



Antibacterial activity of silver nitrate on *Pseudomonas aeruginosa* bacteria using Calvet calorimetry

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Abstract

This study aimed to use microcalorimetry to analyze the real-time effect that silver nitrate has on one of the most common bacteria involved in severe infections in current medical practice. Using a Calvet microcalorimeter, the heat output produced by *Pseudomonas aeruginosa* was determined when exposed to concentrations of silver nitrate from 0 to 3 mol·m⁻³, and a saturated solution. Under physiological human body temperature, for 46 h, thermographic curves were obtained. The maximum voltage peaks (V_{\max}) and the total amount of heat exchanged (Q) were determined. The results showed a bactericidal effect, with a minimum inhibitory concentration (MIC) of silver nitrate of 0.75 mol·m⁻³, showing a V_{\max} of 93 μ V and Q of 193 kJ. With higher concentrations of this metallic solution, the results highlighted the proportional negative effect that silver nitrate has in *Pseudomonas aeruginosa* growth.

Keywords Microcalorimetry · Curves · Metabolism · Bacteria · *Pseudomonas aeruginosa* · Silver nitrate

Introduction

Biofilms of *Pseudomonas aeruginosa* are one of the most common and feared infections in current medical practice. This range of diseases includes chronic resistant periprosthetic infections, even in young or healthy patients after undergoing a joint replacement. This can happen despite all aseptic measures that current medicine offers. These infections often cannot be treated effectively with traditional antibiotic therapy and need further treatment. This also has an important impact to health care costs and therefore, there is a strong need for the development of more effective antibacterial agents and early diagnostic tools.

Infections by gram-negative non-fermenting rods, like *Pseudomonas aeruginosa*, that are frequently seen in orthopedic surgery and other fields [1], are expected to increase in the future. Especially if we consider the increasing number

of elderly, diabetic [2], organ transplant and oncologic patients. According to the United States National Healthcare Safety Network, the infections caused by this type of bacteria rank fourth among the most important pathogens for all device-associated infections in the previous decade [3].

Silver has been known as an effective antimicrobial agent [4, 5]. Therefore, this and other metals have been used in various ways for their antimicrobial properties [6]. As a nanomaterial (Ag NPs) has been reported as agent with higher bactericide capacity than the chlorhexidine [7]. The latter is an antiseptic that is currently widely used in hospitals in the care of wounds and in the prophylactic skin preparation before surgeries. Besinis et al. [7] showed that Ag NPs had the strongest antibacterial activity of the NPs tested, with bacterial growth also being 25-fold lower than that in chlorhexidine.

It has been reported that silver nitrate prevents the separation into two daughter cells of sensitive dividing cells of *Pseudomonas aeruginosa* growing in nutrient broth [8]. In the pharmacopoeia of many countries, silver nitrate, together with silver itself, is used as an antiseptic and disinfectant applied topically [9]. It is also used as a cauterizer in superficial hemorrhages or to refresh sore ulcers, and it has been proposed as a coating material for endoprotheses and other implants in the surgical practice [10, 11]. This metal is an attractive material to be used as a component of fixation

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devices and joint prostheses in orthopedic surgery [12, 13], in order to decrease the postoperative infection rates.

Microcalorimetry is a technique that allows to determine, with high sensitivity, the energy exposed as consequence of any process or transformation [14, 15], which makes it a method of early detection to determine bacterial growth, from the energy exchanged in its metabolism [16]. It has been used in biology, pharmacology, biotechnology and ecology for its high sensitivity, precision and simplicity [17–21], however, clinical use has been very limited [22, 23].

The microcalorimetric analysis of bacterial species allows us to know in real time the growth curves of different microorganisms. Using this technique, the presence of bacteria in a culture medium can be confirmed in less than 10 h, even in samples with few colony-forming units ($10 \text{ CFU}\cdot\text{mL}^{-1}$). These thermograms are characteristic of each bacterial species behaving like a “thermal fingerprint” and therefore, the bacterial species can be identified within a few hours of culture [24, 25]. Furthermore, from the behavioral patterns or “thermal fingerprint”, it is possible to extract the value of the specific parameters of each bacterium, such as growth constant, generation time and amount of heat exchanged [16, 26–28].

