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Efficacy of ozonated water as a PS in photodynamic therapy: A tool for dental caries management? An in vitro study

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Abstract

Background: The most prevalent noncommunicable disease in the world is dental caries; and when it is not adequately treated, it is usually associated with tooth loss or severe dental lesions. In fact, expensive care or tooth extraction may be necessary due to the negative effects dental caries have on general health. This is due to its frequent pain and secondary bacterial infections. The aim of this study was to investigate the activity of ozonated water as such and in combination with appropriate light radiation so as to perform a photodynamic treatment (PDT) against the cariogenic bacterium *Streptococcus mutans*.

Design and methods: This work has been performed in vitro by using an *S. mutans* strain mainly structured in a biofilm status, reproducing the natural condition of the tooth infection. The ozone was tested at three different concentrations by using a commercial device able to generate different O_3 formulations in water. The PDT treatment requires an appropriate light wavelength, evaluated in this work through the UV-Vis adsorption spectrum of the ozonated water.

Results: The obtained results suggested an effective and synergic property of O_3 and light at 460–470 nm against this microorganism. The most antibiofilm activity was observed using a concentration of ozone of 0.06 mg/L alone as well as with PDT treatment.

Conclusions: The results are encouraging for additional research and in vitro/in vivo fresh experimental investigations to perform an exhaustive antimicrobial treatment protocol against the *S. mutans* tooth infection.

Keywords

Ozonized water, photodynamic therapy, PDT against *S. mutans*, ozonated water as a photosensitizer, ozone therapy against *S. mutans*

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Introduction

Topical application of ozone in Dentistry can be managed in different forms: gaseous form, conveyed in the form of oils and in aqueous form, called ozonated water.[1]

The use of ozonated water for the treatment of oral pathologies is widely documented in the literature.

In a recent study, a comparison between ozonated water and topical clotrimazole was performed in order to assess its efficacy in reducing the Candida species colony-forming unit (CFU) count in oral candidiasis.[2] The results showed that ozonated water was more effective than topical clotrimazole in *Candida spp*. CFUs count reduction, with a 60.5% and 32.3% decrease, respectively.

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In addition, the study by *Katti and Chava* evaluated the use of ozonated water subgingival irrigation in patients with periodontal disease. The outcomes of this clinical trial showed that ozonized water was effective in reducing gingival inflammation and in inhibition clinical attachment loss with antimicrobial efficacy.[3]

Moreover, ozone was tested in patients with fixed orthodontic therapy, when gingival inflammation can easily occur. The study by Dhingra and Vandana highlighted that a single subgingival irrigation of ozonated water at baseline efficiently reduced at 2 and 4 weeks of follow-up the gingival inflammation and the lactate dehydrogenase (LDH) enzyme levels in the crevicular fluid.[4]

An in vitro study aimed to assess the antimicrobial effect of ozonated water on *Streptococcus mutans* and *Enterococcus faecalis* considered as the oral microorganisms potentially invading root dentinal tubules. The Viability of *E. faecalis* and *S. mutans* invading dentinal tubules significantly decreased after the two tested irrigants, namely ozonated water and NaOCl 2,5%. When the specimen was irrigated in combination with sonification, ozonated water, showed nearly the same antimicrobial activity as 2.5% sodium hypochlorite (NaOCl). The authors also tested both the irrigant's cytotoxicity on L-929 mouse fibroblasts and ozonated water and showed a low level of toxicity against cultured cells. The authors concluded that the results are supporting the application of ozonated water as root canal irrigation, although further research is needed.[5]

A very recent cross-sectional study compared in a large cohort of patients the efficacy of ozonated water, normal saline, and povidone-iodine after surgical removal of impacted mandibular third molars. The results showed that ozonated water provided the best statistically proven results in reducing the incidence of alveolar osteitis or dry socket and pain at 1 week of follow up.[6]

