



Original Article

## Efficacy of ozonated olive oil against peri-implant microbes isolated from peri-implantitis

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### Abstract

This study aimed to investigate the antibacterial and antimicrobial activity of ozone gel against oral biofilms grown on titanium dental implant discs. The experiment used medical grade five titanium discs on which peri-implant isolated biofilms were grown. The experimental groups were control, *Streptococcus mutans* (*S. mutans*) and *Granulicatella adiacens* (*G. adiacens*), (n = 6). The oral microbes grown on titanium discs were exposed to ozone gel for 3 minutes and the antibacterial activity was assessed by turbidity test and adherence test for the antibiofilm activity test. Bacterial morphology and confluence were investigated by scanning electron microscopy (SEM), (n=3). Two bacterial species were identified from the peri-implant sample, *S. mutans* and *G. adiacens*. The results showed that adding ozone to the bacterial biofilm on titanium dental implants did not exhibit significant antibacterial activity against *S. mutans*. Moreover, there was no significant difference in antibiofilm activity between control and treatment groups. However, significant antibacterial and antibiofilm effect was exhibited by ozone gel against *G. adiacens*. Ozonated olive oil can be considered as a potential antimicrobial agent for disinfecting dental implant surfaces and treating peri-implantitis.

**Keywords:** Dental implants, Oral microbiota, Implant surface, Dental biomaterials

### 1. Introduction

The inflammatory characteristic of peri-implantitis is a significant issue in implant dentistry [1]. The presence of bacteria in the peri-implant tissue results in a destructive immune response and subsequently bone loss [2]. Peri-implantitis is a pathological disease that affects the implant's success due to it deviates from the normal healing process [3]. Peri-implant disease can cause a variety of symptoms, including exudate, increasing pocket depths, and osseous defects that affect the region around the dental implants. Detectable signs include radiographic vertical bone loss, bleeding on probing, swelling and redness of the surrounding tissues [4]. Bacteria play an important role in the continued progression of bone loss. As the condition progresses, an exudate or abscess may appear, indicating an aggravation of peri-implantitis along with potentially accelerated bone loss [5].

Detoxifying the contaminated implant surface is one of the key goals of peri-implantitis therapy [6]. Non-surgical techniques are appropriate and enough for detoxification in the presence of peri-implant mucositis. These include ultrasonic or air polishing, titanium or plastic cures, and mechanical implant cleaning. Additionally, local anti-

septic agents such as chlorhexidine gluconate, hydrogen peroxide, sodium percarbonate, and povidone-iodine, as well as photodynamic treatment, may show an antimicrobial property [7].

Ozone is being used in a variety of dental treatments and specialties [8]. The oxidizing characteristic of ozone contributes to its effectiveness as an antibacterial agent [9]. Researches detected that ozone causes deactivation of the bacteria by destroying the cell envelope [10]. The ozone particles are capable of arresting carious lesions [9], promoting the healing of pre-implantitis by decreasing the number of *P. gingivalis* [8]. In addition, research workers assessed the ability of ozone to enhance the osteointegration process, number of osteoblasts, osteoclast and vascularity after tooth extraction [8]. In addition, ozone's regenerative treatment of peri-implantitis shows a significant outcome on the decantation of implant surfaces [11].

A research conducted by Isler and co-workers investigated the influence of ozone gel on SRT implant surfaces of peri-implantitis, the results demonstrated that ozone therapy reduced the probing depth and improved both plaque and gingival index values [12]. Regarding the healing effect of ozone its stated that ozone enhances the

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healing processes of periodontal/peri-implant wounds and increases the secondary stability of dental implants [13]. A recent review article shows the efficacy of ozone in treating periodontics and peri-implantitis [14]. However, there are different results regarding its antibacterial activity. A study has shown that gaseous ozone significantly increases the implant success rate compared to control (sterile saline), [12]. However, another study found that the use of ozone gel did not exhibit a significant reduction in microbial count compared to CHX [15]. Moreover, ozonated olive oil showed significant antibacterial activity against *P. gingivalis* compared with CHX [16]. Regarding CHX which is commonly used antimicrobial agent in oral cavity, its use as an irrigation solution for treating peri-implantitis is questioned due to its cytotoxicity against human primary osteoblast cells [17]. Moreover, despite its antibacterial efficacy, the use of gaseous ozone might be limited because of the toxicity of the respiratory system when inhaled [18]. Taken together, antibacterial activity of ozone against oral microbes grown on titanium dental implants might be different according to the chemical form of ozone, duration of exposure and type of microbe. Thus, this study aimed to investigate the antibacterial efficacy of ozonated olive oil in preventing oral biofilm grown on titanium implant discs.

