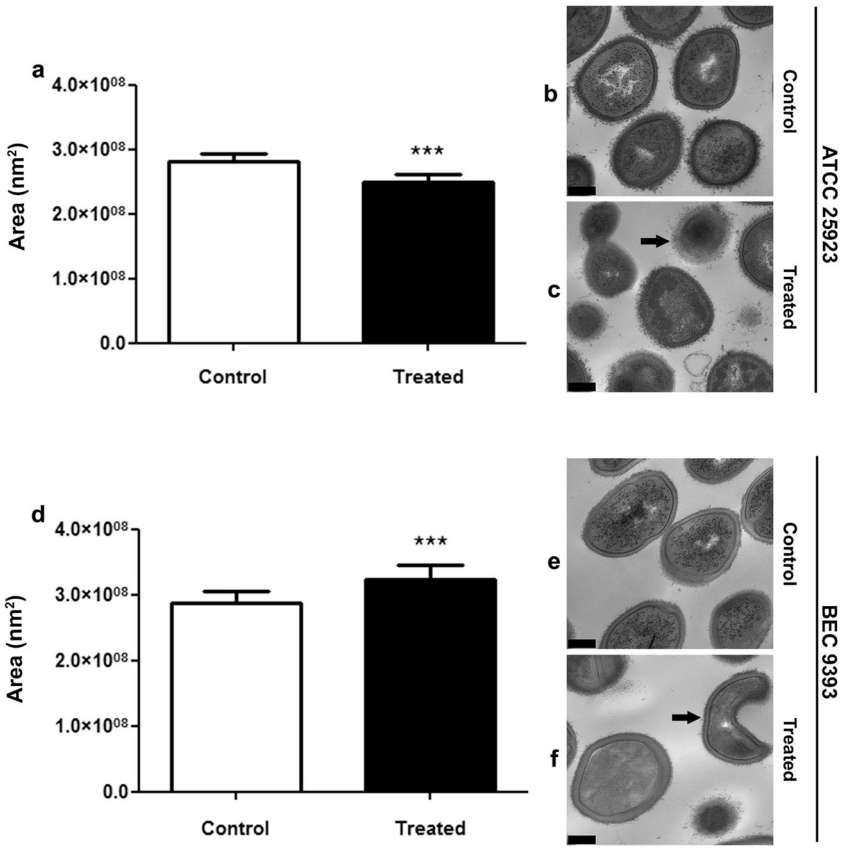

Fig. 5 Morphometric analysis of bacterial TEM images based on the change in bacterial surface area (nm^2) without (control) and with treatment with 0.5 mM GA and 300 MPa HHP and 25 °C for 10 min of *S. aureus* ATCC 25923 (a) and BEC 9393 strain (d). The control and treated images are shown respectively 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.

Acknowledgments The authors thank Prof. Dr. Paulo P. Joazeiro and Adriane C. S. Sprogis for their support during the electron microscopic analysis and for helpful discussions and Stephen Hyslop for editing the English of the manuscript.

Funding information This study was supported by Coordination for the Improvement of Higher Education Personnel (CAPES, grant no. PROEX0232084) and the São Paulo State Research Foundation (FAPESP).

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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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "**High hydrostatic pressure synergism with various physical and chemical agents for biomaterials treatment and inactivation of *S. aureus*, *P. aeruginosa* and *K. pneumoniae* as superbugs.**", desenvolvida no Programa de Pós-Graduação em Biologia Funcional e Molecular do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Synergism between high hydrostatic pressure and glutaraldehyde for the inactivation of Staphylococcus aureus at moderate temperature**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 09.08.2018

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