In this clinical scenario, scanty data are present on ozonated water as a preventive and management tool in dental caries. Dental caries is experienced by more than 90% of all adults in the United States.[7] A very recent review by the European Organization for Caries Research (ORCA) aiming to assess dental caries among European citizens, showed that caries experience (DMFT) was extensive among adults (≥92%). The mean DMFT score ranged from 6.6 to 17.6 (median 12.1) and from 14.7 to 25.5 (median 22.0) in adults and senior citizens of 23 and 21 European countries, respectively.[8]

Baider Bader et al. reviewed the literature in order to provide evidence-based management methods for dental caries. They assessed the level of evidence on the included studies both on primary and secondary preventive measures. The results showed that the strength of the evidence for the efficacy of fluoride varnish for the prevention of dental caries in high-risk subjects was fair, and the evidence for all other methods (chlorhexidine, sucrose-free gum, and combined chlorhexidine- fluoride methods) was incomplete.[9]

Dental caries is defined as a chronic infectious disease with multifactorial etiology and it varies extensively between individuals.[7] The putative pathogen of dental caries is *S. mutans*. Preventive measures aiming to control active and inactive caries progression are focused on developing strategies to reduce a load of this microorganism in the oral biofilm.[10,11] Studies on the antimicrobial effects of ozonized water against *S. mutans* are still few, although promising.[12] Other ozonated topical aids have been shown to be effective in reducing the bacterial load of *S. mutans.*[13] Photodynamic therapy is a treatment that involves the presence of three elements: a photosensitizer, a light with a specific wavelength for that type of photosensitizer; the presence of oxygen. These three elements reacting with each other determine the formation of singlet oxygen and other free radicals which selectively lead to the death of bacterial, fungal, and viral cells.[14] Numerous studies in the literature have demonstrated its antimicrobial efficacy in the most common infections of the oral cavity.[14] Among the most used wavelengths in PDT, we find blue lights with a spectrum between 450 and 470 nm.[15] This type of light, even if not in combination with a photosensitizer, has demonstrated efficacy in vitro against periodontal pathogens.[16] The aim of this in vitro study was to evaluate the efficacy of ozonated water against the main causative agent of caries, *Streptococcus mutans* in terms of viability and biofilm reduction, alone and activated by a LED blue light.

Materials and methods

Strains used in this study

Two different suspensions corresponding to 1×10^8 CFU/ml of *Streptococcus mutans* CIP103220 (Collection of Institute Pasteur), were used as reference strains in this work.

This microorganism was used in its log phase, approximately after 18–20 h of incubation in Schaedler broth at 37°C with 5% CO_2. The suspension was then used to perform different *S. mutans* inoculum as afterward described.

S. mutans behaviors with ozonated water

The in vitro antimicrobial activity procedure was performed using an already published protocol (Casu et al.).[17] In brief, a commercial oral water jet (Aqualab, Sweden Martina, Milan, Italy); was used as an ozonated water generator device following the manufacturer's instructions. This system was able to produce in water different ozone formulations, respectively: 0.025; 0.042; 0.060 [O_3] mg/L.

The experiment was performed in triplicate by using different Eppendorf® tubes, each tube contained 100 µL of *S. mutans* inoculum in Schaedler broth. Then, immediately we add 2 mL of ozonated water containing the different ozone concentrations previously described. The negative