## 2. Material and Methods

### 2.1. Specimen preparation

The titanium alloy chosen for the study is medical, grade five titanium alloy (Ti6Al4V) discs which are frequently used for medical implants. The discs are circular in shape with dimensions of 15 mm in diameter and 1 mm in thickness created by laser cutting and polished with sandpapers of (800–1200 grit) using a rotary tool (Grinder–Polisher, Buehler, UK Ltd, Coventry, England). For the final polish, six and one microns of diamond solution (Diamond solution, Kemet International Ltd, UK) were applied. The discs were then cleaned using an alkaline solution and 5% HCl as described in (19,20).

### 2.2. Ozonated olive oil gel

The ozone used in this study was in the form of gel (OXaktiv, Pharmoxid Arznei, Gmbh & Co KG, Germany). The product contained paraffin liquidum, ozonized olive oil and polyethylene.

### 2.3. Surface roughness values of the specimens

The surface roughness of polished titanium implant disc specimens was measured before and after ozone application. Briefly, the roughness value was measured using a digital profilometer (Surftest-402; Mitutoyo, Kawasaki, Japan). The profiler was set to move a diamond stylus across the specimen surface under a constant load. Each line was scanned for 10 seconds with a constant force of 4 mN (0.4 gf) on the diamond stylus (stylus type = 5  $\mu$ m radius). The value for the surface roughness was obtained from the digital scale. The Ra value ( $\mu$ m) is defined as “the mean value of all absolute distances of the roughness profiles from the mean line within the measuring distance”.

### 2.4. Isolation and identification of *S. mutans* and *G. adiacens* from peri-implantitis

Both bacterial species were isolated from a failed dental implant due to peri-implantitis which was used to replace

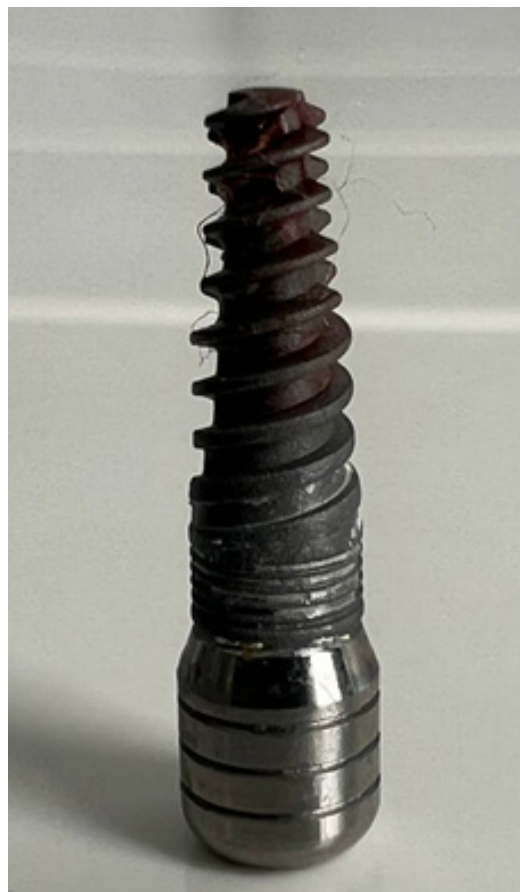
upper right canine of a 23-year-old female (Figure 1). After the implant removal, a swab of peri-implant sample was collected and transported to the lab then cultured on blood agar and incubated for 24 hours at 37 °C. After that, two colonies were identified on the blood agar which were later identified using the VITEK II device (bioMerieux, North Carolina, USA).

