

## *In vitro* inactivation of pathogenic bacteria by the use of ozone in different exposure times

### Inactivación *in vitro* de bacterias patógenas mediante el uso de ozono en diferentes tiempos de exposición

Dora Inés Kozusny-Andreani,<sup>I</sup> Giovanna Andreani,<sup>I</sup> Luiz Fernando Avezum do Prado,<sup>I</sup> Amanda Oliva Spaziani,<sup>I</sup> Rodolpho César Oliveira Mellem Kairala,<sup>I</sup> Felipe Seixas Da Silva,<sup>I</sup> Renato Amaro Zangaro<sup>II</sup>

<sup>I</sup> Universidade Brasil. Fernandópolis - São Paulo, Brazil.

<sup>II</sup> Instituto de Engenharia Biomédica. São José dos Campos, São Paulo, Brazil.

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#### ABSTRACT

**Introduction:** in the area of health, ozone has many therapeutic properties. Several pathologies can be treated with ozone therapy, such as infectious, acute and chronic diseases caused by viruses, bacteria, fungi and parasites, autoimmune diseases, diseases with chronic ischemia, lung diseases, neuropathies, dermatological diseases, dental caries, among others.

**Objective:** to evaluate the effect of ozone applied *in vitro* in the following strains: *Escherichia coli* CCCD E003, *Salmonella enterica* subsp. *enterica* serovar Typhi CCCD S009, *Staphylococcus aureus* CCCD S003, *Pseudomonas aeruginosa* CCCD P013, *Streptococcus mutans* ATCC 25175 and *Enterococcus faecalis* ATCC 18211. For this purpose use was made of different cell concentrations and different times of exposure to ozone.

**Methods:** we used concentrations of  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$  and  $1 \times 10^9$  CFU/mL of NaCl (0.5 % w/v) exposed to ozone for different time intervals (30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 510 and 540 s). Bacterial viability was determined by CFU and the colorimetric method with 2,3,5-Triphenyltetrazolium Chloride.

**Results:** it was found that the species *S. aureus*, *E. coli*, *S. typhi*, *S. mutans* and *E. faecalis* were sensitive to ozone, showing a decrease of 45-80 % of viable cells after 30 s of ozone exposure relative to the initial population, whereas *P. aeruginosa* was reduced 25 % compared to the initial population. The viability of bacteria exposed to ozone was dependent on the cell concentration and time exposure.

**Conclusions:** ozone had a bactericidal effect on the bacteria used in this study and that this effect was proportional to the concentration of bacterial cells and the time of exposure to O<sub>3</sub>. The results show significant efficacy of ozone to control populations of pathogenic bacteria, providing relevant information for its use in different areas, but always taking into account the microorganism involved.

**Keywords:** ozone; bactericidal activity; cell viability; *in vitro*.

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## RESUMEN

**Introducción:** el ozono tiene muchas aplicaciones terapéuticas en la esfera de la salud. Algunas patologías pueden tratarse con ozonoterapia, entre ellas enfermedades infecciosas, agudas y crónicas causadas por virus, bacterias, hongos o parásitos, enfermedades autoinmunitarias, enfermedades con isquemia crónica, enfermedades pulmonares, neuropatías, enfermedades dermatológicas y caries dentales, entre otras.

**Objetivo:** evaluar el efecto del ozono aplicado *in vitro* sobre las siguientes cepas: *Escherichia coli* CCCD E003, *Salmonella enterica* subesp. *enterica* serovar Typhi CCCD S009, *Staphylococcus aureus* CCCD S003, *Pseudomonas aeruginosa* CCCD P013, *Streptococcus mutans* ATCC 25175 y *Enterococcus faecalis* ATCC 18211. Con este propósito se utilizaron diferentes concentraciones celulares y diferentes tiempos de exposición al ozono.

**Métodos:** utilizamos concentraciones de  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$  y  $1 \times 10^9$  UFC/mL de NaCl (0,5 % m/v) expuestas a ozono durante diferentes intervalos de tiempo (30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 510 y 540 s). La viabilidad bacteriana se determinó mediante UFC y el método colorimétrico con cloruro de 2,3,5-trifeniltetrazolio.