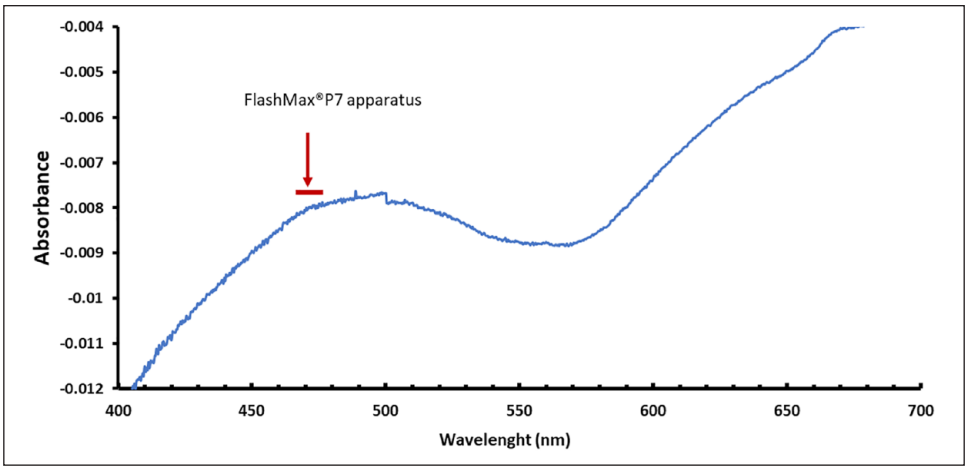

Figure 1. Visible absorption spectra relate to a Ozonate water solution containing an ozone [O_3] concentration of 0.060 mg/L.

control contained the bacterial inoculum plus 2 mL of saline solution [NaCl 0.9%].

After vigorous mixing, each formulation was inoculated on the surface of a Petri dish containing 15 mL of Schaedler agar (Microbiol, Uta Cagliari). The colonies formed after 48 h of incubation at 37°C with 5% CO_2 were counted. In final, the antibacterial activity was calculated by using this formula:

BR% = percentage of CFU reduction versus the negative control (bacterial without ozonated water). [100*CFU_{test}/CFU_{cont}] = BR%

Where:

BR% = bacterial reduction percentage

CFU_{test} = CFU/mL means measured in an ozonated water dish.

CFU_{cont} = CFU/mL means determined in the negative control dish.

Ozonated water absorption spectrum

To determine the ideal assonance between light emission profiles and excitation motifs for use in photodynamic therapy, it was necessary to calculate the UV-vis spectra for [O_3-H_2O].

The absorption spectra of the Ozonate water were investigated using a UV-visible spectrophotometer, the JASCO V600-Bio, (JASCO Europe, Cremella, Italy), in the range of 200–700 nm, with an optical path of $L = 10 \times 10$ mm, using a quartz cuvette (Mettler Toledo, USA Inc.). We looked at the absorption spectra of ozonated water formulations as soon as they were generated by the Aqualab ozonated water apparatus, Figure 1.

Biofilm measurement

A first cultural step was performed in triplicate following these subsequent operational operative phases:

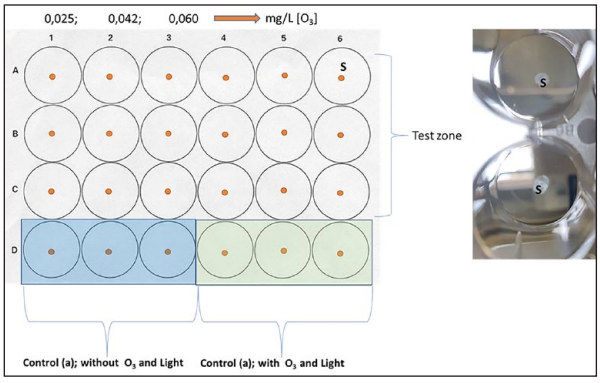

Figure 2. Schematic representation of cultural system used in this work. S = *S. mutans* colony spot used as inoculum and deposed in the bottom of the well plate surface.

i. A colony fragment was deposed to the bottom of the 24-well cell culture microplate, approximately a point of $r = 2$ mm in size, Figure 2.
ii. In each well, inoculated with the corresponding strain as described before, we added 200 µL
iii. of ozonated water at one of three respective ozone concentrations: 0.025; 0.042; and 0.060 [O_3] mg/L, by using an oral ozonated water generator device (Aquolab, Sweden & Martina, Milan, Italy) previously described.
iv. Immediately after, the well was irradiated with wavelength range light from 450 to 470 nm by using FlashMax®P7 apparatus (CMS Dental, Roslev Denmark) following the
v. manufacture instructions (https://cmsdental.com/en/products/flashmax-p7). Two different irradiation times were applied; respectively 30 and 60 s.
vi. 2800 µL of liquid medium (Shaedler or Sabouraud Broth) was added in each well and then the microplates were incubated for 48 h at 37°C with a 5% CO_2.

