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Effect of photoactivated disinfection with a light-emitting diode on bacterial species and biofilms associated with periodontitis and peri-implantitis

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Periodontitis;
Peri-implantitis;
Periodontal pathogens;
Biofilm

Summary

Background: To determine the effect of photoactivated disinfection (PAD) using toluidine blue and a light-emitting diode (LED) in the red spectrum (wave length at 625—635 nm) on species associated with periodontitis and peri-implantitis and bacteria within a periodontopathic biofilm.

Methods: Sixteen single microbial species including 2 *Porphyromonas gingivalis* and 2 *Aggregatibacter actinomycetemcomitans* and a multispecies mixture consisting of 12 species suspended in saline without and with 25% human serum were exposed to PAD. Moreover, single-species biofilms consisting of 2 *P. gingivalis* and 2 *A. actinomycetemcomitans* strains and a multi-species biofilm on 24-well-plates, grown on titanium discs and in artificial periodontal pockets were exposed to PAD with and without pretreatment with 0.25% hydrogen peroxide. Changes in the viability were determined by counting the colony forming units (cfu).

Results: PAD reduced the cfu counts in saline by 1.42 $\log_{10}$ after LED application for 30 s and by 1.99 $\log_{10}$ after LED application for 60 s compared with negative controls (each $p < 0.001$). Serum did not inhibit the efficacy of PAD. PAD reduced statistically significantly ($p < 0.05$) the cfu counts of the *P. gingivalis* biofilms. The viability of the *A. actinomycetemcomitans* biofilms and the multi-species biofilms was statistically significantly decreased when PAD was applied after a pretreatment with 0.25% hydrogen peroxide. The biofilm formed in artificial pockets was more sensitive to PAD with and without pretreatment with hydrogen peroxide compared with those formed on titanium discs.

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Conclusions: PAD using a LED was effective against periodontopathic bacterial species and reduced viability in biofilms but was not able to completely destroy complex biofilms. The use of PAD following pretreatment with hydrogen peroxide resulted in an additional increase in the antimicrobial activity which may represent a new alternative to treat periodontal and peri-implant infections thus warranting further testing in clinical studies.

Introduction

The periodontal disease status impacts markedly on the biofilm composition [1]. Oral microbial-plaque communities are biofilms composed of numerous bacteria on host surfaces. It is generally accepted that a small group of predominantly Gram-negative anaerobic or microaerophilic bacteria is associated with initiation and progression of periodontitis. Organisms strongly associated with chronic and aggressive forms of periodontitis include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* [2]. In the case of *P. gingivalis*, an asaccharolytic anaerobe, high level of proteolytic activity, especially arginine- and lysine-specific cysteine proteases referred to as gingipains [3,4] are considered the most important virulence factors. *A. actinomycetemcomitans* synthesizes many toxins such as leukotoxin and cytotoxin [5]. Moreover, other species such as *Campylobacter rectus*, *Eubacterium nodatum*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Parvimonas micra*, *Streptococcus constellatus* support the pathogenesis of the disease. *Eikenella corrodens*, enterobacteria, staphylococci, *Selenomonas* spp., and yeasts may play a role as superinfecting species [2,6]. Nowadays, dental implants are widely used. Similar microbial colonization patterns observed in periodontitis are found in periimplant diseases [7—9].

Based on the impact of pathogens, the anti-infective regimen is an important component in any treatment of periodontal and peri-implant diseases. Antiseptics, *e.g.* chlorhexidine digluconate are widely used [10,11]. Antibiotics are recommended for severe cases [12,13]. The development of resistance against antibiotics and side effects of the drugs implicate a search for alternatives; among others the light-activated killing might be one option [14,15].

In photoactivated therapy, the photosensitizers are activated by light. Following that, singlet oxygen and other reactive oxygen species known to be highly toxic against tumor cells and microorganisms are released [16]. Low level lasers are commonly used for the activation of the photosensitizers [17—19].

Recently, the use of non-laser red light sources being much cheaper and with no special safety regulations has been suggested as an alternative to activating photosensitizers [20,21]. However, at present the data on the potential effects of this new approach on various microbial species associated with periodontitis and peri-implantitis is scarce. Hence, the aims of the present study were (i) to determine the effect of photoactivated disinfection (PAD) using a light-emitting diode (LED) in the red spectrum and toluidine blue as a photosensitizer on microbial species associated with periodontitis and peri-implantitis, (ii) to analyze the influence of serum on the effect of PAD, and (iii) to evaluate the efficacy of PAD on bacteria within an artificial biofilm.

Materials and methods

Device and photosensitizer

An LED lamp emitting in the red spectrum with a wave length of 625—635 nm and an energy output of about 2 W/cm^2 (FotoSan; CMS DentalApS, Kopenhagen, Denmark) was used in the experiments. Three tips adapted to the possible applications (mucosa, root canal, periodontal pocket) are available; here always ''PERIO'' tips were attached to the LED lamp. The time of exposure was 60 s (2 × 30 s). Only in the experiments testing planktonic bacteria without serum an exposure time of 30 s was used. The photosensitizer was toluidinblue O solution in a concentration of 0.1 mg/ml supplemented with glycerol, xanthan gum (TBO; FotoSan Agent, CMS DentalApS, Kopenhagen, Denmark). In all experiments TBO was used in its highest viscosity.

Microorganisms

Sixteen microbial strains were tested as single species (Table 1). Additionally a multiple species mixture consisting of 12 bacterial strains (*Streptococcus gordonii* ATCC 10558, *Actinomyces naeslundii* ATCC 12104, *F. nucleatum* ATCC 25586, *C. rectus* ATCC 33238, *E. nodatum* ATCC 33099, *E. corrodens* ATCC 23834, *P. micra* ATCC 33270, *P. intermedia* ATCC 25611, *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037, *T. denticola* ATCC 35405, *A. actinomycetemcomitans* Y4) was prepared.

In the assays with serum *F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037, *A. actinomycetemcomitans* J7, *E. corrodens* ATCC 23834, *Enterobacter cloacae* JGr1, *Staphylococcus aureus* ATCC 29213 as well as the multiple species mixture were examined. *P. gingivalis* ATCC 33277 and M5-1-2 as well as *A. actinomycetemcomitans* Y4 and J7 were used to establish single-species biofilms. Further a biofilm consisting of the 12 different species was formed.

