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Research Article

Impact of Diode Laser, Ozonated Water, and Chlorhexidine on Oral Microbiota: Insights from Proteomic Analysis.

Ziba Najmi[1], Emanuele Ruga[2], Andrea Melle[3], Rachele Pertusati[3], Anna Maria Agnone[4], Andrea Cochis[1], Alessandro Calogero Scalia[1*]

1. Department of Health Sciences, Center for Translational Research on Autoimmune and Allergic Diseases CAAD, Università del Piemonte Orientale UPO, Italy.

2. DDS; MSc Oral Surgery, Aesthetic Medicine; Specialist in Oral Surgery, SCDU ODONTOSTOMATO-LO- GIA Osp. S. Andrea, Vercelli. ASLVC., Università del Piemonte Orientale UPO, Italy https://orcid.org/0000-0002-6401-9689

3. RDH; MSc; SCDU ODONTOSTOMATOLOGIA Osp. S. Andrea, Vercelli. ASLVC., Università del Piemonte Orientale UPO, Italy.

4. DDS; Specialist in Oral Surgery; Contract Professor University of Eastern Piedmont. Division of Dentistry, Sant'Andrea Hospital Vercelli, Corso Mario Abbiate, 21, Vercelli 13100, Italy.

*Correspondence to: Alessandro Calogero Scalia, Department of Health Sciences, Center for Translational Research on Autoimmune and Allergic Diseases CAAD, Università del Piemonte Orientale UPO, Italy.

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ABSTRACT

Objectives: *The human oral microbiota is composed of fungi, viruses, protozoa, and more than 700 bacterial species living in eubiosis. Poor oral hygiene, medications, and un-healthy lifestyle may lead to dysbiosis with local (e.g., periodontitis) and systemic diseases(e.g., rheumatoid arthritis, and Alzheimer's). This study evaluates the effectiveness of three professional treatments, diode laser, ozonated water, and 0.20% chlorhexidine, on human cariogenic bacteria present in the oral microbiota extracted from plaque samples, focusing on their ability to target pathogens selectively.*

Materials and Methods: *Oral plaque samples were collected from three healthy volun-teers, pooled and incubated anaerobically for 24 hours. The dentin discs, used as substrate,were inoculated with oral plaque and incubated for 24 hours. Samples were then treated with 20 seconds of diode laser, 30 seconds of ozonated water, or 30 seconds of 0.20% chlorhexidine. Peptides from bacterial proteins were analysed by proteomics, and populations were assessed using the Human Oral Microbiome Database.*

Results: *The bacterial populations isolated from dentin samples after treatments were first compared to those from untreated oral plaque, and then compared to each other to assess shifts in microbial composition due to the specific treatment. In the first comparison, all three treatments led to a marked reduction in several bacterial species, including Fusobac- terium nucleatum and Prevotella oris, commonly associated with oral infections. In the com- parison between treatments, laser and ozonated water appeared to limit the overgrowth of Granulicatella adiacens, which overgrowth may contribute to systemic issues.*

Conclusions: *These findings suggest that while all treatments exert an antimicrobial effect, they differ in their selective pressure on specific bacterial taxa, ultimately influencing the overall microbial composition.*

Keywords: *Oral microbiota, diode laser, ozonized water, chlorhexidine, bovine dentin.*

Introduction

The human oral microbiota is a complex ecological community composed of commensal, symbiotic, and pathogenic microorganisms inhabiting the oral cavity. Among these, more than 700 distinct bacterial species have been identified, coexisting in a typically balanced ecosystem called eubiosis. In addition to bacteria, the oral microbiota includes archaea, fungi, mycoplasmas, protozoa, and a transient viral population. The composition of this microbial community is highly dynamic and evolves in response to changes in the biological environment of the oral cavity [1–5].

The oral microbiota comprises a diverse array of Gram-positive and Gram-negative bacterial species, including both facultative and obligate anaerobes. These bacteria are classified into 13 phyla, which include Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Spirochaetes, Fusobacteria, Synergistes, SR1, TM7, Chloroflexi, Deinococcus, Acidobacteria, and Cyanobacteria [4–6].