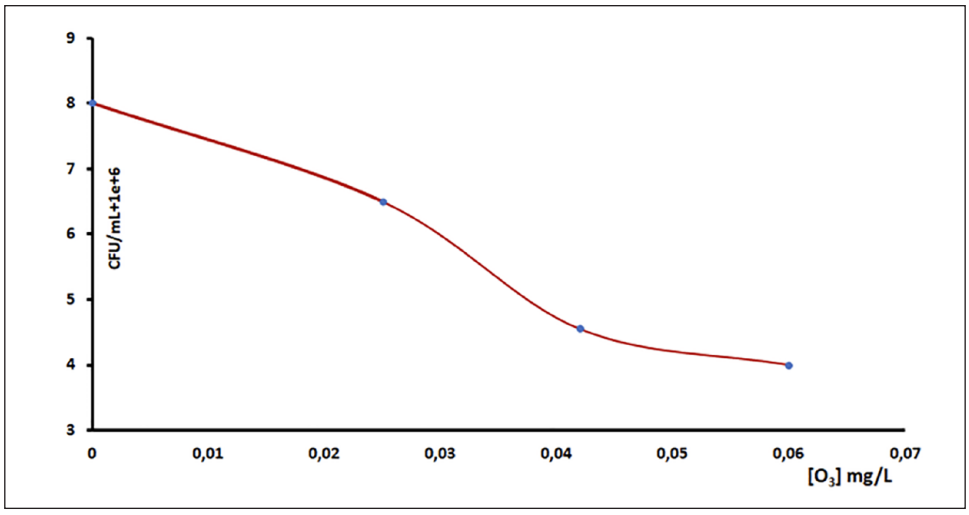

Figure 3. Decrease of *S. mutans* CFU/mL after exposure of different concentrations of ozonated water.

As a second step, after an incubation at 37°C with 5% CO_2 for 3 days, the plate was used for biofilm measurement by using a modified method based on the Montana University Center for Biofilm Engineering. This protocol is based on the crystal violet staining methodology previously described in the literature.[18] In practice, the biofilm that had formed on the well bottom surface was initially gently cleaned three times with PBS solution (Euroclone, Milan Italy) and then stained with a 0.4% crystal violet solution for 2 min after the plates were cleaned three times again with PBS. Finally, 100 µL of 30% acetic acid was then added to each well. From each well 200 µL was collected and deposited in a respective well of a 96-position microplate. The biofilm amount was determined at λ 600 nm by using a Multiscan FC spectrophotometer (Thermo Scientific™, USA).

Statistical analysis

A sample size of three was used for each dilution in each experiment, which was performed three times. All values with a standard deviation (SD) less than 10% of the mean value for the same concentration were deemed significant. To examine the variations between the tested formulations, a Social Science Statistics web calculator (test calculator for two independent means) with a *p*-value estimated at *p* 0.05 was employed.

Results

Antimicrobial activity of pure ozonate water against S. mutans

The antibacterial activity of ozonated water against *Streptococcus mutans* was strongly associated with the ozone concentration for each formulation used in this study, as shown in Figures 3 and 4. While the negative control had an average of 8×10^6 CFU/mL, increasing the ozone concentration in the bacterial mixture from 0.025 to 0.060 mg/L [O_3] resulted in a CFU reduction of 4 million (from 8×10^6 to 4×10^6), the percentage of reduction ranged from 18.8% to 50%.