All strains were precultivated 42 h before the experiments in the appropriate atmosphere. Modified tryptic soy agar [22] was used as cultivation media. Thereafter, microbes were suspended in 0.9% NaCl, washed once, mixed by repeated vortexing and adjusted to the 10^8 bacteria/ml.

Table 1 Tested microbial strains and mixtures.

#	Microbial strains	Gram staining	Group
1	*Fusobacterium nucleatum* ATCC 25586	Negative	Microaerophile/anaerobe
2	*Prevotella intermedia* ATCC 25611	Negative	Microaerophile/anaerobe
3	*Porphyromonas gingivalis* ATCC 33277	Negative	Microaerophile/anaerobe
4	*P. gingivalis* M5-1-2	Negative	Microaerophile/anaerobe
5	*Tannerella forsythia* ATCC 43037	Negative	Microaerophile/anaerobe
6	*Aggregatibacter actinomycetemcomitans* Y4	Negative	Microaerophile/anaerobe
7	*A. actinomycetemcomitans* J7	Negative	Microaerophile/anaerobe
8	*Campylobacter rectus* ATCC 33238	Negative	Microaerophile/anaerobe
9	*Eikenella corrodens* ATCC 23834	Negative	Microaerophile/anaerobe
10	*Eubacterium nodatum* ATCC 33099	Positive	Microaerophile/anaerobe
11	*Parvimonas micra* ATCC 33270	Positive	Microaerophile/anaerobe
12	*Streptococcus constellatus* ATCC 27823	Positive	Microaerophile/anaerobe
13	*Enterococcus faecalis* ATCC 29212	Positive	Superinfecting
14	*Candida albicans* ATCC 76615	Yeast	Superinfecting
15	*Enterobacter cloacae* JGr1	Negative	Superinfecting
16	*Staphylococcus aureus* ATCC 29213	Positive	Superinfecting
17	Multiple species mixture	Mixed	Mixed

Assessment of the efficacy of photoactivated disinfection

Defined inoculates of microorganisms (10^6 in 10 μl NaCl 0.9% each) were given into 1.5 ml tubes of dark color. After short centrifugation, 25 μl of photosensitizer were applied for 1 min and then exposed to the LED light for 30 s (PAD 30) or 60 s (PAD 60), respectively. Controls were 25 μl NaCl 0.9% solution without exposure to light (negative control — con), 25 μl NaCl 0.9% solution followed by 60 s of light exposure (light control — con LED) and 25 μl of TBO without light exposure (con TBO). The numbers of colony forming units (cfu) were determined after addition of NaCl 0.9% solution.

Assessment of the efficacy of photoactivated disinfection in the presence of serum

The experiments were repeated for selected species in the presence of 25% serum (final concentration). The human serum was purchased from Sigma—Aldrich (St. Louis, MO, USA). To inactivate the complement, the serum was heated for 30 min at 60 °C.

In part the samples were pretreated with 0.25% hydrogen peroxide for 1 min. Hydrogen peroxide should be additionally used and therefore only reduce but not completely eliminate the viability of bacteria. The concentration was selected by testing of *F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* J7, *S. aureus* ATCC 29213. The bacteria were suspended in 0.9% (w/v) NaCl solution. Serum was added in a final concentration of 25% and hydrogen peroxide in a final concentration of 0.25%, respectively. After an incubation time of 60 s the numbers of cfu were enumerated.

Biofilm assays

24-Well-plates were covered with 100 μl/well 25% serum in NaCl 0.9% solution for 1 h. Suspensions of bacterial strains were made and mixed with nutrient broth. Each 1 ml was transferred per well. The plates were incubated in appropriate conditions (*A. actinomycetemcomitans* biofilms with 5% CO_2, all other biofilms with anaerobic conditions) for 48 h. Then the medium was exchanged. In case of the multispecies-biofilm *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037 and *T. denticola* ATCC 35406 were added again. After an additional incubation of 48 h, the medium was removed and the biofilms were exposed to treatment with 50 μl 0.25% hydrogen peroxide, 50 μl TBO, LED, PAD and PAD after application hydrogen peroxide (H-PAD) as described above. Finally 1 ml 0.9% NaCl was added to the biofilms. Those were removed from the bottom by scraping, mixing by pipetting and additional vortexing before a serial dilution was made and the cfu counts/well were determined.

Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) photographs were taken to visualize the biofilm results. The photographs for CLSM were prepared by using live-dead staining (Live/dead® *Bac*Light™ Bacterial Viability kit, Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's description. The samples were examined with a Zeiss LSM510 Exciter confocal microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany). For SEM, the samples were fixed in 2% glutaraldehyde in cacodylate buffer for 30 min, washed twice with cacodylate buffer and dehydrated using a graded ethanol series (10 min each concentration). Following critical point drying, the samples were sputtercoated with gold and examined with a ZEISS LEO-1530 Gemini (Carl Zeiss NTS GmbH) equipped with a field emission electron gun at 10 keV.

Additionally, multispecies biofilms were formed on titanium discs with a diameter of 5 mm and a sandblasted and acid-etched (SLA) surface (Institut Straumann, Basel, Switzerland) and in artificial periodontal pockets. Teeth extracted for periodontal reasons were selected. The patients were asked and signed an informed consent for using these teeth in *in vitro*-experiments. Dentin slices of the roots of about 6 mm × 12 mm and a depth of about 3 mm were prepared. The surface properties were standardized by

using silicon carbide disks (Struers GmbH, Willich, Germany) and Gracey curettes (Deppeler SA, Rolle, Switzerland). The slices were placed into silicon blocks by using a placeholder. The artificial pocket had a depth of about 6 mm. Here biofilms were only treated with PAD and H-PAD. Thereafter cfu counts were determined from the titanium discs as described before. Bacteria from the ''periodontal pockets'' were sampled by placing paper points (ISO 50) into the pocket for 30 s. After that the paper points were placed into 1 ml 0.9% NaCl solution and cfus were determined as mentioned above.

