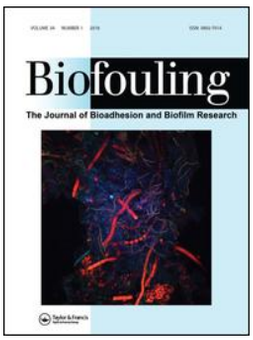

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Antimicrobial effects of a pulsed electromagnetic field: an *in vitro* polymicrobial periodontal subgingival biofilm model

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ABSTRACT

The objective was to test the influence of a pulsed electromagnetic field (PEMF) on bacterial biofilm colonization around implants incorporated with healing abutments. Healing abutments with (test group) and without (control group) active PEMF devices were placed in a multispecies biofilm consisting of 31 different bacterial species. The biofilm composition and total bacterial counts (x10^5) were analyzed by checkerboard DNA-DNA hybridization. After 96 h, the mean level of 7 out of the 31 bacterial species differed significantly between groups, namely *Eubacterium nodatum, Fusobacterium nucleatum* ssp. nucleatum, *Streptococcus intermedius, Streptococcus anginosus, Streptococcus mutans, Fusobacterium nucleatum* ssp. Vicentii and *Capnocytophaga ochracea* were elevated in the control group ($p < 0.05$). The mean total bacterial counts were lower in the Test group *vs* the control group ($p < 0.05$). An electromagnetic healing cap had antimicrobial effects on the bacterial species and can be used to control bacterial colonization around dental implants. Further clinical studies should be conducted to confirm these findings.

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KEYWORDS
Multispecies biofilm; antimicrobial pulsed electromagnetic field

Introduction

Peri-implantitis is characterized by inflammation of the connective tissues and progressive loss of supporting bone around implant-supported restorations (Schwarz et al. 2018). This disease is an increasingly prevalent biological complication associated with a complex dysbiotic microbiota around the implant-supported restoration, in which the incidence of peri-implant diseases may affect 43% of the implants after functional loading for five years (Berglundh et al. 2018; Cosgarea et al. 2019).

Bacterial biofilm continues to be the main etiologic factor of peri-implant diseases. The microbiota associated with peri-implantitis is more complex than that found under healthy peri-implant conditions (Shibli et al. 2008; Retamal-Valdes et al. 2019). Diseased implants harbor higher levels and more diverse types of periodontal pathogens than those found in periodontal diseases, such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Tannerella forsythia*. Therefore, it is of paramount importance to develop alternative options to prevent and/or treat infections in dental implants. Previous systematic reviews (Khoshkam et al. 2016; Tomasi et al. 2019) have shown that the treatment of peri-implantitis is not a simple task and depends on a striking change in the microbial profile around the diseased implants.

Several strategies have been described to reduce or even eliminate the bacterial load in the local peri-implant environment around the implant, such as the use of local or systemic antibiotics associated with mechanical debridement (Heitz-Mayfield et al. 2018; Shibli et al. 2019). However, to date, there is no common consensus about which therapy is more effective. To address this problem, the technology of pulsed electric fields (PEF) has been used in orthopedics for surgical mesh disinfection to eliminate drug-resistant strains.

Magnetic fields are created by electric currents, magnetic dipoles, and changing electric fields. The biological effects of pulsed electromagnetic fields (PEMF) have been evaluated in the past decades resulting in better knowledge about and acceptance of the technique (Shatalov 2012; Yalçin and Erdem 2012). These non-ionizing, non-thermal fields generated by power lines, workplaces, and household electric appliances result in

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Table 1. List of microorganisms used in the biofilm model and respective growing conditions.