Antimicrobial activity of a combination of ozonate water and photodynamic therapy

Following the operative protocol already described, we have evaluated the behavior of *S. mutans* in a biofilm form under ozonate water treatment with photodynamic therapy. This experiment was performed by considering the previous results obtained with single ozonate water in solution. In this experimental step, we assumed that the natural infection of *S. mutans* is supported in a sessile form by a complex microbial biofilm within the dental plaque. If we consider the antibiofilm activity by considering the MBEC "*minimum biofilm eliminating concentration*," in other words the minimum concentration of an antimicrobial able to interfere with a mature biofilm.[18,19]

The results shown in Figure 5 reveal an effective and synergic activity by using the maximum O_3 concentration [0.06 mg/L] plus light in a wavelength range from 450 to 470 nm. This experiment also showed that the photodynamic treatment is ineffective if we use O_3 at a lower concentrations range [0.042 mg/L], in fact, in these conditions, the biofilm inhibition is comparable with only ozone use.

Discussion

The *S. mutans* is considered the causative agent of dental caries and clinical interventions aiming to decrease its presence in the oral habitat are highly encouraged.

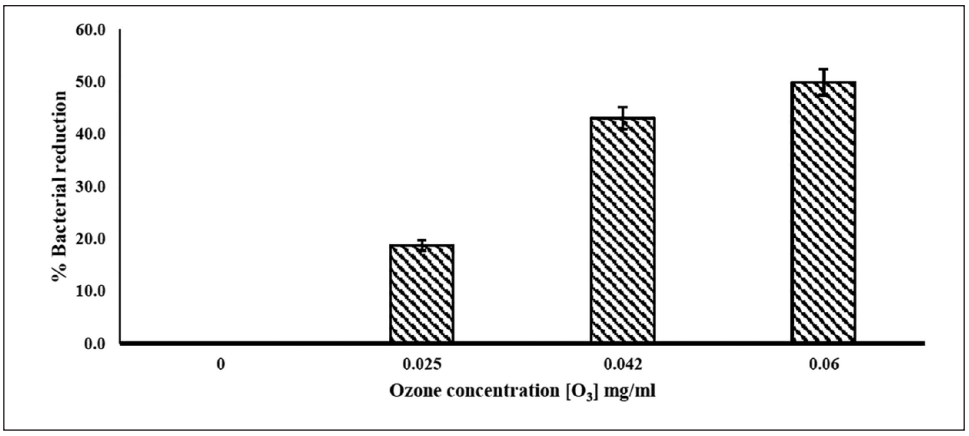

Figure 4. Percentage of bacterial reduction evaluated with different ozone concentrations in water.

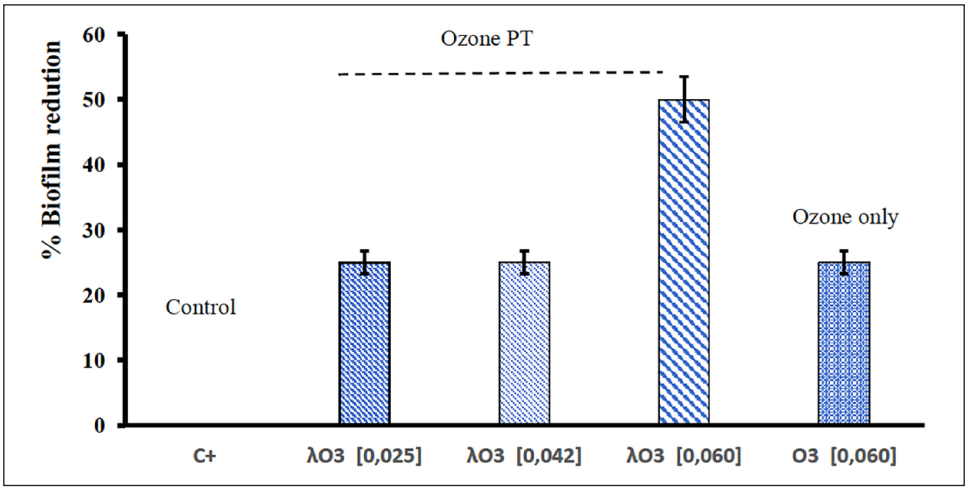

Figure 5. Percentage of *S. mutans* mature biofilm reduction evaluated with different ozone concentrations and light in a wavelength range from 450 to 470 nm.