**Resultados:** se observó que las especies *S. aureus*, *E. coli*, *S. typhi*, *S. mutans* y *E. faecalis* eran sensibles al ozono, mostrando una disminución de 45-80 % de las células viables luego de una exposición de 30 s al ozono en comparación con la población inicial, mientras que la especie *P. aeruginosa* se redujo en un 25 % en comparación con la población inicial. La viabilidad de las bacterias expuestas al ozono dependió tanto de la concentración celular como del tiempo de exposición.

**Conclusiones:** el ozono mostró tener un efecto bactericida sobre las bacterias utilizadas en el estudio, y ese efecto fue proporcional tanto a la concentración de las células bacterianas como al tiempo de exposición al O<sub>3</sub>. Los resultados demuestran la significativa eficacia del ozono para controlar poblaciones de bacterias patógenas, y ofrecen información relevante con vistas a su uso en diferentes áreas, pero siempre teniendo en cuenta el microorganismo en cuestión.

**Palabras clave:** ozono; actividad bactericida; viabilidad celular; *in vitro*.

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## INTRODUCCIÓN

Ozone is a powerful oxidant much used in Europe and the United States, but little used in Brazil.<sup>1</sup> He became notorious in recent decades mainly due to its highly oxidative activity that characterizes it as a potentially biocidal agent to act on

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bacteria, fungi, viruses and helminthes.<sup>2-5</sup> The primary action of ozone on microorganisms occurs on the cell wall, resulting from oxidation of glycopeptides, glycoproteins and amino acids, changing the permeability and causing its rapid lysis.<sup>5-7</sup> By penetrating inside the cell, ozone recombines with elements promoting the oxidation of cytoplasmic amino acids and nucleic acids, resulting in cleavage with consequent cell death.<sup>6,8-13</sup>

The oxidation and inactivation of bacteria by ozone are very fast, reaching different cellular constituents of non-specific manner. It can act on the purine and pyrimidine bases of nucleic acids of *Escherichia coli*.<sup>14</sup> However, the sensitivity of bacteria to ozone is dependent on certain factors such as stage of growth and cell concentration, types of microorganisms, culture media, temperature, exposure time and ozone concentration, among others.<sup>5,10</sup>

Ozone has been used effectively in the food industry,<sup>12-14</sup> water treatment,<sup>11,15</sup> sewage treatment,<sup>16,17</sup> persistent drugs,<sup>18,19</sup> environment deodorization,<sup>20</sup> machine hemodialysis disinfection,<sup>21</sup> dental caries,<sup>22</sup> and in several health human and animal treatment.<sup>23-26</sup>

The use of ozone in the elimination of pathogenic microorganisms in different fields requires the development and implementation of controlled studies to determine specific dosages aiming to improve the techniques employed and the results obtained. The aim of this study was to evaluate the effectiveness of ozone in the inactivation of *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans* and *Enterococcus faecalis*, using *in vitro* model. For this purpose were used different cell concentrations and different times of exposure to ozone.

## METHODS

### BACTERIAL STRAINS AND CULTURE MEDIA

Was used standard strains of *Escherichia coli* CCCD E003, *Salmonella enterica* subsp. *enterica* serovar Typhi CCCD S009, *Staphylococcus aureus* CCCD S003, *Pseudomonas aeruginosa* CCCD P013 (CCCD-Collection of cultures CEFAR Diagnostic, Brazil), *Streptococcus mutans* ATCC 25175 and *Enterococcus faecalis* ATCC 18211 (American Type Culture Collection, Bioscan). The culture media used were Tryptic Soy Agar (TSA, Oxoid®, Cambridge, CB5 8BZ, UK) and tryptic soy broth (TSB, Oxoid®, Cambridge, CB5 8BZ, UK)

### OZONE GENERATION

Ozone was produced by a reactor operating with corona effect (Ozone & Life), fed by pure oxygen. Ozone was generated with the constant flow concentration 4 mg/L. Ozonization of bacterial suspensions (1 000 mL) was performed in controlled temperature of 25 °C, using a gas diffuser connected to the reactor via silicon tube.<sup>27</sup>

