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ORIGINAL ARTICLE

Bacterial reduction effect of four different dental lasers on titanium surfaces in vitro

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Abstract

Compare the effectiveness of selected dental lasers for decontamination of machined titanium surfaces in vitro. Seventy-two sterile machined surface titanium discs were individually inoculated with strains of *Streptococcus mutans* (Sm), *Streptococcus oralis* (So), Aggregatibacter actinomycetemcomitans (Aa), or all three bacteria together (MIX) at 34.0° C, 20.8% O_2 and 5% CO_2 for 12 h. After incubation, the discs were divided into six groups: 1) no treatment, 2) 0.12% chlorhexidine gluconate (CHX), and 3) 10,600 CO_2, 4) 810 nm diode, 5) 2780 nm Er,Cr:YSGG, 6) 1064 nm Nd:YAG laser groups. After treatment, any remaining viable bacteria were liberated from the discs via sonication, transferred onto brain heart infusion (BHI) agar plates for culturing, and colony-forming units (CFUs) were recorded. Statistical analysis was performed. There were statistically significantly differences (SSD) ($p < 0.01$) in bacterial reduction of discs individually inoculated with Aa between the Er,Cr:YSGG and Nd:YAG lasers. There was also a SSD ($p < 0.01$) lower effect with the MIX with the Er,Cr:YSGG compared with all other modalities. Bacterial reduction with the CO_2 was better ($p < 0.001$) than treatment with CHX or the Er,Cr:YSGG laser on killing of So. Although all modalities of treatment showed a mean of 98% or greater viable bacterial reduction, the most consistent bacterial reduction of all titanium discs was with the Nd:YAG laser (100%).

Keywords Laser · Titanium · Biofilm · Decontamination · Implant

Introduction

Dental implants have become one of the most commonly used treatments to replace missing teeth. With an increase in dental implant use, there is and will continue to be an increase in the number of patients suffering from peri-implant diseases, peri-implant mucositis (without bone loss) (PMUC) and/or peri-implantitis (with bone loss) (PIMP). It has been reported that the diagnosis of PMUC is generally based on the sign of bleeding on probing (BOP) without the loss of supporting bone, and the diagnosis of PIMP is generally based on bleeding upon probing and bone loss after 1 year in function. Approximately 47% of patients and 29% of implants exhibit peri-implant mucositis, while approximately 20% of patients and 9% of dental implant sites exhibit peri-implantitis [1].

Numerous studies have identified periodontal pathogenic microorganisms as playing a role in peri-implant infections, both in animals and humans [2–10]. It has also been found that patients with chronic periodontitis have lower implant survival rates and more biological complications than those patients with implants used to replace teeth lost due to reasons other than periodontitis, as well as a similar microbiota being identified around teeth and implants in the same patient [1–7, 11].

The goals of treating PMUC and PIMP revolve around removal of local etiologic factors, reduction of inflammation and potentially establishment of new bone contact (re-osseointegration) with the implant. A basic approach to both

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Table 1 Laser parameters

Laser type	Wavelength (nm)	Average output power (W)	Pulse duration **	Energy per pulse (mJ)	Repetition rate (Hz)	Total applied energy (J)
CO_2*	10,600	4.0	450 µsec	200	20	120
Diode	810	1.5	50 ms	150	10	45
Er:Cr:YSGG*	2780	1.5	140 µsec	50	30	45
Nd:YAG	1064	2.0	150 µsec	100	20	60

Settings used based on manufacturer's recommendations for sulcular debridement/implant disinfection

* No air or water

**—usec = microseconds, msec = milliseconds

The calculations for total applied energy (E_T) were based on the following equations

Energy (J/pulse) = Power (W)/Pulse Repetition Rate (Hz)

Total Pulses = Pulse Repetition Rate (Hz) × Time

E_T = Energy (J/pulse) × Total Pulses

non-surgical and surgical treatment of peri-implant diseases is reduction or removal of the bacterial biofilm that forms on the titanium surface. Several treatments for PIMP have been studied in both animals and humans. The treatments vary from systemic and local antibiotic therapy, antimicrobial therapy, mechanical debridement, osseous resection, attempts at bone regeneration, and laser therapy [12–38].