The oral cavity comprises multiple distinct niches that support the growth of diverse microbial communities. These anatomical habitats include the teeth, the gingival sulcus, the tongue, cheeks, saliva, hard and soft palate, and tonsils [7]. Microbial colonization within these niches is modulated by several physicochemical factors, including oxygen availability, pH, temperature, redox potential, ionic strength, and osmotic pressure [8-10]. Moreover, exogenous factors such as the administration of antibiotics (e.g., tetracyclines) and the use of systemic or topical antiseptics can significantly disrupt the composition and balance of the resident oral microbiota [11]. The oral microbiota predominantly exists in the form of biofilms, which are essential for maintaining oral homeostasis, providing protection against external insults, and limiting the development of pathogenic conditions. Biofilms are structured microbial communities embedded within a self-produced extracellular polysaccharide matrix, which facilitates firm adhesion to both biotic and abiotic surfaces in the oral cavity. Within this complex architecture, microorganisms interact through chemical signaling, metabolic cooperation, physical associations, and molecular communication, promoting the stability and resilience of the community. Mature oral biofilms typically consist of approximately one-third microbial cells and two-thirds extracellular components, including bacterial metabolic byproducts, host-derived exudates, entrapped food debris, and water [12–17].

Under conditions of proper oral hygiene and a balanced diet, the oral biofilm remains in a state of symbiotic equilibrium, contributing to the maintenance of oral health. However, poor oral hygiene practices and frequent intake of fermentable carbohydrates can disrupt this balance, leading to a shift in the microbial composition, a phenomenon known as dysbiosis [18]. This ecological shift favors the proliferation of acidogenic and aciduric pathogenic species at the expense of commensal microorganisms, promoting the development of oral diseases such as dental caries and periodontal disease [19]. The persistence of a dysbiotic biofilm fosters a

pro-inflammatory environment and enhances tissue degradation, ultimately compromising both hard and soft oral tissues [20]. Dental caries is one of the most prevalent oral diseases worldwide and represents a leading cause of tooth pain and tooth loss in humans [15]. Beyond the progressive destruction of dental hard tissues, caries can result in pulpal and periapical infections, potentially leading to systemic complications. The condition exhibits a high incidence and affects individuals across all age groups, from early childhood through advanced age [16,21]. Streptococcus 84 mutans is widely recognized as a key etiological agent in the initiation of dental caries. As 85 the lesion advances, the microbial composition of the biofilm shifts, with a progressive 86 transition from early colonizers such as S. mutans and Actinomyces spp. to aciduric and ac- 87 idogenic genera like Lactobacillus and Bifidobacterium [3,16,22-24].

Periodontitis is a chronic, multifactorial inflammatory disease that affects the supporting structures of the teeth and represents the primary cause of tooth loss globally. In contrast to gingivitis, which is confined to reversible inflammation of the gingival tissues, periodontitis is characterized by the progressive destruction of the gingiva, periodontal ligament, and alveolar bone. The disease is associated with a dysbiotic shift in the subgingival microbiota, favoring the proliferation of pathogenic anaerobic species. Key bacterial taxa implicated in periodontitis include Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Porphyromonas endodontalis, Prevotella denticola, and Dialister spp. Among the modifiable risk factors, tobacco smoking plays a major role in disease onset and progression, exerting both direct immunosuppressive effects and altering the subgingival microbial composition in favor of pathogenic species [3,25].

The oral microbiota has been increasingly linked to the pathogenesis of several systemic diseases, including cardiovascular disease, pneumonia, rheumatoid arthritis, oral squamous cell carcinoma (OSCC), pancreatic cancer, colorectal cancer, esophageal cancer, stroke, and adverse pregnancy outcomes [14,26]. Given its widespread influence on host physiology and its compositional shifts in disease states, the oral microbiota has been proposed as a potential biomarker for various human pathologies. Oral squamous cell carcinoma (OSCC) is among the most prevalent malignant neoplasms of the oral cavity. Emerging evidence suggests that the biofilm associated with OSCC lesions harbors an increased load of both aerobic and anaerobic bacteria. Notably, higher abundances of Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces, Clostridium, Haemophilus, Enterobacteriaceae, and various Streptococcus species have been observed in OSCC related biofilms. Furthermore, salivary profiles of patients with oral can- 111 cer often show elevated levels of Prevotella melaninogenica and Streptococcus mitis, suggesting their potential as microbial indicators of malignancy [3,27].