Microcalorimetric investigations of microbial processes are becoming increasingly popular. Some studies have focused on the study of bacterial growth as single microorganisms [19, 20, 24, 25, 29–37]. Moreover, it has been used to discriminate between strains of the same bacterial species; for example, to differentiate methicillin-sensitive strains of *Staphylococcus aureus* (MSSA) from methicillin-resistant strains of *S. aureus* (MRSA) [38].

Microcalorimetry has also been used as a tool to evaluate the growth and metabolism of bacterial cells in the face of changes in the composition of the culture medium [39–43] and to evaluate the effects of ultrasound waves on the viability of several bacteria that usually occur in mineral waters such as spa thermal waters [44].

In microbiology, the term “minimum inhibitory concentration” (MIC) is the lowest concentration of chemical, usually a drug, which inhibit visible growth of a bacteria. Currently, the MIC is used in antibiotics susceptibility testing. Meanwhile, the term “minimum bactericidal concentration” (MBC) is the minimum concentration of an antibacterial agent that results in bacterial death, therefore resulted in the inability to re-culture bacteria. The closer the MIC is to the MBC, the more bactericidal the compound.

The determination of antimicrobial activity of different drugs and toxic compounds has been carried out using calorimetry [19, 45–49], including de determination of the minimum inhibitory concentration (MIC) of antibiotics [49–54]. Esarte et al. [49] published the microcalorimetric behavior

of *Pseudomonas aeruginosa* and its susceptibility against combination of antibiotics.

Regarding the medical field, microcalorimetry has been used for the detection of microbial contamination in platelet products, diagnosis of meningitis in cerebrospinal fluid of rats [22], detection of bacteria in artificial urine samples [50] or previously sterilized [51]. Furthermore, the microcalorimetry has shown to be a promising and efficient tool to investigate the formation of biofilms in dental implants [52] and bone grafts [53]. It has been used to study the antimicrobial efficacy of wound dressings containing antimicrobial agents [20] and of materials for implant coating [54].

Despite previous studies, there is lack of evidence of the effect of metallic solutions in bacteria growth. Avelledo et al. [55] reported the bacterial growth of *Pseudomonas aeruginosa* measured by microcalorimetry using different concentrations of zinc sulfate. The present paper is a continuation of that research whose objective is to analyze the bacterial growth of *Pseudomonas aeruginosa* under the exposure to silver nitrate.

Materials and methodology

The strains of bacteria that have been used were obtained from the American Type Culture Collection (ATCC) and the Spanish Type Culture Collection (CECT): *Pseudomonas aeruginosa*, ATCC 27,853. This bacterium was inoculated on blood agar plates and incubated at 309.65 K in an incubator for 24 h. The blood agar plates with multiple bacterial colonies were then used to prepare a bacterial suspension. Samples were prepared by adjusting turbidity concentration to 0.5 on the McFarland scale, using Colorimeter Vitek®, as an optical densimeter. This concentration was used to make further dilutions using 0.9% sterile saline solution (physiological), with national Code 641,860, to obtain final concentrations of 10^6 colony-forming units $\text{CFU}\cdot\text{mL}^{-1}$.

As culture medium we have used soy-casein broth digested with CO_2 , which is the most widely used culture medium in hospital clinical practice. The commercial presentation, BACTEC type PEDS PLUS/F, has the reference number 442194. US Pat. No. 4, 63,902; of National Committee for the Clinical Laboratory Standard (2001).

The metallic solutions used were provided by the Laboratory of Chemistry and Applied Physic of the University of Vigo. A saturated concentration of Silver nitrate was prepared, and from this solution, the different concentrations necessary for the experiments were prepared from 0 to $3 \text{ mol}\cdot\text{m}^{-3}$, and saturated solution (Fig. 1). Before each experience, these cells were washed with basic soap, passed through 96° alcohol and autoclaved at 121 °C for 30 min. In this order, sterile environment in the reference cell is