Persson et al. showed re-osseointegration failed to occur to implant surfaces exposed to bacterial contamination, but consistently occurred at "pristine" (new) implant surfaces [39]. The question is whether or not it is possible to decontaminate an implant surface to make it "pristine" and achieve re-osseointegration [30, 39]. Others have also evaluated restoring compatibility as well [15, 34, 35, 40, 41].

Lasers are still a relatively new mode of treatment in dentistry, and there are four main types that are commonly used clinically for periodontal and peri-implant treatment. The active media and their wavelengths of four lasers commonly used in dentistry include near infrared (diodes (810 nm) and Nd:YAG (1064 nm)), mid-infrared (Er,Cr:YSGG (2780 nm)), and far infrared (carbon dioxide (CO_2 10,600 nm)), and these were selected for use in this study.

These different laser wavelengths have different absorption coefficients. The relationship between the primary dental tissue components and their absorption coefficients with respect to the different wavelengths can be summarized into those being absorbed by water and hydroxylapatite and shallow penetrating (Er,Cr:YSGG and CO_2) or absorbed by pigment and blood and more deeply penetrating (diode and Nd:YAG).

Lasers have been used to treat periodontitis and are thought to have bactericidal effects. Lasers may also have an impact as a modality of treatment of PIMP. Laser therapy of PIMP and effect on implant biofilm has been documented in the literature; however, there are only a small number of comparative studies and results tend to be inconclusive [14, 15, 25–27, 31, 35, 41–49]. Successful treatment appears to depend mostly on decontamination of the titanium implant surfaces [14–16, 23, 24, 39, 40, 48]. There does not seem to be any reported method to date that routinely demonstrates complete decontamination of bacteria-laden implant surfaces in order to foster new bone formation and possibly re-osseointegration of dental implants.

The purpose of this study was to compare the effectiveness of 10,600 nm CO_2, 810 nm Diode, 2780 nm Er,Cr:YSGG, and 1064 nm Nd:YAG, lasers for decontamination of machined pure titanium surfaces. Temperature changes and surface effects will be evaluated in separate studies.

Materials and methods

Seventy two machined pure titanium discs with no surface modifications (i.e., acid etching, sand blasting, etc.) with a 5-mm circumference and a 2-mm height were used.[1] Four commonly available dental lasers were included for this experiment, 10,600 nm CO_2[2] 810 nm Diode,[3] 2780 nm Er,Cr:YSGG,[4] and 1064 nm Nd:YAG.[5] Settings for each laser used were based on the manufacturer's recommendations for either sulcular debridement or implant disinfection.

[1] All Metal Sales, Inc., 29,260 Clemens Road, Westlake, Ohio 44,145

[2] 10,600 nm CO_2 laser, Lutronic Denta 2®, Great Plains Technology, Inc, Fairfield, NE

[3] 810 diode, Picasso®, AMD Lasers, West Jordan, UT

[4] Er,Cr:YSGG, WaterLase MD®, Biolase, Inc., Irvine, CA

[5] Nd:YAG, PerioLase MVP-7®, Millennium Dental Technologies, Inc., Cerritos, CA

The parameters recorded for each laser were wavelength (nm), average output power (W), pulse duration, energy per pulse (mJ), and pulse repetition rate (Hz/pps) (Table 1).

The bacterial strains that were used for this experiment were Streptococcus mutans (Sm) (ATCC 25,175) and Streptococcus oralis (So) (ATCC 9811), both Gram-positive staining; and Aggregatibacter actinomycetemcomitans (Aa) (ATCC 43,718), Gram-negative staining. All are facultative anaerobic species; and all are bacteria commonly found in the oral cavity and on dental implants [3–6, 8–10, 13, 24, 36, 42, 50–55].