Chlorhexidine is considered the gold standard disinfectant for antimicrobial rinses due to its broad-spectrum activity and prolonged substantivity [27]. It is highly effective in controlling bacterial proliferation and preventing dental caries. However, its effect on already established carious lesions is limited. Ozonated water

and diode lasers present several advantages, and studies have demonstrated their efficacy not only as preventive agents but also as therapeutic tools [28-30]. In particular, they are recommended as supplementary antibacterial surface pretreatment methods to enhance the removal of cariogenic bacteria.

Diode lasers, in particular, offer multiple benefits in both periodontal and surgical applications. They are increasingly employed in periodontal therapy as adjuncts to conventional treatments such as scaling and root planing (SRP) [30]. Diode lasers have shown antibacterial effects against a variety of oral pathogens and are commonly used before and after cavity preparation to reduce postoperative sensitivity and microbial load. The 915 nm diode laser wavelength is especially noted for its beneficial properties, including enhanced bacterial reduction, decreased inflammation, and accelerated healing. These effects are partly attributed to its biostimulatory impact on the viability 130 and regenerative capacity of human gingival fibroblast cells [31].

This research project aimed to evaluate the effectiveness of three professional treatments diode laser, ozonated water, and 0.20% chlorhexidine each with distinct mechanisms of action, on cultures of human cariogenic bacteria on dentin slices by assessing their impact on bacterial populations collected from the oral plaque of healthy donors. Furthermore, using a high-throughput analytical approach, the study identified which pathogenic and commensal bacterial species were most affected by each treatment, thereby highlighting their potential targeted effects.

Results

Population analysis

The results of the comparison between the original bacterial populations identified in the oral plaque exploited for the infection of the dentin slices and those present on the surface after infection's eradication with the diode laser, ozonated water (O_3 water), or chlorhexidine (CHX) are presented in the Figure 1. Only bacterial populations with a relative abundance greater than 1% were considered. A total of 19 bacterial populations were included in the comparison between the original plaque and the treated samples, with the results shown in Figure 1. Similarly, 8 populations were selected for the comparison among the three treatments, as reported in Figure 2. The complete list of identified bacterial species is provided in Supplementary Table 1 (ST1).

In general, according to the results shown in Figure 1, all three treatments substantially reduced the abundance of several bacterial strains, including Fusobacterium nucleatum and Prevotella oris, both of which are commonly associated with endodontic and periodontal infections [32]. Conversely, an increase was observed in other taxa such as Granulicatellav adiacens and Veillonella atypica.