Dental caries still is a global burden with a high prevalence in young, adults, and elderly populations, It is considered as a multifactorial and behavior-dependent disease affected by oral hygiene measures. Figuero et al. demonstrated that regular brushing with fluoride toothpaste is strongly related to lower caries experience[20] and brushing twice a day controls the rate of caries progression more effectively than brushing only once a day.[21] A recent review by ORCA documented that only 33%–85% of adults in some European countries follow the recommendation to brush twice a day with fluoridated toothpaste.[8] Moreover, ORCA documented epidemiologic data on DMFT values over the period of 1996–2016 with a reduction of the range of mean DMFT values from 13.4 and 20.8 to 6.6 and 17.6, which clearly indicates a decline in caries.[22] Therefore, DMFT values for the European adults population ranging from 6.6 to 17.6 are far from reflecting a caries-free population and highlight the need for a more effective strategies for oral hygiene procedures.

S. mutans is an obligate human Gram-positive pathogen with a biofilm-dependent lifestyle. It is an acidogenic bacteria associated with caries in primary and secondary dentition. Dental caries is the outcome of a shift in the oral biofilm community with acidogenic bacteria that colonize the tooth surface and cause enamel demineralization in the presence of fermentable carbohydrates.

The natural habitat of *S. mutans* is the human oral cavity, more specifically, the oral biofilm formed on the hard surfaces of the tooth. The *S. mutans* cariogenic potential resides in four core attributes: (i) adherence to enamel surface; (ii) production of acidic metabolites; (iii) the capacity to build up glycogen reserves; (iv) the capacity to synthesize extracellular polysaccharides (EPSs).[23] *S. mutans* can modify the local environment through the synthesis of insoluble glucans from sucrose for the formation of a stable biofilm matrix that facilitates bacterial colonization of the tooth surface and, at the same time, functions as a diffusion barrier helping to maintain the acidic setting within

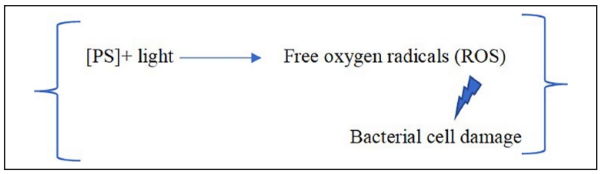

Figure 6. Summury diagram of the working mechanism of photodynamic process.

which other acidogenic and aciduric species thrive.[24,25] *S. mutans* is a therapeutic target in caries management and prevention. The mechanical removal of biofilm during the at-home oral procedures may be insufficient, especially in those patients presenting with a high caries risk. Additional topical use of antimicrobial products may help by: (i) reducing the rate of existing plaque; (ii) reducing the rate of plaque growth; (iii) inhibiting of the growth of the species associated with the disease; (iv) inhibiting of the production of EPS. Among the antibacterial agent chlorhexidine is the gold standard and it has been used for the last 40 years. However, its use for a prolonged period of time is not recommended due to side effects such as discoloration, taste alterations, and oral desquamation.[26] A preventive strategy is thought to be used day by day with efficacy and without side effects. Therefore, chlorhexidine cannot be used as a preventive tool for caries management. Moreover, studies demonstrated that: (i) chlorhexidine didn't show long-term effects with *S. mutans* subsequent increase after weeks or months[27]; (ii) the chlorhexidine anti-caries effects are minor.[28]

New products, considered safer for the environment and to the patient, such as propolis,[29] curcumin,[30] cranberries,[31] and green tea extracts[32] showed to be effective against oral biofilm and in caries management and prevention, however, none of these products proved to be selective against *S. mutans*. A new therapeutic strategy against *S. Mutans* based on alkali generation, through ammonia production from arginine and urea was described in a review by Burne and Marquis. The authors showed that alkali plays a major role in pH homeostasis in oral biofilms and may moderate the initiation and progression of dental caries.[33] These scientific hypotheses are fascinating, but far away from being reality in the everyday dental practice.