Approximately 1×10^7 cells of Sm, So, and Aa were individually grown in sterile, 14 ml polypropylene tubes with 2 ml of sterile brain heart infusion (BHI) broth and incubated at 34.0 °C, 20.8% O_2, and 5% CO_2 for 12 h. The bacterial concentrations and growth period were determined from growth curve data collected prior to the experiment, showing the maximum colony-forming units per milliliter (CFU/ml) for all three bacterial species were reached at approximately 12 h. Sm, So, Aa, or a mixture of all 3 (MIX) was inoculated onto titanium discs at similar concentrations ranging from 1×10^5 CFU/ml to 1×10^7 CFU/ml each. After 12 h of static growth, 100 µl containing approximately 1×10^7 cells of Sm, So, and Aa was either individually dispensed, or combined (MIX) and then dispensed into 0.9 ml of BHI broth in each well of a 24 well polystyrene plate as follows: one sterile titanium disc was placed into each well containing the bacterial suspension in BHI using sterile cotton forceps. The wells were then incubated at 34.0° C, 20.8% O_2, and 5% CO_2 for 12 h in order to inoculate the discs. A separate 24-well plate was prepared by placing 0.9 ml of sterile phosphate buffer solution (PBS) into each well and labeled identically to the plate with inoculated discs, in order to transfer the discs into the appropriate corresponding well after treatment. After incubation, the discs were divided into six groups: 1) no treatment, 2) 0.12% chlorhexidine gluconate (CHX), 3) 10,600 nm CO_2 laser, 4) 810 nm diode laser, 5) Er,Cr:YSGG laser, and 6) Nd:YAG laser. Treatments were allocated based on a computer-generated randomization scheme.

For groups 3 through 6, each of the lasers was used in a similar manner. Laser safety precautions were followed and each laser's power output was verified with a PowerMax 600f power meter.[6] Four titanium discs were removed from individual wells with the designated forceps for the respective bacterial suspensions and individually placed onto 2 × 2 inch sterile cotton gauze for treatment. Each disc was individually treated at the previously mentioned settings with the laser's delivery tip held at a distance of 5 mm above the discs and at a 90° angle. Figure 1, the laser energy was applied for 15 s per side of each disc, using vertical, horizontal and circular passes. Each titanium disc was then placed into its corresponding PBS well with sterile forceps for transfer.

The discs were then individually transferred into 1.5-ml microfuge tubes using sterile forceps. The remaining 0.9 ml of PBS solution was also transferred with a pipette into the tubes with the corresponding discs. Each tube containing a disc with its PBS solution was sonicated at a setting of 1 W for a total of 15 s in order to liberate the bacteria from the disc into solution, without causing lysis of remaining live bacteria.

Each of the tubes containing the discs with supernatant PBS solution was individually serially diluted by transfer of 100 µl of PBS-supernatant with a pipette into 5 separate sterile 1.5-ml microfuge tubes containing 0.9 ml of PBS solution. One sterile BHI agar plate per disc in each treatment group and the control group was prepared and labeled.

Each plate was then incubated at 34 °C, 20.8% O_2, and 5% CO_2 and monitored for 24-48 h to allow for bacterial colony formation. The BHI agar plates were then placed over a black background for ease of identification of colonies due to their light color and the translucency of the BHI agar. A fine tip marker was used to "spot count" the colonies for all plates and the total CFUs were manually recorded.

This experiment was repeated based on the randomization code so that each treatment had three biological replicates, resulting in 12 each of untreated negative control, CHX positive control, and CO_2, diode, Er,Cr:YSGG, and Nd:YAG irradiated discs.

Statistical analysis of all collected data was conducted to compare treatment modalities with respect to each other in terms of viable bacterial cell reduction. This was performed with the Prism[7] statistics program and included one-way analysis of variance (ANOVA) with Tukey's multiple comparison test at a statistical significance of $p < 0.05$.