### 2.5. Assessment of antibacterial activity of ozone gel using disc diffusion method

Prior to the main antibacterial experiment of ozone against grown oral microbes on titanium discs, antibacterial efficacy of ozone against *S. mutans* and *G. adiacens* was assessed using a disc diffusion assay which is a conventional method used for testing microbial growth sensitivity against antimicrobial agents. Briefly, 6 mm diameter filter papers were soaked in ozone gel for 3 hours, after that, the conditioned filter paper was transferred to Mueller Hinton agar where either *S. mutans* or *G. adiacens* were newly sub-cultured (n = 3), unit of replication the agar plate. After 24-hour incubation, the agar plates were examined for the presence of microbial growth inhibition zone around the filter papers.

### 2.6. Preparation of bacterial suspension

The microbial sample which was isolated from the failed dental implant was put in blood agar and then incubated for 24 hours at 37 °C to acquire bacterial growth. Then a swab of the microbial colony in the blood agar was cultured in brain heart infusions (BHIB). The sample in BHIB was put in anaerobic jars and incubated at 37 °C for 24 hours to activate the bacteria.



**Fig. 1.** The failed dental implant fixture from which the microbial swab was collected.

## 2.7. Experimental design (Experiment on Titanium Dental Implant Discs)

Following disc diffusion assay, two sets of experiments were conducted to test antibacterial and antibiofilm activity of ozonated olive oil against for *S. mutans* and *G. adiacens* grown on titanium dental implant discs. The experimental groups were; *S. mutans* grown on titanium discs (control), ( $n = 6$ ), ozone gel-treated *S. mutans* grown on titanium discs (treatment). The same method was applied for the other species (*G. adiacens*). Basically, each species in 1.5 BHIB was added to the titanium discs in sterile glass tubes and incubated over-night at 37 degrees in an anaerobic jar. After 24 hours, the BHIB was removed and 5 ml ozonated gel was added to the discs (treatment group) to cover the entire surface for 3 minutes. The ozone gel was then washed away, and the specimens were rinsed three times with distilled water. Fresh BHIB was then added to the specimens and incubated for 24 hours. Another triplicate of control and treatment group for each species was also prepared for assessing bacterial morphology after 24 hours under the SEM.

## 2.8. Assessment of antibacterial activity

Optical density measurement (turbidity test) was conducted to assess the antibacterial activity of ozone against suspended bacteria in the BHIB. Briefly, aliquots of 100  $\mu$ l of the nutrient broth of blank, control and treatment groups were added to a 96-well plate with a flat bottom and a lid. After that, 100  $\mu$ l of the fresh BHIB was added to each well. The 96-well plate was then placed in a plate reader (BioTek ELX800) and the absorbance values were read at 630 nm to determine the turbidity of the samples.

## 2.9. Assessment of antibiofilm activity

Antibiofilm assay used in this study followed the protocol used by (21). After the experiment, the media was removed and crystal violet 1% was used to stain the biofilm on each specimen for 10 minutes. The intensity of the stain was used to determine the strength of the biofilm, with darker staining indicating stronger biofilm. After staining, the specimens were rinsed with distilled water to remove excess stain and left at room temperature to dry. In order to remove the biofilm, 3 mL of ethanol was added to each specimen. Finally, the biofilm mixed with the ethanol solution was withdrawn and placed in 96 well-plated and the turbidity was measured using a plate reader (BioTek ELX800) at 630 nm.

## 2.10. Assessment of the bacterial morphology and confluence using SEM

SEM was used to visualize the morphology of the bacterial cells after the experiment. The bacterial cells were examined in situ on the titanium discs. After the experiment, the media were discarded, and the specimens were washed with phosphate buffer. After that, the bacterial biofilm on the discs was immersed in ethanol solutions (30, 50, 70, and 95%) for 20 min each then 100% ethanol for 1 h. Later, the specimens were left to dry overnight and then sputter-coated with chromium. The bacterial cells on the discs were then examined under SEM for morphology and confluence.