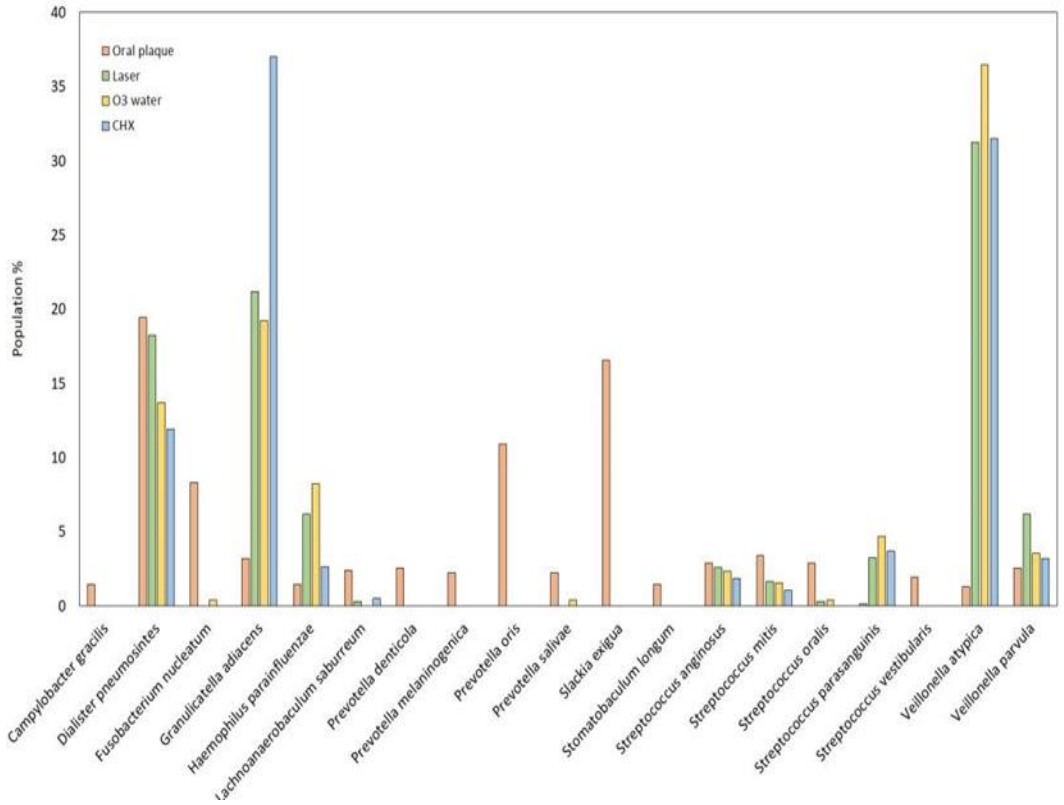

Figure 1. Comparison between the untreated oral plaque and oral plaque exposed to Laser, O3 water, and Chlorhexidine. Results represent the % of the single species over the total plaque.

Then, the proteomic analysis was focused on the impact of each treatment in terms of targeted bacteria. Figure 2a shows the relative abundance, in terms of percentage of total population, of bacterial phyla identified in plaque samples treated with Laser (left panel), O_3 water (middle panel), and CHX (right panel). Firmicutes emerged as the dominant phylum across all treatments, representing 95.16%, 94.89%, and 97.52% of the total biomass, respectively. Further analysis at the species level (Figure 2b) highlights notable shifts in the bacterial population composition, indicating that each treatment distinctly influences the microbial profile in a different manner beyond the general antibacterial effect.