Another important issue of the preventive strategies against the caries putative agent *S. mutans* is biocompatibility. The biocompatibility of ozone was evaluated by in-vivo and in-vitro studies. No cytotoxic signs were observed, and aqueous ozone showed the highest biocompatibility among the tested antiseptics. Moreover, a very recent clinical study on a mouthwash based on ozonated olive oil used for a period of 3 months showed no side effects correlated with its daily use.[26]

This in vivo study, for the first time, documented the efficacy of ozonated water in reducing *S. mutans* vitality. In addition, the antibacterial activity resulted strongly correlated with the ozone concentration, but the first approach by using only ozonated water resulted in not being completely exhaustive in the bacterial biofilm reduction. In these conditions, the maximum reduction index was observed around 25%; the same result was obtained at a lower ozone concentration by using a photodynamic approach. However, a considerable synergic effect was shown by using a [O_3] concentration of 0,06 mg/L plus light with an inhibition index of 50%. This dose-dependent in antibiofilm activity is probably due to at least two conditions:

(a) The biofilm matrix could be made waterproof to PS, able to restrict it diffusion versus bacterial cells.
(b) Free radicals generated by the photodynamic process are related to PS concentration following the subsequent reaction (Figure 6):

In practice, bacterial cellular inactivation by PDT requires a minimum level of ROS, the reason that they should exceed the cytoplasmatic anti-ROS molecules level. Thus, these results suggest that in PDT, there may be a minimum concentration of PS at which biofilm inactivation increases significantly due to light radiation addition. This aspect, following recent literature data, is little studied but should be better investigated for each PS in all PDT-used protocols.

Conclusions

In spite of this work appearing preliminary, the obtained results are encouraging for further studies and in vitro/in vivo new experimental investigations; this is because different questions result open. First of all could be necessary to evaluate other light wavelengths, for example, the maximum absorption peak range shown in Figure 1, that is, around 500 nm and over 650 nm in the infrared zone. In addition, other cariogenic microorganisms could be evaluated, in particular *Streptococcus sobrinus* and different *Lactobacillus spp*.

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Authors' contributions

CC: primary author of the manuscript, critical appraisal and editing of the manuscript. FS maintained the isolates, and the strains, prepared cultures, and performed experiments. GO critical analysis of the manuscript, editing, and final presentation. R G and RFG: assisted in writing the paper and critical analysis of the manuscript. GN: analyzed the experimental data and critical analysis of the manuscript. All authors read and approved this manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics approval and consent to participate

Ethical approval was not required for this in vitro study.

Patient consent for publication

Not applicable. No individual-level personal data were collected, and data were reported only in the aggregate.

Informed consent

The manuscript does not contain any individual person's data in any form

Institutional review board statement

Not applicable.

Significance for public health

The incidence of caries in the general population is very high, and represents one of the most frequent oral pathologies, with different values between the various countries. The main cause of caries is *S. mutans*, so acting on this factor is extremely important from the perspective of caries prevention. Many oral antiseptics are contraindicated in children and during pregnancy, such as chlorhexidine for example. Hence the need arises to find a device free from side effects, usable on children and in pregnancy capable of lowering the charge of *S. mutans* and therefore significantly reducing the incidence of caries. Photodynamic therapy (PDT) and ozonated water have been shown to have no side effects, and safe even during pregnancy. These in vitro results on the PDT inactivation of S. mutans carried out with ozonated water as a photosensitizer, show that these aids could make a valid contribution to the achievement of this aim.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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