## 2.11. Statistical analysis

Data are expressed as mean  $\pm$  S.E.M. and analyzed

using stat graphics version 16. To locate the significant difference between the groups, data were subjected to one-way ANOVA, then Tukey's test. All statistical analyses used a 95% confidence limit, p values  $< 0.05$  were considered statistically significant.

## 3. Results

The results showed that ozonated olive oil applied to the microbial biofilms grown on titanium dental implant discs significantly inhibited the growth of *G. adiacens* but did not show a significant effect against *S. mutans*. The same result was obtained while testing the antibacterial activity of ozone using disc diffusion method.

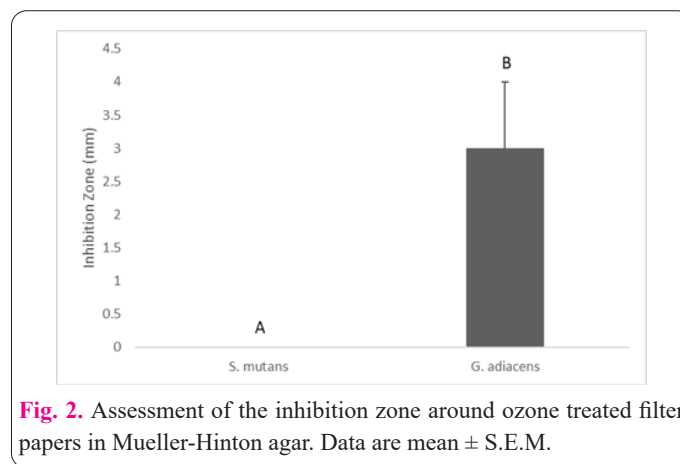
Regarding the microbial identification of the collected sample from the failed implant surface, it was found that the sample contained *S. mutans* (96% probability) and *G. adiacens* (97% probability), (Table 1).

When the antibacterial activity of ozone was tested against those species using disc diffusion method, it was found that the growth inhibition zone of *G. adiacens* around ozone gel was 3 mm while no *S. mutans* growth inhibition zone was detected around the ozone-conditioned filter papers (Figure 2).

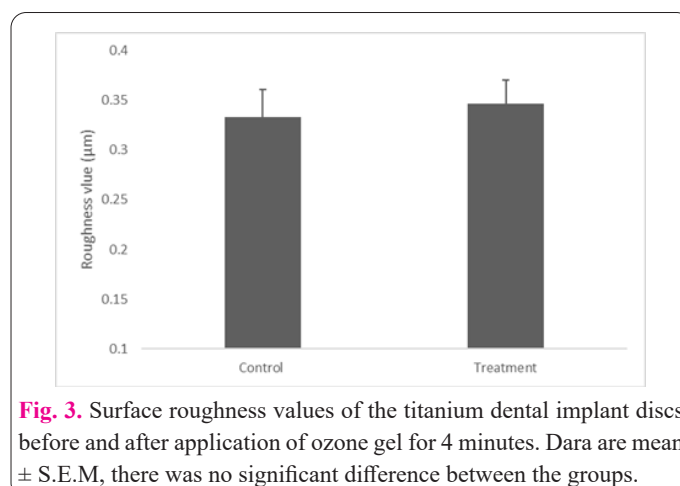
Furthermore, the surface roughness measurement before and after ozone gel application, results showed that the roughness values of both groups were around 3  $\mu$ m.

**Table 1.** Identification of the bacterial species in the peri-implant biofilm.

Type of species	Percentage probability
<i>Streptococcus mutans</i>	96%
<i>Granulicatella adiacens</i>	97%



**Fig. 2.** Assessment of the inhibition zone around ozone treated filter papers in Mueller-Hinton agar. Data are mean  $\pm$  S.E.M.



**Fig. 3.** Surface roughness values of the titanium dental implant discs before and after application of ozone gel for 4 minutes. Data are mean  $\pm$  S.E.M, there was no significant difference between the groups.

No statistically significant difference was found between the variables (Figure 3).