Effect of photoactivated disinfection on interaction of multi-species biofilm with monocytic cells

For these experiments the Mono Mac 6 cells (DSMZ, Braunschweig, Germany) as a monocytic cell line was used. The cells were maintained in RPMI 1640 medium (Invitrogen Corporation) supplemented with 10% fetal bovine serum (Invitrogen Corporation). The monocytic cells were washed once with RPMI 1640 immediately before they were added to the biofilm. After this step, the monocytic cells were adjusted to 10^6 cells/ml RPMI 1640.

Multi-species biofilms were prepared in 24-well plates as described above. After treatment of the biofilms with TBO, PAD and H-PAD 10^6 Mono Mac 6 cells per well were added. The cells were incubated with 5% CO_2 for 6 h and 20 h. Afterwards, the media were collected, centrifuged 4 min at $400 \times g$ and the supernatants were analyzed for the levels of released IL-1β by using an ELISA-kit (R&D Systems Europa Ltd., Abingdon, UK) according to the manufacturer's description. The detection level of the kits was 1 pg/ml IL-1β.

All experiments were made in independent quadruplicates.

Statistical analysis

In all statistical analysis $\log_{10}$ cfu values were used. In the experiments which analyzed the effect of PAD on planktonic bacteria (incl. in the presence of serum) the analysis was made by using Student's *t*-test for dependent samples. The Student's *t*-test for independent samples was used to compare Gram-positive and Gram-negative bacteria. One way ANOVA test followed by *Post Hoc* Bonferroni analysis was the method to determine differences between the different groups of microbes.

Biofilms of different strains within one species were compared by using the Student's *t*-test for independent samples. All other analysis of the biofilm experiments was made by using the one way ANOVA test followed by *Post Hoc* Bonferroni.

Results

Effect of photoactivated disinfection on different micro-organisms

Compared with the negative controls, an addition of TBO reduced the cfu counts by 1.04 ± 1.19 $\log_{10}$ cfu. On the other hand, the exposure to LED alone did not change the numbers of viable microorganisms.

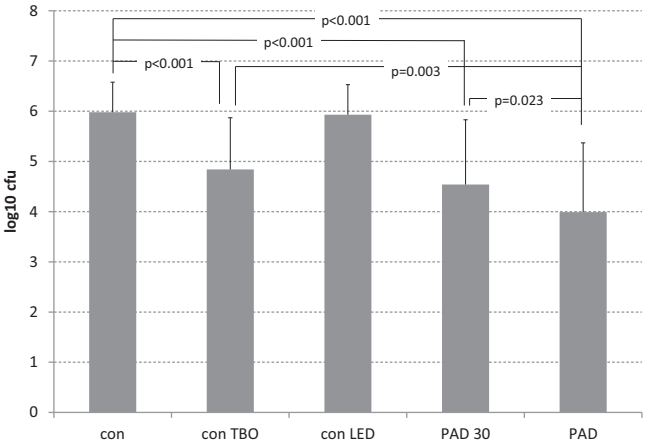

Figure 1 Numbers of viable microorganisms determined by colony forming unit counts (cfu; mean and SD) in the negative control (con), photosensitizer control (con TBO), LED control (con LED) and after photoactivated disinfection using 30 s LED exposure (PAD30) and 60 s LED exposure (PAD 60) incl. statistical differences.

After using PAD 30, the cfu counts were 1.36 ± 1.02 $\log_{10}$ lower compared to negative controls. PAD with an exposure time of 60 s LED further reduced the numbers of viable bacteria, the differences being 1.96 $\log_{10}$ compared to the negative control, 0.84 log-compared to the TBO con and 0.53 $\log_{10}$ compared to the PAD with an exposure time of 30 s. The cfu counts and statistical significances are presented in Fig. 1.

Considering the Gram staining of the bacteria (Fig. 2), the reducing effect of TBO con was more visible on Gram-negative bacteria (mean difference -1.47 ± 1.42 log) than on Gram-positives (mean difference -0.58 ± 0.44 $\log_{10}$ steps). Focusing on group of microbes, differences in the sensitivity were visible after TBO con, PAD 30 and PAD 60. The Bonferroni analysis confirmed a higher efficacy against microaerophilic/anaerobic species compared to superinfecting species after TBO con ($p = 0.033$), PAD 30 ($p = 0.001$) and PAD 60 ($p < 0.001$; Fig. 2).

Efficacy of photoactivated disinfection in the presence of serum

In the suspensions containing 25% serum the reduction by PAD was 2.00 ± 1.47 $\log_{10}$. In these samples without serum, PAD reduced the cfu counts by 2.36 ± 1.43 $\log_{10}$. The LED exposure of 60 s alone did not have any influence on the viability of bacteria. In contrast, application of TBO alone clearly reduced the cfu counts, the difference was 1.99 ± 1.47 $\log_{10}$ being in the range of PAD. Pretreatment with 0.25% hydrogen peroxide was very effective without LED, yielding a decrease of 2.90 ± 1.46 $\log_{10}$ compared with the controls. H-PAD killed nearly all bacteria (Fig. 3).

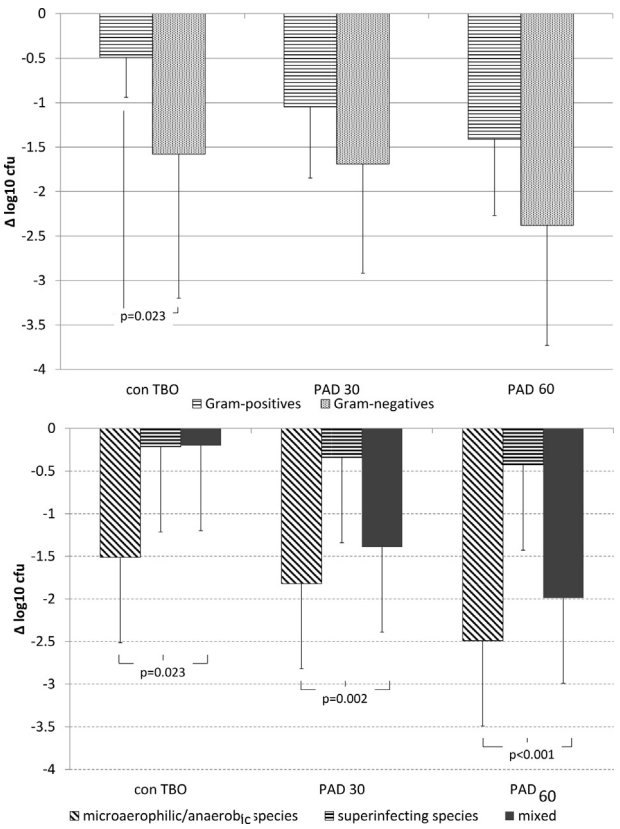

Figure 2 Differences in the mean counts of viable bacteria according to their Gram-properties and groups determined by colony forming unit counts (cfu; mean and SD) after application of photosensitizer (con TBO) and after photoactivated disinfection using 30 s LED exposure (PAD30) and 60 s LED exposure (PAD 60) incl. statistical differences.