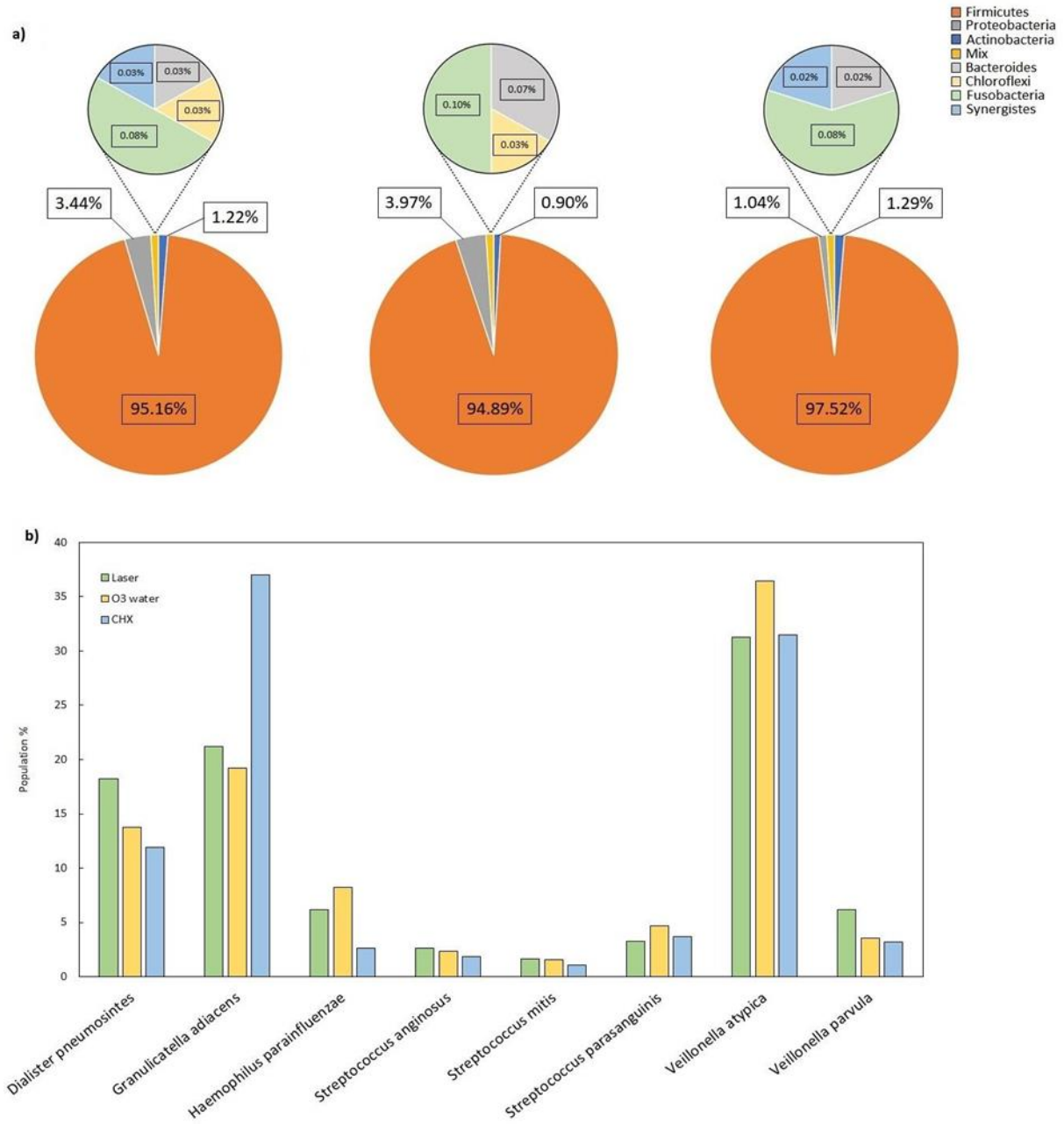

Figure 2. Changes in the most abundant bacterial species (>1%) following oral plaque exposure to Laser (left), O_3 water (middle), and CHX (right). a) Distribution of bacterial phyla; b) composition at the species level. *Statistically significant differences between groups ($p < 0.05$).

Discussion

Diode lasers, ozonated water, and chlorhexidine are widely used in dental clinics for the management and treatment of periodontal disease and related conditions. Among these, chlorhexidine remains the most commonly used agent. However, long-term use of chlorhexidine can lead to several side effects, including tooth discoloration, dysgeusia (altered taste perception), epithelial desquamation, mucosal burning sensation,

and increased formation of supragingival calculus. In a study by Bernardi et al. [33], conducted on perio- 180 dontally healthy volunteers with experimentally induced gingivitis, rinsing with 0.20% chlorhexidine for 15 days resulted in noticeable tooth staining, particularly in areas with exposed root surfaces. The study also reported an increase in surface roughness of the teeth [34]. Diode laser treatment offers several significant advantages, including reduced bleeding and swelling, faster healing, no need for anesthesia, effective decontamination of implant sites, and safe use in patients with pacemakers. Moreover, it ensures high patient compliance due to its painless nature. However, some disadvantages are associated with the initial investment and the need for a suitable clinical environment [30,35]. Regarding the use of ozonated water, its main advantages include antimicrobial, anti-inflammatory, analgesic, and detoxifying effects, as well as the ability to perform painless procedures. A further benefit is that ozonated water treatments can also be carried out at home. In recent years, ozone-based products have become increasingly available on the market in a variety of formats. However, the literature reports rare cases of ozone toxicity, particularly when O_3 concentrations exceed 0.0007% per application. Possible side effects include coughing, headache, nausea, dyspnea, cardiovascular disturbances, vomiting, and respiratory tract irritation [36,37]. Based on these premises, the primary objective of this study was not to compare the antimicrobial efficacy of different well-known and effective treatments, but mostly to assess their impact on the oral microbiota in terms of target bacteria in order to better understand the rationale for their clinical success and choosing the best therapy based on the pathology to be treated. For this preliminary study, bovine dentin has been used as substrate for the adhesion of the oral plaque that was collected from 3 healthy donors (both M/F origin, age means=25) and pooled to obtain a non-patient-specific microbiota. In dental research, bovine dentin is commonly used for in vitro studies, as the use of human teeth is restricted by ethical considerations and the limited availability of suitable samples. Additionally, bovine dentin has long been recognized for its compositional and biological similarities to human dentin, making it an increasingly preferred substitute [38-40].