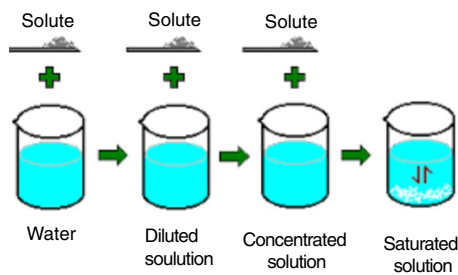


Fig. 1 Illustration of diluted, concentrated and saturated solutions

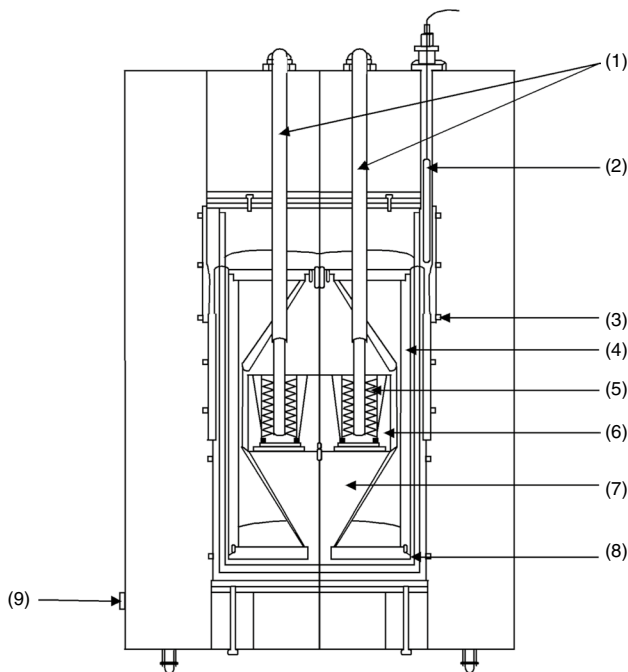
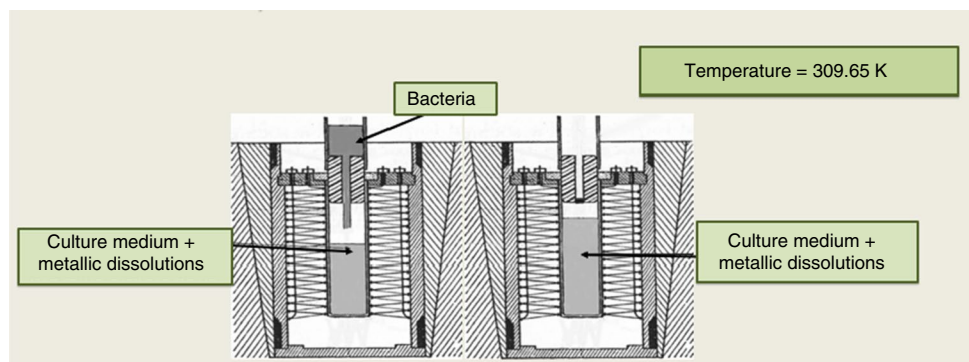


Fig. 2 Structure of Calvet microcalorimeter [14]: (1) Cell access (2) Regulator system detector. (3) Heating resistance. (4) Aluminum tray. (5) Thermopiles. (6) Calorimetric block. (7) Equal heat distribution cone. (8) Multiple enclosure thermostat. (9) Direct heating

guaranteed. Many autoclaves are used to sterilize equipment and supplies by subjecting them to pressurized saturated

Fig. 3 Experimental (left side) and reference cell (right side) placed inside the Calvet microcalorimeter internal block [14], adapted to our experience



steam at 121 °C (249 °F) for around 15–20 min depending on the size of the load and the contents [56].

The measures were carried out in the Calvet microcalorimeter designed by Professor M. I. Paz Andrade [15]. It is equipped with a device allowing operation in the absence of vapor phase, and having two Teflon® screw capped stainless steel cells (experimental and reference) of approximately 10 cm³ [57]. In each experiment, both cells were introduced, from the upper part of the microcalorimeter into the internal thermopile chamber through two parallel aligned cylindrical holes (Fig. 2), which extended from the top of the microcalorimeter toward the internal thermopile chamber. The large distance that separates the cells from the inlet ensures the minimization of heat flow to the outside. As shown in Fig. 3, the reference cell was filled with 6 mL of culture medium, 1 mL of the metallic dissolution and 1 mL of mineral-medical water. In the experimental cell, the mineral-medical water was replaced by the bacterial suspension. At any time, a constant temperature of 309.65 K was maintained in the outer chamber of the calorimeter.

The use of a Calvet microcalorimeter allows the monitoring of numerous physical, chemical and biological processes of long duration. The system used to collect data from the Calvet microcalorimeter is a Philips PM2535 multimeter, which registers the potential differences, connected through an RS232 interface to a data acquisition and control system consisting of a Raspberry PI minicomputer. Thus, using various modules, the design can be endowed with various functionalities. The operating system that runs on the minicomputer is LINUX, which provides the system with great robustness and reliability against failures. The precision in calorimetric signal achieved was $\pm 1 \mu\text{V}$.