Microorganism	Growth Conditions*
Actinomyces gerencseriae ATCC 23840	TSA + 5% sheep blood
Actinomyces israelii ATCC 12102	TSA + 5% sheep blood
Actinomyces naeslundii ATCC 12104	TSA + 5% sheep blood
Actinomyces odontolyticus ATCC 17929	TSA + 5% sheep blood
Actinomyces oris ATCC 43146	TSA + 5% sheep blood
Aggregatibacter actinomycetemcomitans ATCC 29523	TSA + 5% sheep blood
Campylobacter gracilis ATCC 33236	TSA + 5% sheep blood
Campylobacter showae ATCC 51146	TSA + 5% sheep blood
Capnocytophaga sputigena ATCC 33612	TSA + 5% sheep blood
Capnocytophaga gingivalis ATCC 33624	TSA + 5% sheep blood
Capnocytophaga ochracea ATCC 33596	TSA + 5% sheep blood
Eikenella corrodens ATCC 23834	TSA + 5% sheep blood
Eubacterium nodatum ATCC 33099	TSA + 5% sheep blood
Eubacterium saburreum ATCC 33271	TSA + 5% sheep blood
Fusobacterium periodonticum ATCC 33693	TSA + 5% sheep blood
Fusonucleatum polymorphum ATCC 10953	TSA + 5% sheep blood
Fusonucleatum vincentii ATCC 49256	TSA + 5% sheep blood
Gemella morbillorum ATCC 27824	TSA + 5% sheep blood
Parvimonas micra 33270	TSA + 5% sheep blood
Porphyromonas gingivalis ATCC 33277	TSA + YE + 1% hemin + 5% menadione + 5% sheep blood
Prevotella intermedia ATCC 25611	TSA + 5% sheep blood
Probionibacterium acnes ATCC 11827	TSA + 5% sheep blood
Selenomonas noxia ATCC 43541	TSA + 5% sheep blood
Streptococcus anginosus ATCC 33397	TSA + 5% sheep blood
Streptococcus constellatus ATCC 27823	TSA + 5% sheep blood
Streptococcus gordonii ATCC 10558	TSA + 5% sheep blood
Streptococcus mitis ATCC 49456	TSA + 5% sheep blood
Streptococcus oralis ATCC 35037	TSA + 5% sheep blood
Streptococcus sanguinis ATCC 10556	TSA + 5% sheep blood
Streptococcus intermedius ATCC 27335	TSA + 5% sheep blood
Tannerella forsythia ATCC 43037	TSA + YE + 1% hemin + 5 % menadione + 5% sheep blood + 1% N-acetylmuramic acid

*All the microorganisms were grown at 37 °C under anaerobic conditions (85% nitrogen, 10% carbon dioxide, and 5% hydrogen.). TSA: triptone soy agar; YE: Yeast extract

physiological outcomes in living organisms (Simko and Mattsson 2004; Shatalov 2012; Yalçin and Erdem 2012) and therefore, can be used in the biomedical area.

PEMF could affect cytoplasmic membranes, leading to pore formation, leakage of cell components, and consequently, to cell death. Pore formation can be reversible or irreversible, depending on the treatment conditions and the parameters used (Ulmer et al. 2002). PEMF used on cells induces changes in biological membrane permeability, also called electroporation. Electroporation impacts on the formation of aqueous pores in the lipid bilayer that enable molecular transport between cells and the environment (Rubin et al. 2019). Low-frequency electromagnetic fields can promote alteration in the bacterial membrane and can, therefore, change bacterial metabolism and cell growth (Oncul et al. 2016). These authors concluded that PEMF affected the crucial physico-chemical processes in both Gram-positive and Gram-negative bacteria and could be a potential tool that can be used to overcome bacterial contamination and infections.

Furthermore, electromagnetic fields are useful in clinical applications such as bone healing and have been shown to be promising in other areas such as wound repair and neuroregeneration (Madkan et al. 2009). A positive effect was reported in the improvement in the bone mineral density of osteoporotic women (Tabrah et al. 1990). A relatively recent published study (Barak et al. 2016) found that in rabbits, PEMF devices stimulated early osseointegration and ingrowth of bone onto dental implants by more than 3-fold.

The project hypothesis was that PEMF could be useful for improving osseointegration and in controlling microbial colonization around implant-supported restorations. Thus, the aim of this *in vitro* study was to evaluate the antimicrobial effects of pulsed electromagnetic fields (PEMF) on the *in vitro* polymicrobial subgingival periodontal biofilm model.