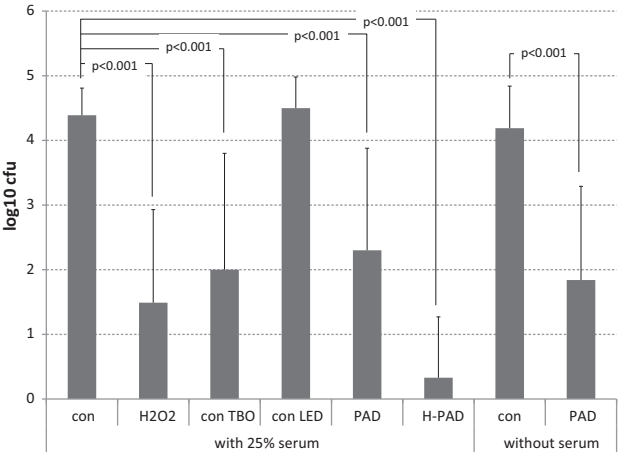

Figure 3 Numbers of viable microorganisms determined by cfu counts (mean and SD) in the untreated control (con), after exposure to 0.25% H_2O_2 (H_2O_2), after application of photosensitizer (con TBO), after application of 60 s LED (con LED) and after photoactivated disinfection using 60 s LED exposure without (PAD) and with pre-exposure to 0.25% H_2O_2 (H-PAD) in samples containing 25% serum (con and PAD additionally without serum).

Efficacy of photoactivated disinfection on bacteria within biofilm

P. gingivalis biofilms

The biofilms without any treatment contained on average 9.73 ± 0.51 $\log_{10}$ of viable bacteria. There was no difference between the two tested strains. An application of hydrogen peroxide significantly reduced the cfu counts. The TBO alone and the LED light alone did not change the viability. PAD eliminated many viable bacteria. H-PAD killed nearly all bacteria within the *P. gingivalis* biofilms (Fig. 4). The M5-1-2 strain tended to be less sensitive to the action of hydrogen peroxide, but PAD killed more bacteria of that strain in comparison with the ATCC strain ($p = 0.030$).

A. actinomycetemcomitans biofilms

The biofilm of *A. actinomycetemcomitans* Y4 contained more viable bacteria than those of *A. actinomycetemcomitans* J7 ($p = 0.007$). Only H-PAD was able to reduce significantly the cfu counts within biofilms (Fig. 4).

Multi-species biofilms

The mixed populations contained less cfu counts in comparison with the *A. actinomycetemcomitans* and with the *P. gingivalis* biofilms (each $p = 0.010$; Fig. 6). This might be caused by species not cultivable on the used agar (*e.g. T. denticola*) or by a higher matrix content. In multispecies biofilms, a treatment with 0.25% hydrogen peroxide significantly reduced the cfu counts. PAD alone tended to decrease the viability of the biofilm, whereas H-PAD reduced significantly the counts by 1.41 $\log_{10}$ (Fig. 4).

The comparisons of the different biofilms resulted in statistically significant differences of the untreated controls ($p < 0.001$), after treatment with 0.25% hydrogen peroxide ($p < 0.001$), after exposure to light alone ($p < 0.001$) and after H-PAD ($p < 0.001$). After exposure of light alone the differences were exactly the same as in controls. After application of TBO and PAD alone no difference was found suggesting a higher susceptibility of the *P. gingivalis* biofilms. After treatment with 0.25% hydrogen peroxide with and without PAD the *P. gingivalis* counts were lower in comparison with *A. actinomycetemcomitans* and with the multi-species biofilm (each $p < 0.001$).

Additionally, a biofilm was grown on titanium discs simulating an implant surface and in artificial periodontal pockets. In both models, the cfu counts were significantly reduced after exposure to PAD as well as after using H-PAD (Fig. 5). A comparison of the multi-species biofilms showed differences for the cfu counts after PAD ($p = 0.032$) between the plates and the pockets ($p = 0.031$) as well as after H-PAD ($p < 0.001$) between the pockets with the plate ($p = 0.017$) and with the titanium discs ($p < 0.001$).

SEM and CLSM photographs were taken from the biofilms formed on the plates. CLSM photographs show a high viability of bacteria within the untreated biofilm. The percentage is clearly reduced after application of TBO and also after PAD. The counts of bacteria are higher which might be explained by a disruption of the biofilm matrix leading to denser located bacteria within one layer. The percentage of the viable bacteria seemed not to be additionally reduced after H-PAD but there are less total counts of bacteria (Fig. 6).