The heat voltage difference versus time was collected and recorded by the data acquisition and the processing system, at intervals of 22.2 s for approximately 48 h. The bacterial growth curves were obtained at different concentrations of the metallic dissolutions, represented as thermograms. The calorimetric curve is defined by the series of electromotive force points recorded by the data acquisition system, and corresponds to the energy exchange

that occurs during the culture period. The differences in shape of curves allow us to visualize the trend of bacterial growth when increasing the concentration of the different metallic solutions. Therefore, a qualitative analysis can be made analyzing the aspect of the thermograms.

Samples were subjected to pH control, both before and after each experiment, using a basic 20 + pH meter, in order to measure the modification of the medium pH. In addition, this device shows the temperature of the sample that is being measured.

The maximum voltage peak (V_{\max}) was located in each thermograms, in order to carry out a quantitative analysis. Furthermore, the area under the curve (AUC) and the amount of heat exchanged (Q) during the time of the experiences of all the experiences were calculated. As the period of time of data collection varied between thermograms (46 to 52 h), the minimum time frame recorded of each group of metallic solution was used to calculate all the area under the curves (AUC), thus the total amount of heat exchanged (Q), for each group. In this way, we can make accurate comparisons by taking the data in a same period of time.

Using the data of the curves displayed by the microcalorimeter, the amount of heat released (Q) during the culture time can be determined using Eq. 1:

$$Q = K \cdot A \quad (1)$$

where A ($\mu\text{V}\cdot\text{h}$) is the area under the curve (AUC), calculated by trapezoidal method, and K represents a constant, whose value of $23.8 \mu\text{V}^{-1}\cdot\text{h}^{-1}$ was calculated from the electric calibration performed by the Joule effect of the equipment [55]. A Setaram EJP30 stabilized current source was used to perform an electrical calibration.

Results and discussion

The results obtained are shown as heat flow versus time curves in Fig. 4. The bacterial growth of *Pseudomonas aeruginosa* follows a characteristic pattern when it is not exposed to any external agent. This growth pattern is represented in the thermogram of the concentration of $0 \text{ mol}\cdot\text{m}^{-3}$ (not added) of silver nitrate (Fig. 4a) [25]. The curve is characterized by the presence of one phase. It shows an initial ascending part with two main leaps. Then, a descending curve showing an exponential shape, which is prolonged over time. After 35 h, the changes of voltage signal were minimum, therefore the heat produced by bacteria metabolism remained almost without change on the last period of the experiment.

The thermograms' curves remained stable (no significant changes or effects were appreciated) when the

bacteria were exposed to concentrations of silver nitrate lower than $0.75 \text{ mol}\cdot\text{m}^{-3}$. Therefore, this value was the minimum inhibitory concentration (MIC) of silver nitrate against this bacterial strain.

The maximum voltage peak (V_{\max}), the area under the curve (AUC_{46}), and the amount of heat exchanged (Q_{46}) during 46 h of the experiment of *Pseudomonas aeruginosa* with the different solutions of silver nitrate with the different solutions of silver nitrate that caused any effect on bacteria growth are shown in Table 1.

The voltage levels observed during these experiments were significantly low using higher concentrations of metallic dissolutions, with a strictly inverse proportionality with respect to the relation silver nitrate concentration/heat released. Among the thermograms obtained, the highest voltage peak (V_{\max}) was $119 \mu\text{V}$ using the bacterial suspension in the culture medium without the exposure of silver nitrate. Then, when increasing concentrations higher than $0.75 \text{ mol}\cdot\text{m}^{-3}$ were added, the voltage signal decreased gradually; showing a V_{\max} of $93 \mu\text{V}$ using a concentration of $0.75 \text{ mol}\cdot\text{m}^{-3}$, and $63 \mu\text{V}$, $36 \mu\text{V}$ and $14 \mu\text{V}$ using concentrations of $1 \text{ mol}\cdot\text{m}^{-3}$, $1.5 \text{ mol}\cdot\text{m}^{-3}$ and $3 \text{ mol}\cdot\text{m}^{-3}$, respectively. No signal was detected when the saturated dissolution of silver nitrate was used. The total amount of heat exchanged showed the same pattern.