Results

All of the bacteria grown individually and as a mixture increased in CFU/ml by at least one log in the untreated group (data not shown). Each modality of treatment had surprisingly different results (Fig. 2) with individual comparison of bacterial kill results shown in Fig. 3.

Discs treated with a standard 1-min soak in CHX showed a mean overall viable cell reduction for Sm, So, Aa, and MIX of 94.6%, 99.8%, 99.9%, and 99.9%, respectively (Fig. 2A, Chlorhexidine).

The discs treated with the CO_2 laser had the most variability in overall bacterial count reduction. The CO_2 laser had its greatest effect on So, where the bacterial counts

[6] PowerMax 600, Coherent, Santa Clara, CA

[7] Prism 7® version 5.0d, GraphPad Software, Inc., La Jolla, CA

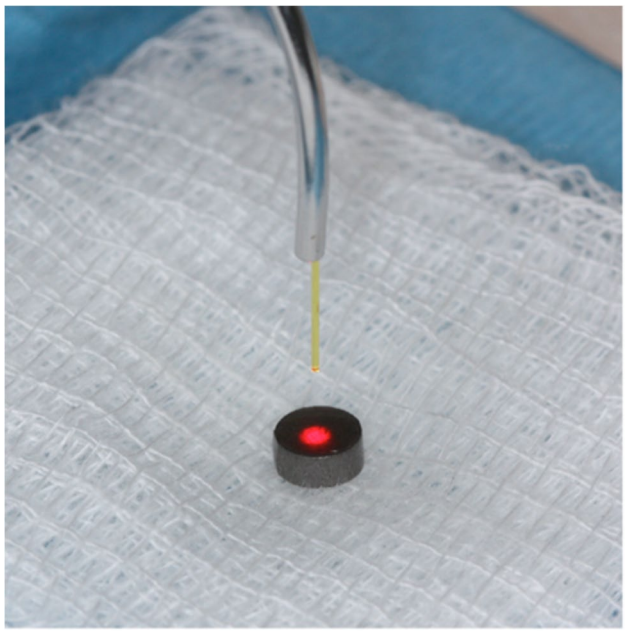

Fig. 1 Laser (Nd:YAG) energy application to the titanium alloy discs for 15 s per side. The emission of red light onto the disc is a direction indicating light used to direct and focus the laser's emission of near infrared light. Similar application was used with the other lasers

were reduced to 0.01% of the initial inoculum (Fig. 2E), (Fig. 3C). In contrast, the CO_2 laser had the least effect on Aa in that the total CFU went from 2 X 10^7 to 5.0 X 10^6 after treatment, a very modest reduction (Fig. 2E), (Fig. 3A). The CO_2 reduced the amounts of Sm and MIX infected discs to 95.9% and 98.8% of the starting inoculum. The CO_2 was the least effective laser at killing Sm and the MIX biofilm on the discs (Fig. 3B, D).

The discs irradiated with the Diode laser showed a mean overall reduction of Sm, So, Aa, and MIX by 99.6%, 99.9%, 99.7%, and 99.9%, respectively (Fig. 2C, diode). The diode was most effective against MIX compared to the individual bacteria (Fig. 3).

The discs irradiated with the Er,Cr:YSGG laser showed a small reduction in CFU/ml for Sm, So, Aa and MIX from initial inoculums that ranged from 1×10^7 (for Sm, Aa and MIX) to 1×10^5 (for So) to 1.2×10^5, 5.7×10^6, 8.5×10^6, and 4.5×10^6 CFUs, respectively. (Fig. 2D). In terms of mean overall percentage kill, discs treated with the Er,Cr:YSGG laser showed the least amount of killing when compared to the other treatments (Fig. 2D, Er,Cr:YSGG).

All the discs treated with the Nd:YAG laser showed no organisms detected. This was an overall viable cell reduction of 100% for all bacteria on all the discs (Fig. 2B, Fig. 3A–D).