The comparison between the untreated oral plaque and the plaque exposed to the three treatments evealed a marked reduction in several opportunistic bacterial species, including Fusobacterium nucleatum and Prevotella denticola. In the context of oral dysbiosis, these species are known to contribute to both local and systemic health complications. Beyond their established role in periodontal and endodontic infections, they have also been associated with chronic systemic conditions such as rheumatoid arthritis and inflammatory bowel disease when a systemic worsening occurs [41]. Notably, F. nucleatum has been implicated in the exacerbation of Alzheimer's disease pathology and has also been linked to an increased risk of colorectal cancer [42,43]. Conversely, the reduction of the above-mentioned bacterial species was balanced by an increase in Granulicatella adiacens and Veillonella atypica, both belonging to the Firmicutes phylum. Although G. adiacens is generally considered a commensal organism, its excessive proliferation has been

associated with endodontic infections and dental abscesses [44].

However, the concomitant rise of the probiotic bacterium V. atypica may mitigate the pathogenic potential of G. adiacens [45]. The analysis of the bacterial populations isolated from the dentin surface after exposure to the three treatments revealed a predominant presence of Firmicutes, accounting for approximately 95% in samples treated with the diode laser and ozonated water, and 98% in those treated with chlorhexidine (CHX). While many Firmicutes contribute to oral health by preventing pathogen colonization and supporting the synthesis of vitamins (such as B-complex vitamins and vitamin K), some species within this phylum can act as opportunistic pathogens, particularly when host defenses are compromised [46]. In addition to Firmicutes, members of the Proteobacteria and Bacteroidetes phyla are recognized as components of the stable and resilient community of the oral microbiota, often referred to as the core microbiota [47]. Among the three treatments, chlorhexidine (CHX) was the only one to induce a marked reduction in the Proteobacteria population, lowering it to 1%,compared to 3% in samples treated with the diode laser or ozonated water. Proteobacteria play an important role in maintaining oral health. They are involved in nitric oxide production, help regulate oral pH, and contribute to controlling the excessive proliferation of pathogenic bacteria [48]. A species-level comparison reveals notable differences among the treatments in their ability to reduce or maintain specific bacterial populations, thus confirming the starting hypothesis that each of the treatments tested may have a different target. Both the diode laser and ozonated water appeared to limit the excessive proliferation of Granulicatella adiacens, with its relative abundance reaching approximately 20% under these treatments. In contrast, treatment with chlorhexidine (CHX) resulted in a markedly higher abundance, exceeding 35%. Although G. adiacens rarely causes infections beyond the oral cavity, its excessive and uncontrolled proliferation, particularly in immunocompromised individuals, can lead to bacteremia and infective endocarditis. In rarer cases, it has also been associated with vertebral osteomyelitis, pancreatic abscesses, otitis media, and genitourinary tract infections [49-52]. The analysis also revealed a differential impact of the treatments on the relative abundance of the Proteobacterium Haemophilus parainfluenzae. De Palma et al. [53] reported that H. parainfluenzae is associated with good oral health and may contribute to the mainte- 251 nance of a healthy host status. Moreover, Tseng et al. [54] demonstrated that H. parainfluenzae could exert a protective effect against Sjögren's syndrome (SS), a systemic autoimmune disorder characterized by inflammation and dysfunction of the exocrine glands, potentially supporting immune homeostasis in the host. In the present study, the application of chlorhexidine resulted in a marked reduction in the abundance of H. parainfluenzae, which dropped to 2.65% of the microbial community. This value was substantially lower compared to the 6.2% observed following laser treatment and the 8.23% detected after exposure to ozonated water.

Ozonated water and diode laser treatments did not significantly reduce any of the other bacterial species considered in the analysis. This outcome may be viewed positively, as it suggests that these treatments do not substantially impact the commensal species naturally present in the oral microbiota.

Materials and Methods

Preparation of bovine dentine discs

For the preparation of dentin disks, two bovine mandibles were used. Dental elements were extracted and stored in a 50% ethanol solution in physiological saline. Afterward, the teeth were rinsed with water to remove ethanol and any residues, then milled with a tungsten laboratory bur to eliminate the enamel and cement layers. This procedure exposed a dentin surface along the entire length of the tooth. Bovine dentin disks, each with a diameter of 5 mm and a thickness of 4 mm, were obtained using an implant core drill (Esthetic Implant, Henry Schein Krugg, New York, United States). The disks were stored in a 50% ethanol solution. Sterilization was performed via three immersion cycles in 70% ethanol under stirring for 2 hours. A final rinse with saline solution was conducted to remove any residual ethanol. A total of 96 samples, 32 for each treatment group, were used for the analyses.