The total heat exchanged expressed as Q_{46} , which was obtained using the area of the thermograms (AUC_{46}) in Eq. 1, gradually decreased as the concentrations of silver nitrate increased. The sample with the bacteria alone produced 198 kJ , while the sample with $0.75 \text{ mol}\cdot\text{m}^{-3}$, $1 \text{ mol}\cdot\text{m}^{-3}$, $1.5 \text{ mol}\cdot\text{m}^{-3}$ and $3 \text{ mol}\cdot\text{m}^{-3}$ of silver nitrate, had a heat generation of 193 kJ , 112 kJ , 94 kJ and 11 kJ , respectively.

The shape of the thermograms during the experiments with silver nitrate (Fig. 4) showed similarity to the normal pattern of the growth of *Pseudomonas aeruginosa* only with the two lowest concentrations of this metallic dissolution; with the typical ascending phase with two main leaps, and the descending curve showing an exponential shape, which is prolonged over time. However, with higher concentrations of silver nitrate, where the bacteria growth was significantly inhibited, the bacteria growth curves did not follow the regular pattern. They did not show a sharp leap in the ascending curves. The concentration of $3 \text{ mol}\cdot\text{m}^{-3}$ of silver nitrate did not produce a prolonged exponential curve in the final phase, as no voltage signal was detected after 29 h of data collection. This was the minimum concentration of silver nitrate that caused total voltage suppression ($0 \mu\text{V}$) during the experiments, therefore, this concentration of silver nitrate corresponds to its minimum bactericidal concentration (MBC) against this bacterial strain.

The bactericidal effect of silver nitrate has already been reported, and our MIC and MBC values were similar to such published studies [58, 59]. However, according to the