## BACTERIAL VIABILITY

The bacterial strains were grown in tryptic soy agar (TSA, Oxoid®) incubated at 37 °C for 24 h. A colony of each species was inoculated into 1000 mL of tryptic soy broth (TSB, Oxoid®) and incubated at 37 °C for 24 h. The initial bacterial density was determined by absorbance at 550 nm using McFarland standard (BioMerieux, Marcy-l'Étoile, France) which corresponds to a concentration of  $1.0 \times 10^8$  CFU / mL. For the ozone treatment, the cell density of cultures were adjusted to concentrations of  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$  and  $1 \times 10^9$  CFU/mL solution of NaCl (0.5 % w/v). As a control (no ozone treatment) was used a sample of each bacterial species at a concentration of  $1 \times 10^6$  CFU/mL. The samples of 0.1 mL, untreated and treated with ozone were collected at different periods of time (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 510 and 540 s),<sup>28</sup> and inoculated into tryptic soy agar and incubated at 37 °C for 24-48 h and then colonies were counted. In those same periods of time, in order to verify the inhibitory efficiency of ozone were withdrawn 1mL from the sample, aiming to confirm the presence of viable microorganisms. Each sample was added to 50 mL of the dye (2,3,5 - Triphenyltetrazolium Chloride (TTC , Merck KGaA , Darmstadt , Germany), to show the activity of the enzyme dehydrogenase, involved in the breathing process. Hydrogenation of TTC in living cells produces the triphenyl formazan, which is a red substance, stable and non-diffusible. It became possible to distinguish the live samples stained in red, from the inactive samples that remains in the same color.<sup>29</sup>

The experiment was repeated four times and then obtained the average CFU/mL and percentage of cell viability for each time interval.

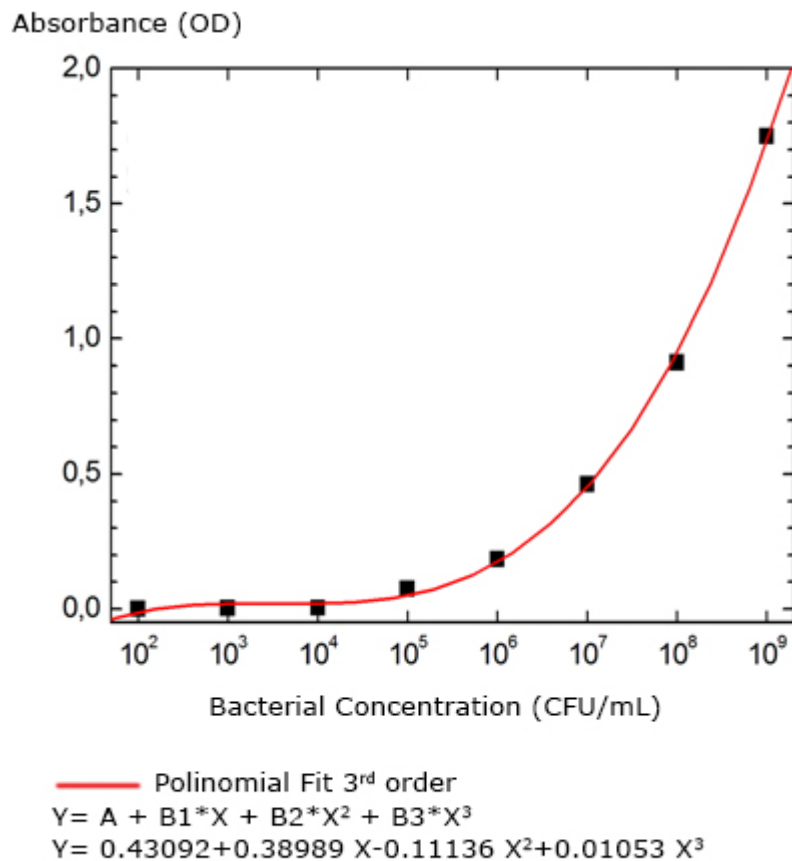
## STATISTICAL ANALYSIS

The results were expressed as the means  $\pm$  SD of three independent measurements for each experiment. The statistical evaluations were performed using the statistical software SPSS ver. 10. Significance was defined as a P value  $< 0.05$ .