Treatments used

This project aimed to characterize the antimicrobial effects of three different treatments commonly used in clinical practice: (i) a diode laser (PocketLaser, 88dent, Pero, Italy) 915 nm wavelength operated at 1.5 watts, 100 Hz for 20 seconds in unfocused mode with a 400 µm inactivated optical fiber ; (ii) ozonated water produced using an ozone injector (Ozonette, Sedecal, Madrid, Spain) through 4 cycles of 5 minutes each at a flow rate of 30 L/min; and (iii) 0.20% chlorhexidine (Curasept, Saronno, Italy). These treatments will hereafter be referred to as Laser, O3 water, and CHX, respectively.

Plaque preparation

Oral plaque samples were collected from 3 healthy volunteers (both M/F origin, age means=25), with prior informed consent, through a non-invasive procedure. Specifically, the samples were collected from the coronal surface of the premolars or molars through the use of sterile Gracey curettes using a scaling procedure. None of the volunteers underwent courses of antibiotics in the 3 months preceding the collection. After collection, the samples are combined and kept in Cooked Meat medium (Merck, Darmstadt, Germany) for 24 h in a hood suitable for the cultivation of anaerobic bacteria (Concept 400, Baker Ruskin, Maine, United States). After the incubation period, the plaque samples were frozen at -80°C to preserve the initial microbial population. Before

each experimental assay, a vial containing the plaque suspension was thawed and maintained at 37°C to allow the bacteria to reach the exponential growth phase and used immediately to avoid a switch in the bacterial population [55].

Microbiological analysis

To test the three treatments described above, sterile dentin samples are moved to a 48- well plate. Subsequently, a bacterial suspension containing 105 cells per well is placed in contact with the samples for 24h under anaerobic conditions. After incubation, the samples (32 for each treatment) were exposed to one of the following: Laser for 30 seconds, O3 water for 20 seconds, and CHX for 20 seconds.

Figure 3. Representation of the treatments used (diode laser, ozonated water, chlorhexidine 0.20%) on the dentin disks contaminated with plaque and subsequently observed with Alamar Blue for the observation of the metabolic activity.

Evaluation of bacterial populations

To evaluate whether the three treatments affected microbial populations, bacteria adhering to the dentin surface were detached using a protocol involving 3 sonication cycles (27% amplitude for 5 minutes), interspersed with three 30-second vortexing steps. The resulting bacterial suspension was centrifuged at 14,000 × g for 20 minutes at 4°C to form a bacterial pellet. This pellet was washed with physiological solution, then centrifuged again (14,000 × g, 20 minutes, 4°C), and exposed to a lysis buffer (8 M urea in Tris-HCl), which disrupted the bacterial membranes. To enhance lysis efficiency, 6 sonication cycles (27% amplitude, 10 seconds each) were performed, interspersed with 10 seconds of cooling on ice. Protein concentration was determined using Bradford reagent (Merck), and 80 µg of total protein, representing the minimum threshold for downstream analysis, was diluted in 100 mM ammonium bicarbonate. For efficient protein reduction, the samples were incubated at 60°C for 30 minutes in a 1:1 solution of trifluoroethanol (final concentration to be specified) and dithiothreitol (200 mM) (Merck). Subsequently, proteins were alkylated with iodoacetamide for 30 minutes at room temperature in the dark to enable peptide mapping. The final solution was digested overnight with trypsin at 37°C. Enzymatic activity was halted by adding formic acid, and the digest was dried using a speed vacuum concentrator. Peptide analysis was performed by mass spectrometry, and the resulting data were analyzed using the Human Oral Microbiome Database (version 3) [46], which enables the association of detected peptides with specific bacterial species. The bacterial species identified following the three treatments were then compared to those found in the original oral plaque sample. Furthermore, the treatments were compared with each other.

Conclusions

From the analysis of the bacterial populations, a similarity between the three proposed treatments (laser, ozonized water, 0.20% chlorhexidine) was highlighted, and it was observed that the use of the diode laser and ozonized water favors the development of commensal bacteria in the oral cavity. It is therefore to be hoped that further research into the antimicrobial effect of these treatments will increasingly extend their use in dental clinical practice.

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Data Availability Statement: Dataset available on request from the authors

Conflicts of Interest: The authors declare no conflicts of interest.

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