Discussion

This study sought to compare the bacterial reduction capacity of four dental lasers on titanium discs. The four lasers used were selected based on availability in our clinics and general use in periodontal and peri-implant therapy. Overall, the findings in this study parallel some and conflict with other reports, which tend to show variable results with regards to bacterial reduction with lasers [14, 21, 29, 33, 38, 41, 42, 45–47, 49, 50, 53–58]. This may relate to different laser settings and power densities used by others, a point made by Kamel et al. [31], as well as different model systems used [59].

Efforts were made to identify literature documenting the minimal infectious dose of bacterial cells necessary to cause infection or re-infection, but this information was not available. One challenge was to apply clinical validity to this study with respect to the species of bacteria used to create a viable biofilm on the titanium disc surfaces. Steps were employed during this study to mimic the cultivation of applicable bacteria and formation of a biofilm similar to those mentioned in previous articles [13, 53, 55, 59]. Standar et al. evaluated biofilm formation behavior of mixed-species cultures with dental and periodontal pathogens via SEM and found that combinations of Streptococcus mitis with either Sm or Aa revealed bacterial interactions influencing biofilm mass, biofilm structure and cell viability in vitro. The latter two bacterial species were chosen for use in this study due to their aerobic/facultative nature and ease of handling, as well as their capability for in vitro biofilm formation [59]. Persson et al. cultured bacterial species from peri-implantitis patients and of the seventy-nine bacterial species identified via DNA hybridization, three of these were Aa (identified by the authors as the most prevalent bacterial species identified), Sm and So [8]. Other studies also give clinical validity to the bacteria chosen for use in this study as being among the population of species involved with peri-implantitis [9, 36, 50, 51, 53–55, 58].

One concern was that the MIX might incur species competition that would alter or decrease the viability of the MIX. As stated at the beginning of the Results section, all cultures grew robustly, so species competition did not appear to have occurred.

Physical characteristics of the bacterial biofilm on the titanium discs were different during treatment with each of the lasers. During irradiation with the Nd:YAG laser, an evaporation of the BHI broth was observed with each pass until there was no remaining fluid on the surface of any disc. This reaction was not noted with the CO_2, diode, or Er,Cr:YSGG lasers, which had intact fluid on the disc surfaces after 15 s of irradiation. However, for each laser