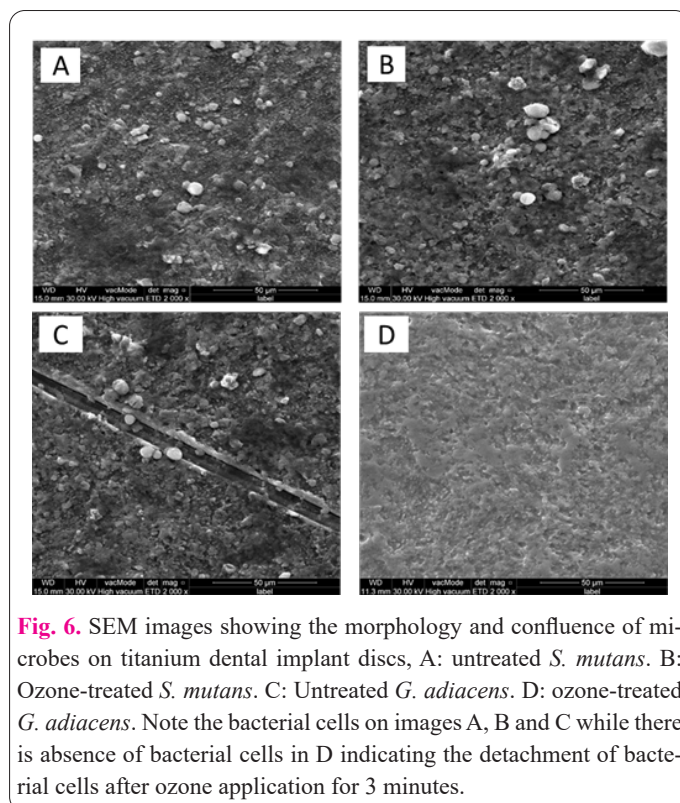
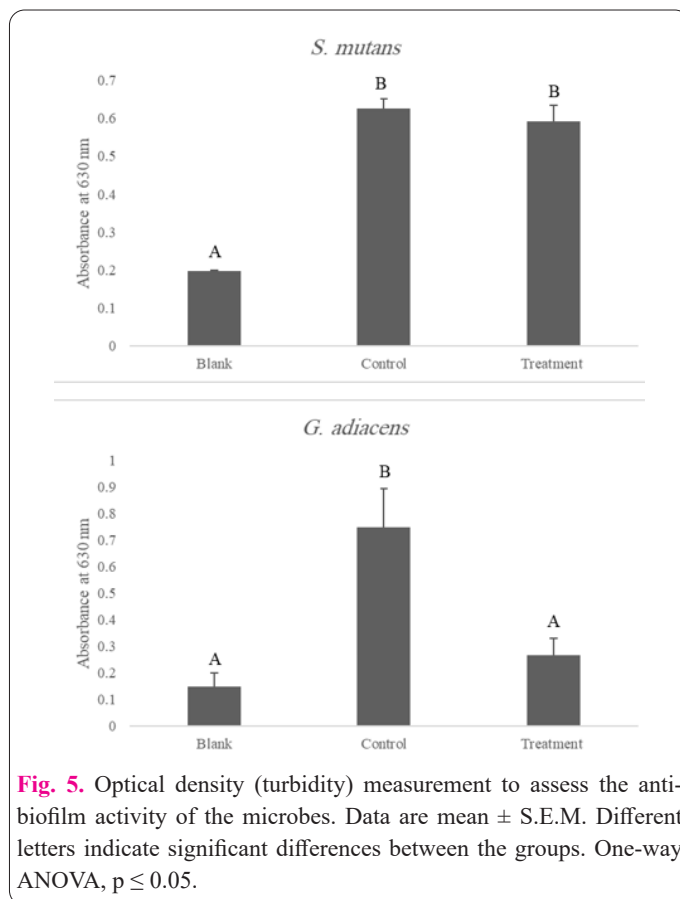
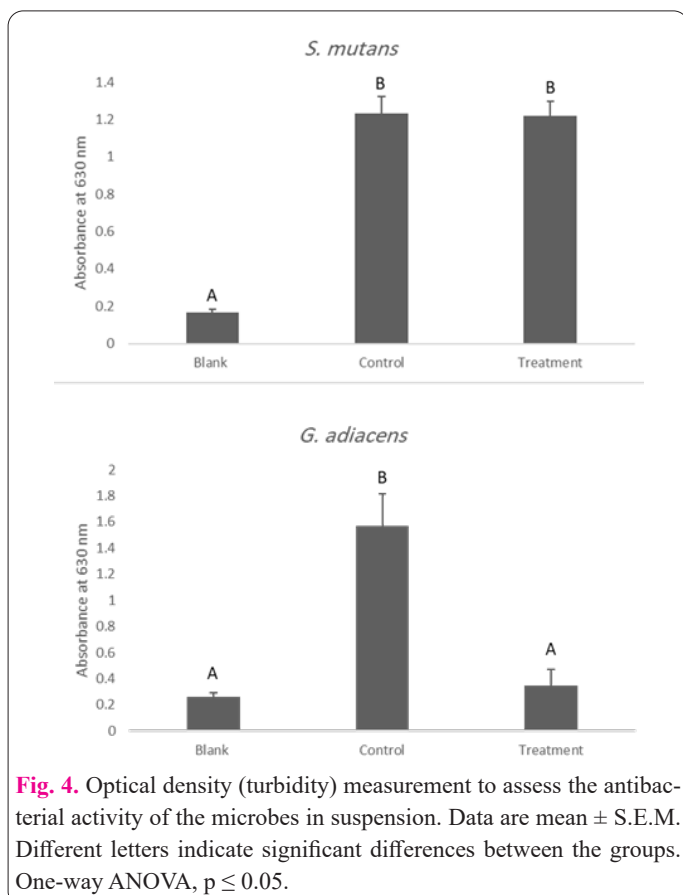
Regarding the antibacterial and antibiofilm activity of ozone gel against *S. mutans* and *G. adiacens* separately grown on titanium dental implant discs, the findings revealed that the turbidity of the BHIB of *S. mutans* was  $1.23 \pm 0.08$  for the control and  $1.21 \pm 0.07$  for the treatment, the difference between them was not statistically significant (Figure 3). However, the turbidity of BHIB of *G. adiacens* was significantly higher in the control compared to the treatment measuring  $1.54 \pm 0.24$  and  $0.32 \pm 0.11$  respectively (Figure 4).