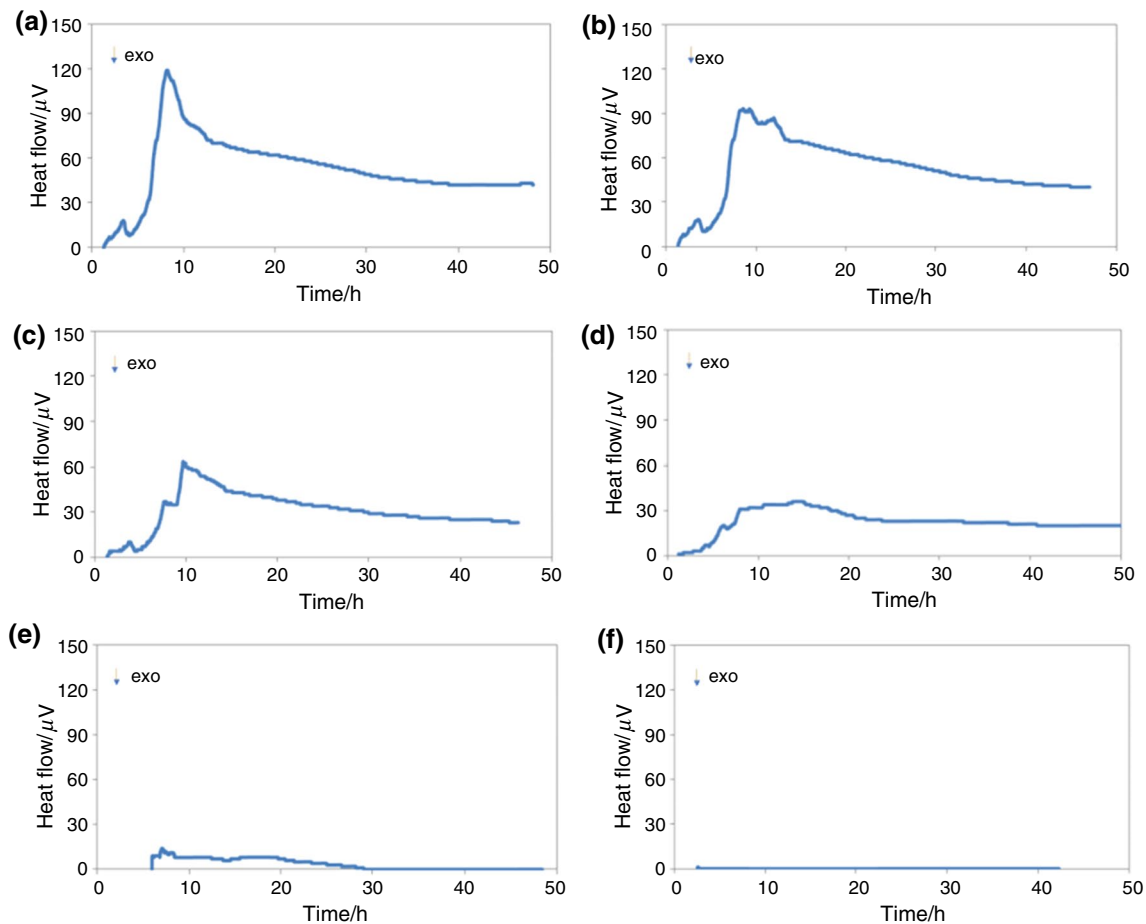


Fig. 4 Calorimetric heat flow versus time for: **a** *Pseudomonas aeruginosa* [25], **b** *P. aeruginosa* + 0.75 mol·m⁻³ silver nitrate, **c** *P. aeruginosa* + 1 mol·m⁻³ silver nitrate, **d** *P. aeruginosa* + 1.5 mol·m⁻³

silver nitrate, **e** *P. aeruginosa* + 3 mol·m⁻³ silver nitrate, **f** *P. aeruginosa* + Saturated silver nitrate

Table 1 Maximum voltage peak (V_{\max}), Area under the curve (AUC_{46}) and heat generated during 46 h of *Pseudomonas aeruginosa* (Q_{46}) with different concentrations of silver nitrate

Silver Nitrate concentration/mol·m ⁻³	$V_{\max}/\mu\text{V}$	$AUC_{46}/\mu\text{V}\cdot\text{h}$	Q_{46}/kJ
0	119	8348.77	198.699
0.75	93	8128.27	193.452
1	63	4732.03	112.622
1.5	36	3955.96	94.151
3	14	500.18	11.904
Saturated	0	0	0

Table 2 pH values and temperature of the samples before and after each experiment of *Pseudomonas aeruginosa* with different concentrations of silver nitrate

Silver nitrate concentration/mol·m ⁻³	Initial pH	$T_i/^\circ\text{C}$	Final pH	$T_f/^\circ\text{C}$
0	7.09	26.7	6.96	29.4
0.75	6.13	27.2	6.80	30.0
1	6.35	29.3	7.01	31.2
1.5	6.29	26.9	6.74	29.5
3	6.79	30.0	7.04	32.1
Saturated	6.42	24.3	6.53	28.1

literature review performed, this is the first study that has assessed the bactericidal effect of silver nitrate using thermodynamic analysis.

Regarding the outcome of the temperature of the samples, measured before and at the end of the experiments, it increased in all experiences with different proportions (Table 2). The basal and final mean were 27.4 °C and

30.5 °C, respectively. This pattern was logically expected as, in order to recreate the physiological human body medium, a constant temperature of 309.65 K (36.5 °C) was maintained in the outer chamber of the calorimeter all the time. However, no significant differences or tendencies patterns were observed between the experiments using different concentrations of silver nitrate.

The outcome of the pH of the different samples after 46 h tended to increase or, in other words, they were more alkaline at the end of most experiences (Table 2). However, unlike the voltage signal/heat produced, the pH values were not proportional to the different concentrations of silver nitrate. Before starting this study, an overall acidifying effect was expected as the residues produced by the bacteria metabolism are usually acid, resulting in a decreased pH in culture medium. However, when reviewing the literature, studies have revealed the particular property of *Pseudomonas aeruginosa* to increase the pH when biofilm is produced [60].

Conclusions

Using microcalorimetry, this study highlighted the proportional negative effect that silver nitrate has in *Pseudomonas aeruginosa* growth. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), of this metallic solution against this bacterial strain, were defined. This thermodynamic technique supports the use of silver nitrate in the medical industry, among others, in order to take advantage of its bactericide property.

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Author contributions JLL conceived the original idea and supervised the project. NL, MMM helped supervise the project. JLL, NL, MMM planned the experiments. MMM wrote the manuscript with support from RA, JLL. RA prepared the samples, carried out the experiments, performed the calculations, designed the figures, analyzed the data and interpreted the results with support from MMM, NL, JLL. AA contributed to sample preparation. All authors discussed the results and contributed to the final manuscript.

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