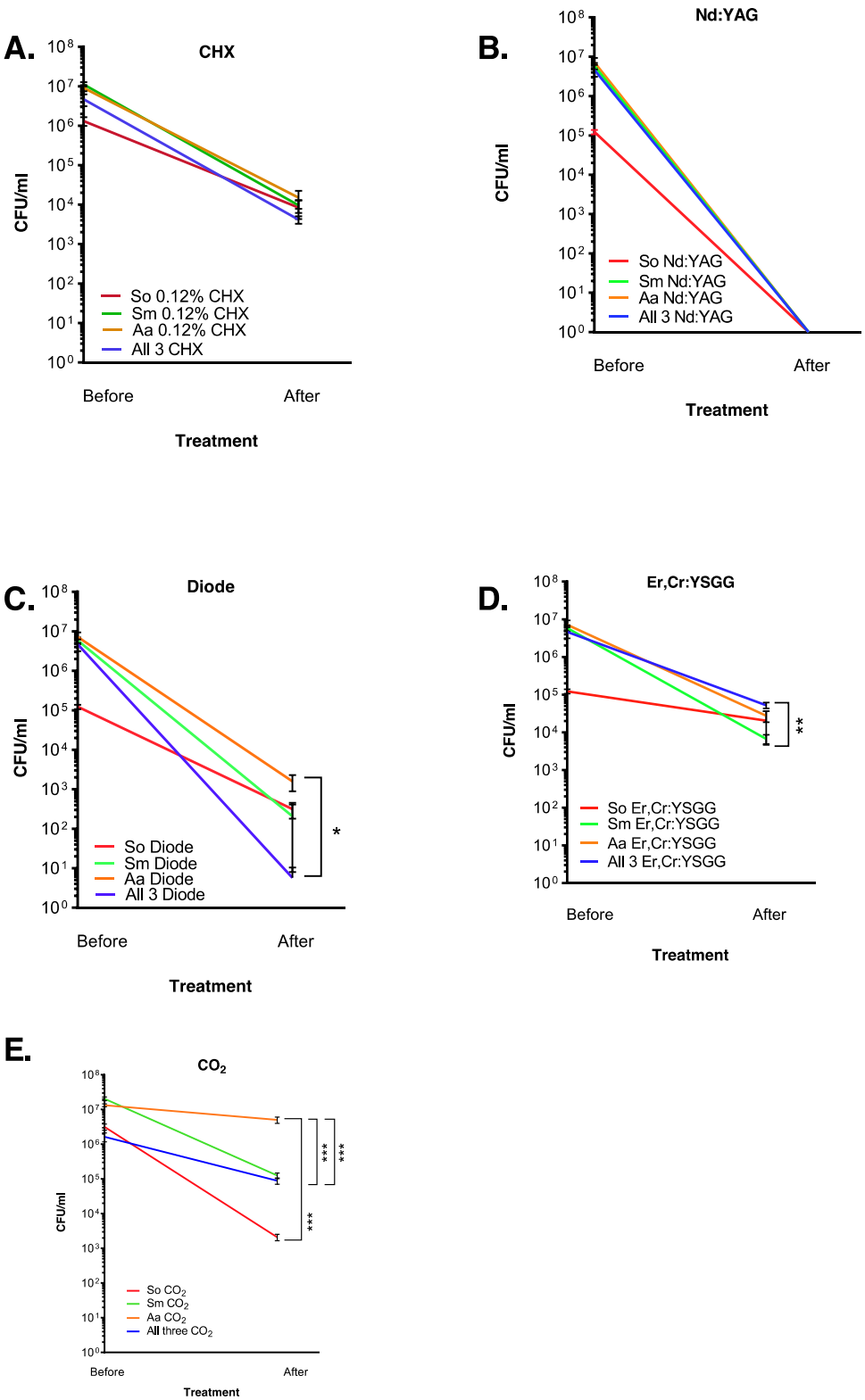

Fig. 2 Effects of chlorhexidine, Nd:YAG, 810 nm Diode, Er,Cr:YSGG, and 10,600 nm CO_2 lasers on *S. oralis*, (red line) *S. mutans* (green line), A. actinomycetemcomitans (orange line), and all three bacteria (blue line) grown on titanium discs. **A**) Discs treated with a 1-min soak in 0.12% chlorhexidine gluconate (CHX). **B–E**) Titanium discs irradiated for 30 s with Nd:YAG (**B**), 810 nm diode (**C**), Er,Cr:YSGG (**D**) or 10,600 nm CO_2 (E) in triplicate

application, the titanium discs were placed on 2 × 2 inch sterile gauze for 15 s per side, which acted to absorb the BHI broth possibly containing viable bacterial cells in suspension. Thus, when the irradiated discs were transferred into their corresponding PBS wells for transfer, they were essentially dry and free of any remaining BHI broth. If this action were to remove all of the suspended viable cells from the titanium discs, the remaining bacteria could