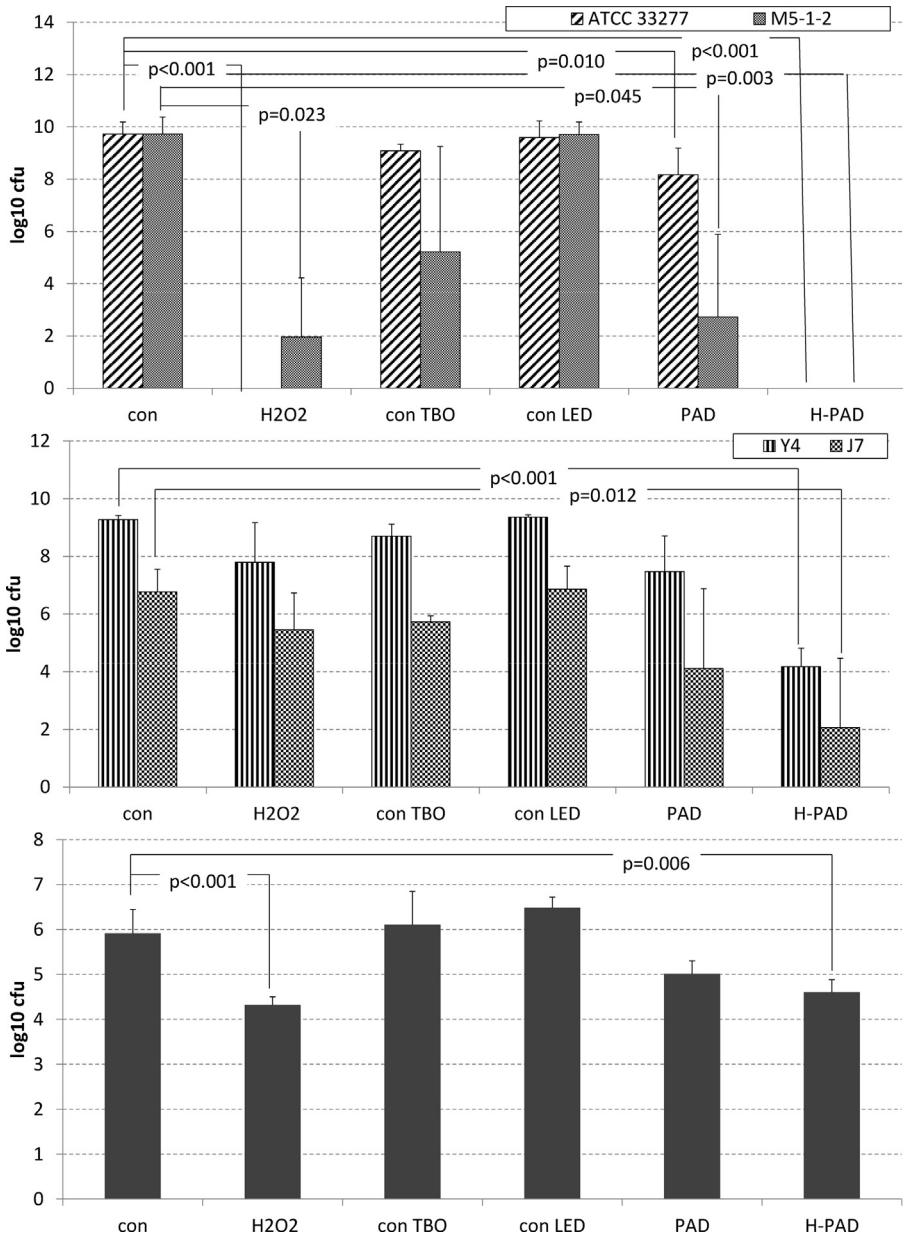

Figure 4 Numbers of viable microorganisms determined by cfu counts (mean and SD) in the untreated control (con), after exposure to 0.25% H_2O_2 (H_2O_2), after application of photosensitizer (con TBO), after application of 60 s LED (con LED) and after photoactivated disinfection using 60 s LED exposure without (PAD) and with pre-exposure to 0.25% H_2O_2 (H-PAD) in *P. gingivalis*, *A. actinomycetemcomitans* and multi-species biofilms.

SEM photographs show single large pores in the bacterial cell walls after application of TBO and after PAD (Fig. 7).

Non-stimulated Mono Mac 6 cells did not release any IL-1β after 6 h, whereas after 20 h moderate levels were detectable. Different exposures of the cells resulted in significant differences after 6 h ($p = 0.003$) and after 20 h ($p = 0.001$), respectively. After treatment of the biofilms with TBO as well as with PAD, higher levels of IL-1β were assessed in comparison with non-stimulated cells at the 6 h time-point. If the biofilms were treated with 0.25% H_2O_2 followed by PAD, no IL-1β was detectable. After 20 h of incubation, more IL-1β was measured in the supernatants when the biofilm was exposed to TBO in comparison with non-stimulated cells and cells exposed to the untreated biofilm (Fig. 8).

Discussion

The present study evaluated the possible antimicrobial activity of LED light when used after application of a photosensitizer using a commercially available system. The findings showed that PAD using a LED was effective against periodontopathic species and reduced viability in biofilms.

We have previously demonstrated that red light inactivates certain porphyrin producing bacteria [23]. The present

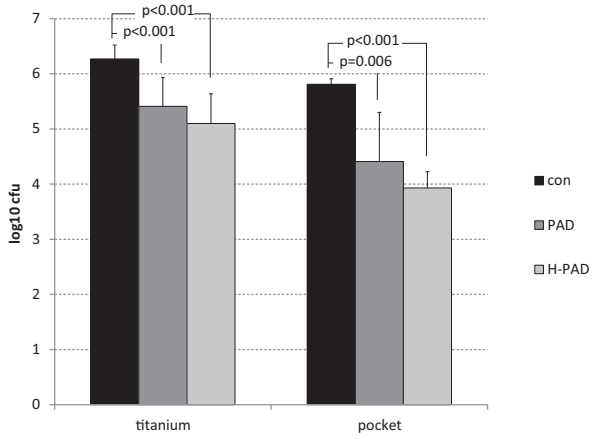

Figure 5 Numbers of viable microorganisms determined by cfu counts (mean and SD) in the untreated control (con) and after photoactivated disinfection using 60 s LED exposure without (PAD) and with pre-exposure to 0.25% H_2O_2 (H-PAD) in multispecies biofilms established on titanium discs and in an artificial pocket.

results confirm a potential of using red light when combined with application of a photosensitizer in the treatment of bacterial infections. A reduction of up to 2 $\log_{10}$ cfu with being more pronounced on microaerophiles/anaerobes was found. An irradiation time dependent effect was visible, the difference between 30 s and 60 s irradiation after application of TBO was 0.6 $\log_{10}$ cfu. Following that, an irradiation time of 60 s may be suggested. In a pilot study photoactivated disinfection using an irradiation time of 10 s only after application of TBO did not show any favor against scaling and root planing alone [24]. Another study showed that PAD using a conventional light source was at least as effective as a Helium—Neon laser, there a reduction of cfu counts up to 5 $\log_{10}$ was reported for several periodontopathic species [25]. The device used in the present study was tested before for endodontic treatment by using extracted teeth. PAD was successful in reducing counts of *Streptococcus intermedius* and *Enterococcus faecalis* within root canals [21,26].