Furthermore, the turbidity of the *S. mutans* biofilm showed no significant difference between the control and the treatment measuring  $0.63 \pm 0.57$  and  $0.73 \pm 47$  respectively (Figure 5). Whereas there was a statistically significant difference in turbidity measurements between the control and the treatment of *G. adiacens* biofilm measuring  $0.75 \pm 0.14$  and  $0.26 \pm 0.06$  respectively (Figure 5).

The morphology and confluence of the bacterial cells were evaluated after exposure to ozone gel. SEM images showed that there was confluent *S. mutans* coverage on the titanium implant discs for both control and treatment specimens without noticeable alteration in cellular morphology following ozone application (Figure 6 A & B), indicating that the ozone treatment did not induce a bactericidal effect against *S. mutans*. However, *G. adiacens* were very difficult to find on titanium implant discs after ozone treatment (Figure 6 D), but confluent cells were detected on the control (Figure 6 C).

#### 4. Discussion

This study investigated the antibacterial and antibiofilm effect of ozonated olive oil against *S. mutans* and *G. adiacens* isolated from the surface of a failed implant due to



peri-implantitis. Results showed that the ozone treatment caused significant damage to *G. adiacens* in suspension and biofilm, however, no significant effect on *S. mutans* was detected. Prior to the antibacterial activity experiment, the polished titanium dental implant discs were subjected to ozone treatment to investigate the effect of ozone on the surface roughness of the specimens. The results showed that ozone did not induce a significant change in surface roughness compared to control. This finding is supported

by another study which found that ozone-treated teeth surface did not show a significant change in surface roughness [22].

Regarding the efficacy of ozone against peri-implant microbes, this study used oral microbial species derived from an infected per-implant surface. After sample isolation and identification, it was found that the sample consisted of *S. mutans* and *G. adiacens*. Studies have shown that *S. mutans* is commonly detected in peri-implantitis and considered as an early colonizer, whereas, *G. adiacens* is less commonly detected in peri-implantitis [23]. When the colonies were subjected to ozone-treated filter papers in Mueller-Hinton agar, it was found that ozone inhibited the growth of *G. adiacens* for about 3 mm, while no growth inhibition was detected in *S. mutans*. This can be explained by the fact that the sensitivity of microbes to the antimicrobial agents can be different between different species due to the difference in cell wall structure, morphology, type of biofilm they produce... etc.