## RESULTS

The bacterial viability was assessed by the methods of counting colonies on plates and color changing using the dye 2,3,5 triphenyl tetrazolium chloride (TTC). It was found that both methods were effective in determining the effect of ozone in bacteria, differing between them the period of time to obtain the results. Using the TTC we can get results in 10 min to *Staphylococcus aureus* CCD S003, *Streptococcus mutans* ATCC 25175 and *Enterococcus faecalis* ATCC 18211. The reaction occurred in 20 min when evaluated *Escherichia coli* CCD E003, *Salmonella typhi* CCD S009 and *Pseudomonas aeruginosa* CCD P013. The CFU counts occurred 24 h after inoculation on tryptic soy agar and incubated at 37 °C. The results obtained from the same cell bacterial dilution and periode of ozonization were analyzed by the two methodologies and proved be consistent among them, indicating that the no bacteria growth in Petri dishes was accompanied by no color change of the medium contained in tubes. The standard optical density for the TTC was obtained from different concentrations of bacteria (Fig. 1), showing an exponential increase in optical density as a function of increasing concentration of bacteria, fitted by a third order polynomial. It was observed that different bacteria showed no significant change in this pattern. TTC dye has determined qualitatively

(living cells/dead cells) cell viability after treatment with ozone in a short period of time. In 10 m was possible to obtain effective results of ozonation on *E. coli*, *S. aureus*, *S. mutans*, *P. aeruginosa* and in 20 min on *S. typhi* and *E. faecalis*.



**Fig. 1.** Tetrazolium Standard for Bacterial Concentration.

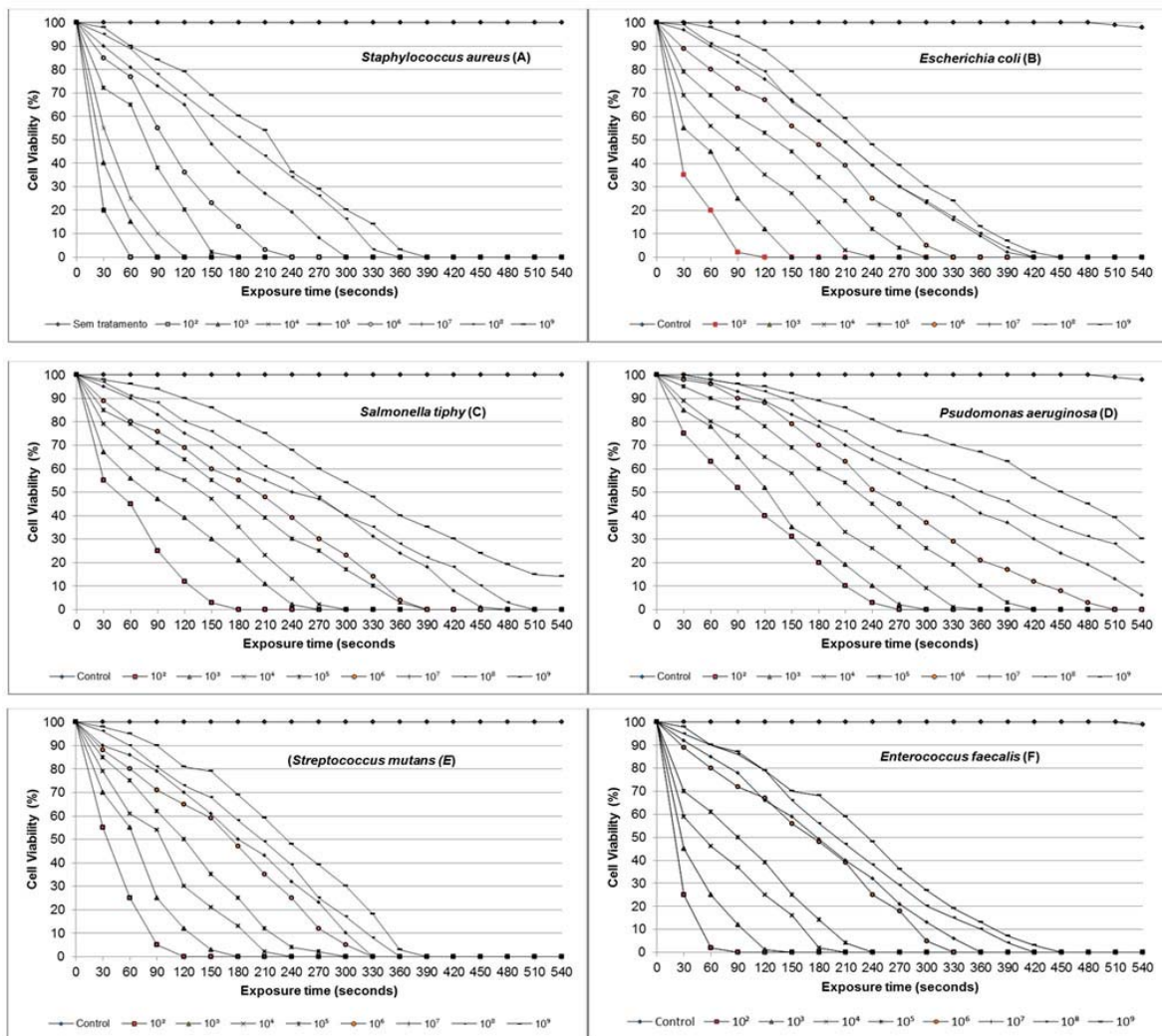
The counts of CFU provided quantitative data on the viable cells relative to the initial number of each dilution, but was required 24 h to obtain the results. Both methods are effective for evaluation of the ozone effect on bacteria and the use of each one depends on the purpose of the study.