TBO was used as photosensitizer. The concentration of 0.1 mg/ml was pointed out to be most effective on selected oral species when activated with red light emitted by light-emitting diodes [20]. TBO interacts more with LPS than methylene blue which may explain its good efficacy on Gram-negative bacteria [27]. Our live-dead staining and cfu counting results clearly demonstrate that application of photosensitizer alone for 60 s reduced viability of microbes. However an antimicrobial effect of LED alone was not found. In contrast, laser systems are able to reduce bacterial viability to some degree [28,29]. The antibacterial effect of toluidine blue was described before by using an incubation time of 1 h [30]. In the present study the reduction rate was lower despite using similar concentrations of the dye. The exposure to TBO was only 1 min which is probably more similar to a clinical situation. Differences in the susceptibility of the used microbial strains and mixtures were found. Most sensitive were microaerophilic/anaerobic Gram-negative species. Superinfecting species which are normally found after non-successful initial treatment are more resistant. These findings may favor an application of PAD in the treatment of periodontitis and peri-implantitis.

Subgingival bacterial biofilms are surrounded by gingival crevicular fluid, which is rich of serum [31]. Serum may inhibit efficacy of antimicrobials [32]. Thus, the influence of serum was considered. A concentration of 25% was used. Human serum albumin concentration in GCF was found to be between 3.4 and 35.6% of those in serum [33]. PAD was still active in the presence of 25% human serum. The difference to PAD in saline was only − 0.27 $\log_{10}$ cfu. This confirms the results of others reports about the activity of PAD in the presence of 100% serum but to a less extent in comparison with saline [25,34].

Bacteria in the oral cavity form biofilms. Therefore, the activity of PAD on bacteria within biofilms was of interest. We have chosen four single-species (2 *A. actinomycetemcomitans* and 2 *P. gingivalis* strains) and a multi-species biofilm consisting of 12 species. The biofilms were formed over a period of four days. The media were exchanged to provide new nutrients and to remove bacterial metabolic products. This ensured optimal conditions for biofilm growth. Instead of saliva we used serum to form a protein layer for attachment of the microbes. This might be more relevant for subgingival plaque with the crevicular fluid as being the most essential fluid in that region. PAD and H-PAD were effective in reducing viability of the *A. actinomycetemcomitans* and *P. gingivalis* biofilms. The bacteria within the *P. gingivalis* biofilms were completely killed by H-PAD. Differences were found between the two used *P. gingivalis* strains. The biofilms of *P. gingivalis* M5-1-2 tended to be less sensitive to the action of hydrogen peroxide but PAD killed more bacteria than in the biofilms formed by *P. gingivalis* ATCC 33277. It can be suggested that these differences are due to capsule components. The ATCC 33277 strain is characterized by missing capsule formation [35,36], whereas the M5-1-2 strains forms a large capsule. A reduction by about 2 $\log_{10}$ cfu was observed in the *A. actinomycetemcomitans* biofilms. Comparing with a similar study the bactericidal effect by applying for 1 min the TBO as a photosensitizer followed by 60 s LED was 1 $\log_{10}$ higher than described for 30 min preincubation of an *A. actinomycetemcomitans* biofilm with rose bengal followed by activation through visible light [37]. In contrast, using a methylene-blue based photosensitizer followed by irradiation with a diode laser resulted in decreased viability (up to 5 $\log_{10}$) of single-species biofilms of *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* [38].

Subgingival plaque consists of hundreds of different species [39]. Our multi-species biofilm formed by 12 species showed a striking reduced sensitivity to PAD and H-PAD in comparison with the single-species biofilms although the reduction of cfu counts was still more than 90% (1.41 $\log_{10}$ cfu for PAD, 1.88 $\log_{10}$ cfu for H-PAD). The efficacy of PAD (toluidine blue followed by 60 s LED) was higher than described in a multispecies biofilm model consisting of six different species, where photodynamic therapy using methylene blue as photosensitizer followed by application of a diode laser failed to reduce remarkable bacterial counts [40]. Nevertheless, PAD in combination with an application of hydrogen peroxide solution was unable to completely eradicate a periodontopathic biofilm in this *in vitro* study. This underlines that H-PAD can only be effective after