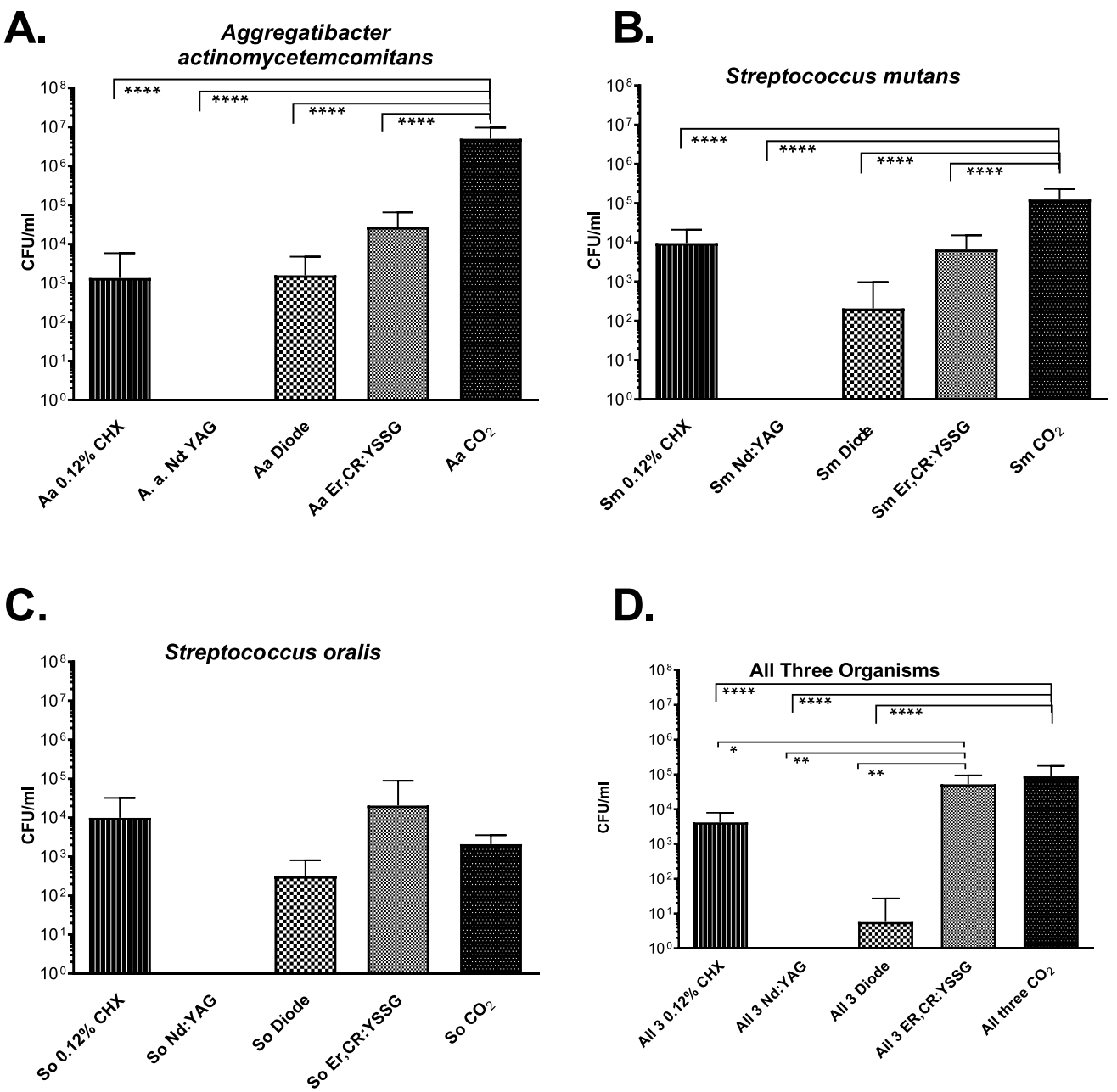

Fig. 3 Treatment effects of chlorhexidine or Nd:YAG, 810 nm diode, Er,Cr:YSGG, and 10,600 nm CO_2 lasers on dental pathogens grown on titanium discs. Each panel shows bacteria recovered (CFU/ml) after each treatment. One-way ANOVA, **** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$

be assumed attached to the discs at the time of transfer and liberated by way of the sonication used. Evidence that all bacteria were not absorbed by the gauze during treatment rests in the culture and count of viable CFUs on the BHI agar plates after treatment with the CO_2, Diode and Er,Cr:YSGG lasers. This also strengthens the finding that no organisms were detected on titanium discs irradiated with the Nd:YAG laser.