In the current study, ozonated olive oil showed no significant activity against *S. mutans* both in suspension and in a biofilm on titanium dental implant discs. However, it showed a significant antibacterial activity against *G. adiacens* both in suspension and in a biofilm on titanium dental implant discs. Similar studied studies have been conducted by other researchers which found either positive or negative results regarding antibacterial activity of ozone. For example, research was conducted comparing the antibacterial activity of ozonated olive oil and chlorhexidine gluconate against *S. mutans*, results showed that ozone did not demonstrate antibacterial activity [24]. Another study investigated the efficacy of ozone gel against *Enterococcus faecalis* by applying ozone for 1 and 2 minutes, results showed that the antibacterial and antibiofilm effect of ozone was negligible [25]. Moreover, another study was conducted to test the efficacy of ozonated water against plaque microbes, it was found that ozonated water containing mouthwash did not significantly affect the supra and sub gingival biofilm formation [26]. In contrast, a study has proved that ozonated sunflower oil induced significant growth inhibition against streptococci [27]. Another research has demonstrated that gaseous showed significantly more antibacterial properties compared to the aqueous form after applying for 3 minutes [28,29]. This could be explained by the fact that antimicrobial efficacy of ozone strongly depends on its chemical form. It could be argued that the reason for better efficacy of gaseous ozone compared to aqueous or oil form is that gaseous ozone is chemically active and oxidizes more easily compared to other forms. However, although incidence rate is very low, gaseous ozone's clinical use is limited due to the possible respiratory toxicity resulting from inhalation. Undoubtedly, the unique properties of olive oil [30-32] have caused these effects.

The efficacy of ozonated olive oil against oral microbes used in this study was different. It was found that ozone induced significant growth inhibition and toxicity against *G. adiacens* using disc diffusion method and optical density method in suspension and biofilm on titanium discs. However, opposite result was observed when *S. mutans* were subjected to ozone. The scientific interpretation of this finding is rather complicated, it has been found that the sensitivity of ozone is different against different species. For example, petricola and co-workers found that

ozone gel demonstrated significantly more antibacterial activity against *P. intermedia* compared to *S. mutans* [24], based on this finding, it was concluded that gram-negative species might be more sensitive to ozone gel compared to gram-positive species as the later have thicker peptidoglycan in the cells wall. However, in the current study both species used were gram-positive, so different sensitivity to ozonated olive oil might not be due to the gram staining, it is rather caused by other factor(s) that influenced the antibacterial efficacy of ozone against *S. mutans*. Since *S. mutans* is widespread in oral cavity with a relatively high proportion in saliva, gingivitis, periodontitis as well as peri-implantitis opposite to *G. adiacens* which takes a very small proportion of oral microbiota, so development of a resistant species against ozone gel might be the explanation. More in-depth microbiological studies are needed to further elucidate the antibacterial activity of ozonated olive oil against microbes that are incorporated in peri-implantitis. Providing that ozonated olive oil is affordable and not risky compared to gaseous ozone, it might have the potential to be used to disinfect a contaminated peri-implant surface due to peri-implantitis.

## 5. Conclusions

Within the limitations of this study, it could be concluded that ozonated olive oil has the potential to be used clinically as a disinfectant to decontaminate the infected dental implant surface. However, the efficacy of ozonated olive oil varies according to the type of bacterial species. Thus, further studies are needed to elucidate the efficacy of ozonated olive oil against mixed oral biofilms having both gram-positive and negative species.

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## Author contributions

Ranj Salaie, wrote the main manuscript, designed the experiments and conducted the methodology, Shehab Ahmed, developed the article's main idea and conducted part of the methodology, Zhala Meran arranged and analyzed the data.

## Conflict of Interest

The author declares no conflict of interest.

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