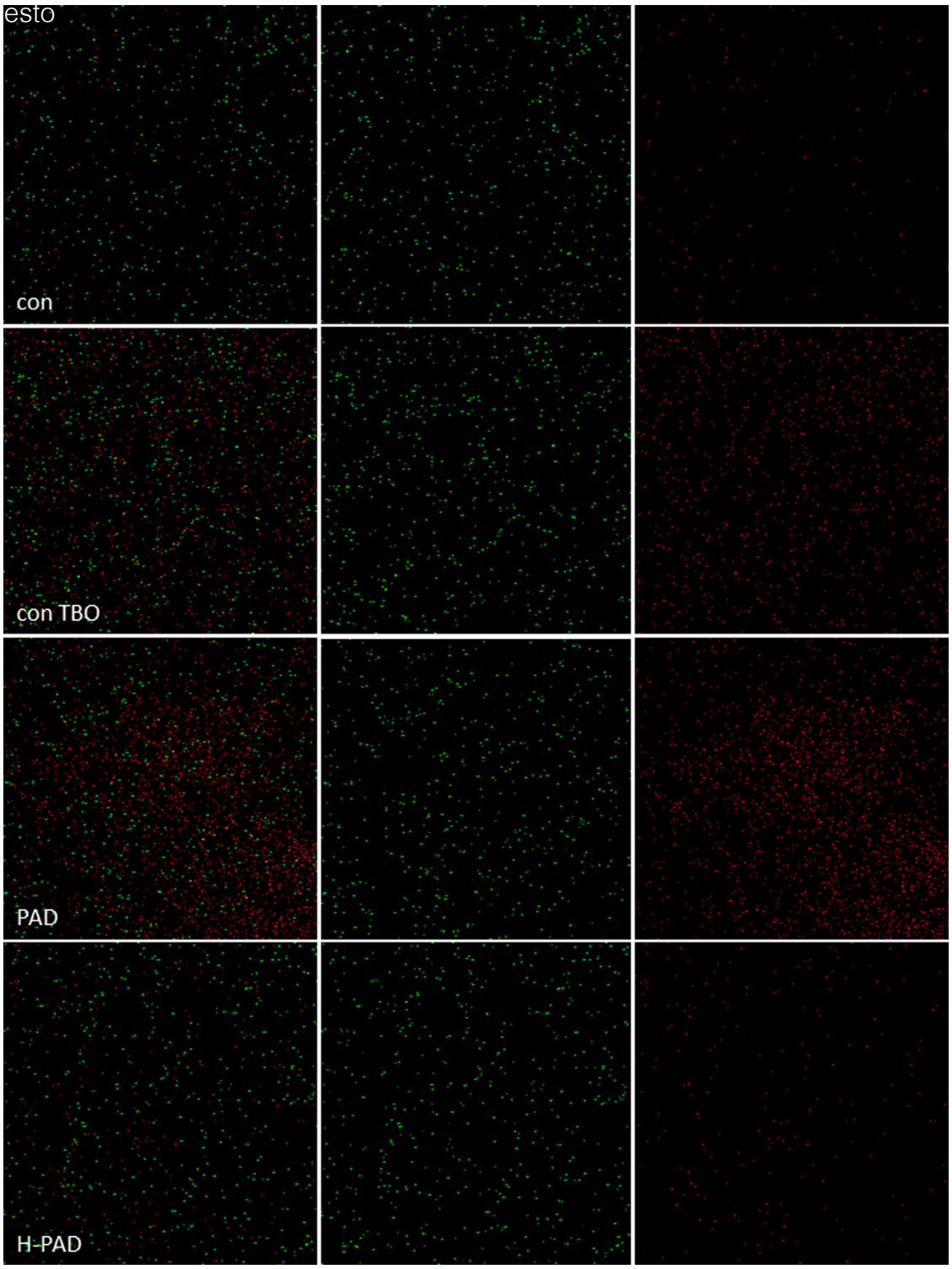

Figure 6 Live-dead staining of the multi-species biofilms by using CLSM in the untreated control (con), after application of photosensitizer (con TBO) and after photoactivated disinfection using 60 s LED exposure without (PAD) and with pre-exposure to 0.25% H_2O_2 (H-PAD) (left: viable (green) and dead (red) bacteria; middle: viable (green) bacteria; right: dead (red) bacteria).

mechanical disruption of a biofilm and thus, it should be used following scaling and root planing.

CLSM photographs confirmed a higher percentage of dead bacteria after application of TBO and after PAD. After H-PAD no differences were visible in comparison to the untreated controls. CLSM shows only one layer of the biofilm. A complete removal or destruction of bacteria by the application of 0.25% hydrogen peroxide which are not stained any longer might be suggested. SEM photographs show large pores of the bacterial cell wall after application of TBO and PAD.

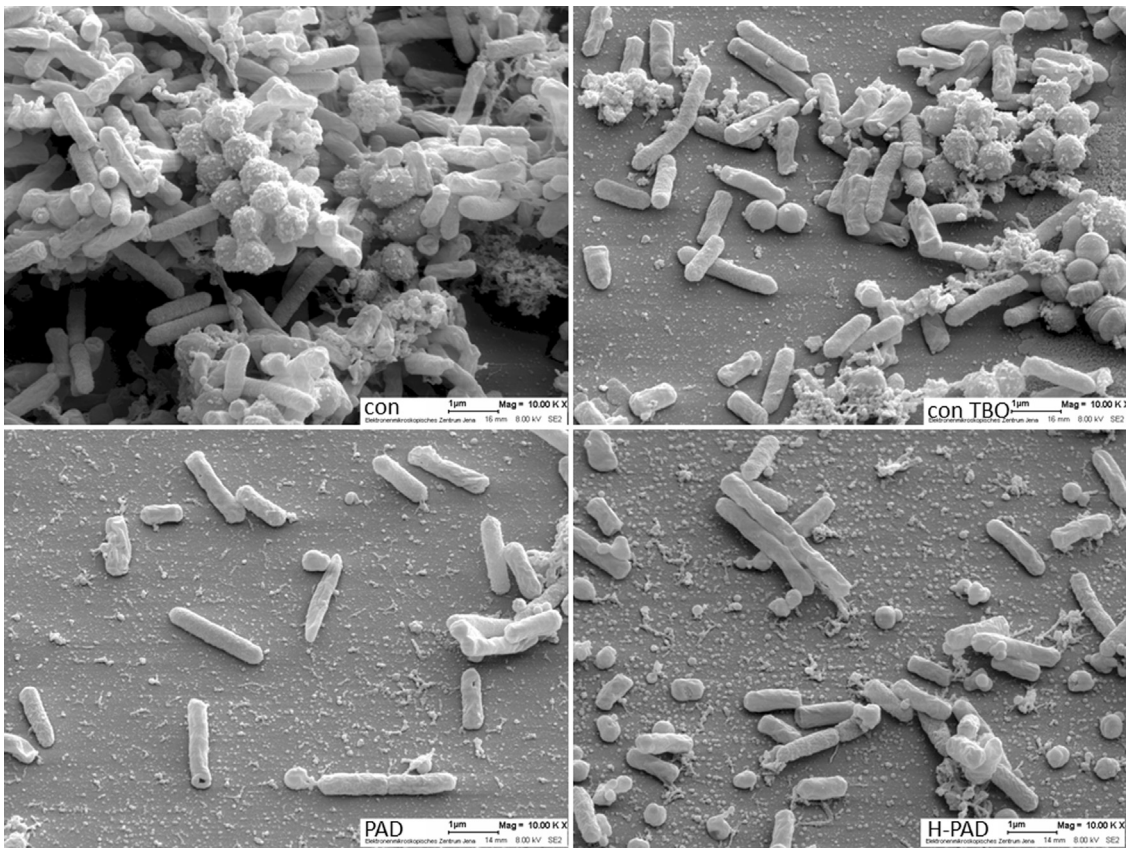

Figure 7 SEM photographs of the biofilms in the untreated control (con), after application of photosensitizer (con TBO) and after photoactivated disinfection using 60 s LED exposure without (PAD) and with pre-exposure to 0.25% H_2O_2 (H-PAD).