CHX was chosen as a positive control based on previously published information that found 0.12–2.0% CHX killed oral bacterial species up to 94.6% [13, 20, 22, 24, 25, 33, 35, 45]. This is comparable to the percent kill with the 0.12% concentration available in the US used in this study, which was 99.8% for Sm, 94.6% for So, 99.9% for Aa, and 99.9% for MIX.

Each laser was used at the manufacturer's recommended setting for sulcular debridement/implant disinfection. Different parameters (power, pulse duration, repetition rate, etc.) may have yielded different results, but that was not

the focus of this experiment. Future studies are underway to evaluate these aspects.

Although studies have demonstrated the capacity of lasers to kill bacteria, the fundamental mechanism of the laser effect remains unclear. Hibst et al. evaluated whether bacterial killing is caused by the light itself (photochemical effect) or by a photothermal process. The authors heated suspensions of Escherichia coli in a water bath or with a diode laser and found no SSD when comparing the killing rates between laser and water-based heating, concluding that the most important parameter is the maximum temperature [60]. This did not seem to apply to the results of this study as the laser with the highest E_T (CO_2) was not as effective as the other lasers, all of which had lower E_T. Other mechanisms such as O2 singlet production, OH- radical formation, protein denaturation, etc. have been proposed [50]. Temperature changes will be addressed in a subsequent study.

Even though all modalities of treatment reduced bacterial levels substantially, the most consistent result was irradiation with the Nd:YAG laser with respect to all bacteria, whether individually cultured or grown together. The overall bacterial reduction of the Nd:YAG laser was 100% for all four biological replicates in this study. These results coincide with Goncalves et al., who compared the efficacy of both Nd:YAG and 980 nm diode lasers and found a 100% reduction of bacteria on machine surfaced implants at 2.5 and 3.0 Watts of power output and a total treatment time of 5 min with both lasers [49], both parameters greater than those used in the current study. The effectiveness of Nd:YAG was also shown by Giannini et al. [14].

No water or air was used with the Er,Cr:YSGG or the CO_2 so that the biofilm would not be blown nor flushed away. This may not reflect typical clinical usage of these devices. However, whether water was used or not with the Er,Cr:YSGG has been shown to make no difference [38]. Also, the perpendicular energy application would only be clinically achievable during surgical flap treatment of peri-implantitis. Non-surgical or certain minimally invasive surgical approaches would utilize a parallel approach. Further studies should evaluate the effect of angle of laser application.

All the lasers used in this research were somewhat effective (Figs. 2, 3), but there was variability in how effective they were at reducing/eliminating the bacteria on the titanium discs. Laser use during PMUC or PIMP treatment may provide an improved level of decontamination in a relatively short application time. More research is needed both in vitro and clinically to determine the most effective laser(s) and the most appropriate settings and parameters, as well as any detrimental effects related to laser use with dental implants, such as increased temperature or surface changes (studies underway at our facility). It must be recognized that laser use is only one aspect of PMUC and PIMP treatment. Improved oral hygiene, occlusal management, restorative design, and other aspects are part of any periodontal or peri-implant therapy.

Conclusions

All of the disinfecting treatments used showed appreciable decontamination of machined pure titanium discs. CHX was more effective than the 10,600 nm CO_2 and Er,Cr:YSGG lasers. The laser treatments were inconsistent in their ability to eliminate the bacteria species used and to completely decontaminate the titanium discs used in this study. From least effective to most effective were the Er,Cr:YSGG, 10,600 nm CO_2, 810 nm diode, and Nd:YAG. The most consistent results in bacterial reduction on all titanium discs infected with any individual or a mixture of bacterial species were with the Nd:YAG laser, with 100% total bacterial reduction 100% of the time.

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Declarations

Ethics approval Not applicable. This article does not contain any study with human or animal participants.

Conflicts of interest The authors declare no competing interests.

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