Inactivation of different bacterial species after action of ozone showed dependence on cell concentration and exposure time as shown in [figure 2](#). The results showed that the strains of *Staphylococcus aureus* CCCD S003, *Escherichia coli* E003 CCCD, *Streptococcus mutans* ATCC 25175 and *Enterococcus faecalis* ATCC 18211 were sensitive to the effect of ozone like shows the [figures 2A, 2B, 2E e 2F](#), indicating that inactivation cell varied according to the initial bacterial concentration. It took 60 s exposure to ozone at a concentration of  $10^2$  CFU/mL for *S. aureus* and *E. faecalis* were completely inactivated. For the same concentration, *E. coli* and *S. mutans* demanded 90 and 120 s respectively for total inactivation. With increasing bacteria concentration of the samples, ozone exposure time required for complete inactivation was also increased. Using  $10^9$  CFU/mL, 390 s were required for complete inactivation of *S. mutans* and *S. aureus*, and 450 s for the complete inactivation of *E. coli* and *E. faecalis*.

The species *Salmonella typhi* CCD S009 (Fig. 2, C) and *Pseudomonas aeruginosa* CCD P013 (Fig. 2, D), showed a relatively lower response to ozone, requiring longer periods of exposure to ozone to inactivate completely their species. Was required 510 s to completely inactivate the *S. typhi* concentration of  $10^8$  UFC/mL, whereas concentration of  $10^9$  CFU/mL remained still 15 % viable bacteria as shown in figure 2, C.

Inactivation of  $10^2$  CFU/mL of *P. aeruginosa* occurred after 270 s of exposure to ozone, and demanded 510s to inactivate completely  $10^6$  CFU/mL. After exposing to ozone concentrations of  $10^7$ ,  $10^8$  and  $10^9$  for 540 s respectively remain viable, 6, 20 and 30 % of cells (Fig.2, D)

For bacterial concentrations ranging from  $10^2$ - $10^9$  CFU/mL and exposure to ozone by 510 s at 4 mg/L, allows completely inactivate species (2A) *Staphylococcus aureus* CCD S003, (2B) *Escherichia coli* CCD E003, (2E) *Sptreptococcus mutans* ATCC 25175 and (2F) *Enterococcus faecalis* ATCC 18211.



**Fig. 2.** Comparison of the effects of ozone exposure on cell viability. Six strains of bacteria were exposed to ozone at 4 mg/L for a period of 0-540 s. The data represent the mean values of the percentage of bacterial survival. (A) *Staphylococcus aureus* CCD S003, (B) *Escherichia coli* CCD E003, (C) *Salmonella typhi* CCD S009, (D) *Pseudomonas aeruginosa* CCD P013, (E) *Sptreptococcus mutans* ATCC 25175, (F) *Enterococcus faecalis* ATCC 18211.