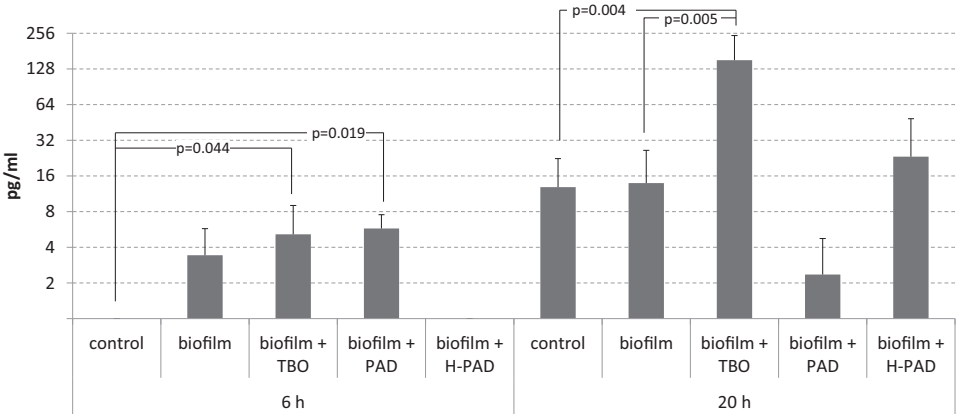

Figure 8 Released IL-1β from Mono Mac 6-cells without (con) and with pre-exposure to multispecies biofilms. Biofilms have been pretreated with photosensitizer (TBO), photoactivated disinfection using 60 s LED exposure without (PAD) and with pre-exposure to 0.25% H_2O_2 (H-PAD).

This is different to earlier observation testing the effect of chlorhexidine on periodontopathogens, where leakages all over the cell wall were seen [41]. Atomic force microscopic analysis indicated damage of the bacterial cell membrane and loss of cytoplasmatic materials by photodynamic therapy [42].

In part we included a pretreatment of the bacteria with hydrogen peroxide before exposure to photosensitizer and light. Hydrogen peroxide was highly active against the microorganisms. A synergism by the action of hydrogen peroxide with PAD was shown. We combined a pretreatment of hydrogen peroxide with PAD. Although the photodynamic therapy is discussed as an alternative to application of antibiotics another possibility might be to use PAD additionally to antibiotics. *In vitro* application of vancomycin to *S. aureus* biofilms after photodynamic therapy showed a synergistic effect [43].

Photodynamic therapy may influence the host response to the microbial challenge. In Wistar rats it reduced neutrophil migration and decreased tumor necrosis factor (TNF)-α level in gingival tissue [44]. In clinical studies lower levels of inflammatory cytokines were measured in gingival crevicular fluid after photodynamic therapy. However, there no differences to scaling and root planing alone were observed [45,46]. *In vitro*, photodynamic therapy directly inactivated the inflammatory cytokines TNF-α and IL-1β [47]. We did not test the direct effect of PAD on inflammatory cells. The monocytic Mono Mac 6 cell line which exhibits many characteristics of mature blood monocytes including the production of IL-1β [48] was exposed to the biofilm after PAD. Biofilms as the bacterial stimulus induced the release of IL-1β from monocytic cells initially. Later the biofilm treated with TBO only increased remarkably the amount of IL-1β. Periodontopathogens induce the release of IL-1β from monocytic cells [49—51]. TBO attached to cell walls may act as an additional stimulus. In another study it was shown that diode laser and LED irradiation were able to reduce *in vitro* the inflammatory response of mouse macrophages to *P. gingivalis* LPS adherent to a titanium surface [52]. Also, the response of other cells to the photoactivated biofilms is of interest. Treatment of *S. aureus* biofilms enhanced phagocytosis by blood neutrophils [43]. Further research is needed to specify the immune-modulatory effects of PAD in periodontitis and peri-implantitis therapy.

The biofilm formation on titanium discs with an SLA surface should mimic the peri-implant biofilm. The results of antimicrobial activity were comparable to those obtained in 24-well plates. This indicates a possible application of PAD using a light-emitting diode in mucositis and peri-implantitis as an adjunctive treatment to mechanical biofilm removal. Data about using photodynamic therapy in treatment of peri-implant diseases are rare. It was shown that photodynamic therapy might be beneficial in peri-implantitis treatment [53]. Recently we have shown that photodynamic therapy was as active as the application of local antibiotics in the treatment of moderate peri-implantitis [54].

Further, an artificial periodontal pocket was created. The cfu counts decreased more than using the other multi-species biofilm models after PAD. The surface area treated by the LED-light was smaller which lead to a higher energy per surface. On the other hand the biofilm was deeper which suggests even an activity of PAD and H-PAD in the deeper areas of the periodontal pocket. These results appear promising in view of a possible adjunctive application in periodontitis treatment. Photodynamic therapy by using a diode laser was shown to be clinically effective in untreated and in maintenance periodontitis patients. However, microbiologically no clear effect on the major periodontopathogens was found [17,18]. It might be of interest to test the effect of adjunctive H-PAD by using a LED on clinical and microbiological variables in clinical trials.

Taken together, the present findings indicate that: (a) PAD using LED is effective against periodontopathic microbial species even in the presence of serum, (b) PAD and H-PAD reduce viability in single-species biofilms. Multi-species biofilms are less sensitive than the single-species biofilms. Complete elimination of multi-species biofilms appears to be impossible thus underlining the importance of mechanical biofilm removal prior to treatment with H-PAD and (c) the increase in antimicrobial activity following the use of H-PAD may bear potential relevance as an adjunctive antimicrobial treatment in periodontal and peri-implant infections thus warranting further clinical testing.

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