## DISCUSIÓN

The bacteria used in this study are generally micro-organisms involved in infectious diseases or as contaminants of medical equipment and hospital environments. In addition to the involvement of these microorganisms in infectious diseases in recent years been isolated multiresistant bacteria to different antibiotics, making complicated the treatment of infected persons.<sup>30,31</sup> These problems led to numerous studies in the search for alternative treatments for the control of bacterial infections. This study evaluated the effect of ozonization on *S. aureus*, *E. coli*, *S. typhi*, *P. aeruginosa*, *S. mutans* and *E. faecalis*, checking cell viability at different periods of time, using the colorimetric tetrazolium (TTC) and CFU counts on plates. The results obtained by the colorimetric tetrazolium to verify the viability of bacterial species evaluated were confirmed by the counts, showing the efficiency of the method. The tetrazolium colorimetric test as described by Sylvester<sup>29</sup> is an established method for determining the number of viable cells in proliferation and cytotoxicity studies. The assay provides a rapid, convenient, economical and thus has become a very popular technique for qualify viable cells in culture, providing reliable data.

Ozone is a powerful biocidal agent, capable of inactivating innumerable micro-organisms including Gram-negative and Gram-positive sporulated forms vegetative cells, spores, fungal or viral capsids, severely reducing the populations in a short exposure time.<sup>2-6</sup> The reduction or inactivation of microbial population depends on ozone concentration, time of application and the micro-organisms involved.<sup>3,6,21</sup>

The results show that ozone inactivation of the bacterial species studied depends on the species and their concentration and exposure time to the gas (Fig. 2, A, B, C, D, E, F), showing to be effective for both, Gram-positive and Gram-negative species. there were significant differences between the bacterial strains and exposure times ( $p < 0.05$ ). Similar results were obtained by Thanomsu<sup>28</sup> evaluating the Gram-negative (*E. coli* and *Salmonella*) and gram positive bacteria (*S. aureus* and *Bacillus subtilis*). These authors found that the effectiveness of ozone depends on the bacterial concentration and exposure time, and when evaluated by scanning electron microscopy observed damage and deformity in the wall of the bacteria treated with ozone, and no change was observed in the structures of control samples without exposure to the gas. Prabakaran<sup>32</sup> also show that ozone was effective for inactivating *E. coli*, *S. typhi*, and *Pseudomonas fluorescens*.

Cells of *S. aureus*, *E. coli*, *S. typhi*, *S. mutans* and *E. faecalis* at a concentration of  $1 \times 10^2$  UFC/ml were highly sensitive to ozone, showing a decrease of viable cells ranging from 45 to 80 % (Fig. 2, A, B, C, D, E, F) for the first 30 min of exposure to the gas, whereas *P. aeruginosa* in the same condition decreased by only 25 % of the initial population (Fig. 2, D). The results obtained for *P. aeruginosa* is probably related to the characteristics of bacterial species that has the ability to survive in poor nutrient and grow in water with low solids and dissolved organic compounds.<sup>33</sup>

Studies have shown variation in the sensitivity of the different microorganisms when exposed to ozone, and the temperature variation can be responsible for this effect, because the increase of temperature reduces the stability and solubility of the gas.<sup>8,30,34</sup> In order to ensure reproducibility of the results, this study was conducted in an environment with controlled temperature at 25 °C. The culture medium was standardized for all bacteria, which were subjected to the same exposure time and concentration of ozone.

The results showed that ozone was effective in reducing the population of *Staphylococcus aureus* CCCD S003, *Escherichia coli* CCCD E003, *Salmonella typhi* CCCD S009, *Pseudomonas aeruginosa* CCCD P013, *Sptreptococcus mutans* ATCC 25175, *Enterococcus faecalis* ATCC 18211, leading to the conclusion that ozone had a bactericidal effect on the bacteria and that this effect was proportional to the concentration of bacterial cells and the time of exposure to O<sub>3</sub>.

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*Dora Inés Kozusny-Andreani*. Universidade Brasil. Fernandópolis - São Paulo, Brazil.  
Correo electrónico: [doraines@terra.com.br](mailto:doraines@terra.com.br)