Material and methods

Miniaturized electromagnetic device (MED) and PEMF

Twenty MEDs (Magdent ltd, Bnei-Brak, Israel) shaped as dental healing abutments were used in the present study. PEMF generated by the miniaturized electromagnetic device was active within a radius of 2 mm, with an exposure ratio of 1/500–1/5000, an intensity of 0.05–0.5 mT and a frequency of 10–50 kHz. All MED healing abutments were installed into dental implants

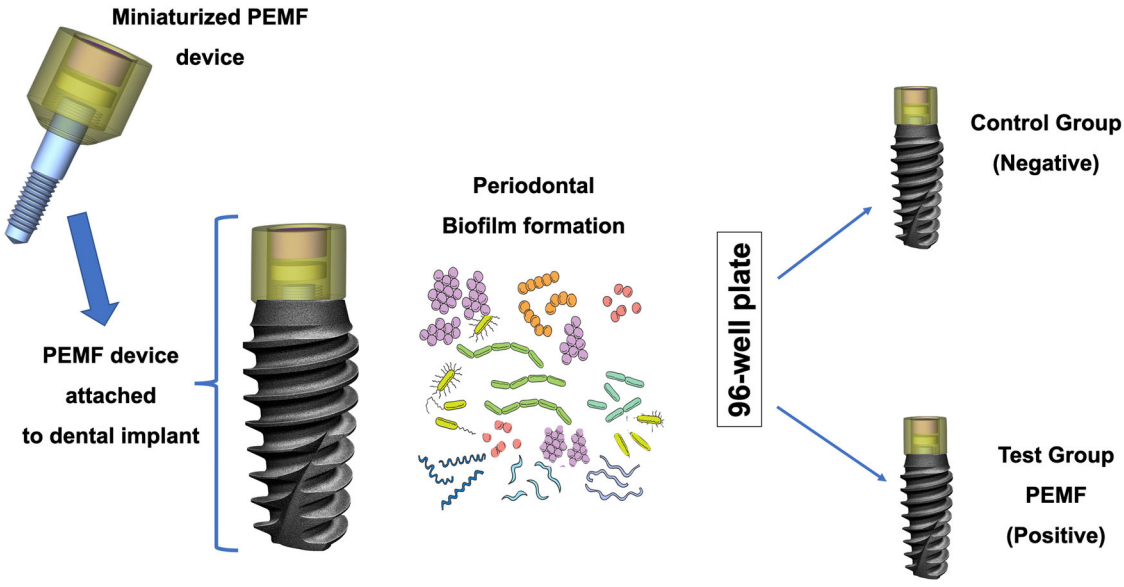

Figure 1. Experimental design. The miniaturized PEMF (pulsed electromagnetic field) was attached to the dental implant and after biofilm formation for 7-days, 96-well plates were prepared for the test group (PEMF) and control group (without PEMF activation).

(or replicas) with a 3.75 mm diameter x 10 mm height. Half of these healing abutments included an electromagnetic healing cap that consisted of a battery, an electronic device, and an induction coil and was activated (test group, $n = 10$ samples) before being inserted in the biofilm model; the remaining MEDs were not active (control group, $n = 10$ samples).

Biofilm formation

The species used to form multispecies biofilm were *Actinomyces gerencseriae* ATCC 23840, *Actinomyces israelii* ATCC 12102, *Actinomyces naeslundii* ATCC 12104, *Actinomyces odontolyticus* ATCC 17929, *Aggregatibacter actinomycetemcomitans* ATCC 29523, *Campylobacter gracilis* ATCC 33236, *Campylobacter showae* ATCC 51146, *Capnocytophaga sputigena* ATCC 33612, *Capnocytophaga gingivalis* ATCC 33624, *Capnocytophaga ochracea* ATCC 33596, *Eikenella corrodens* ATCC 23834, *Eubacterium nodatum* ATCC 33099, *Eubacterium saburreum* ATCC 33271, *Fusobacterium periodonticum* ATCC 33693, *Fusonucleatum polymorphum* ATCC 10953, *Fusonucleatum vincentii* ATCC 49256, *Gemella morbillorum* ATCC 27824, *Parvimonas micra* 33270, *Parvimonas micra* ATCC 33270, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Probionibacterium acnes* ATCC 11827, *Selenomonas noxia* ATCC 43541, *Streptococcus anginosus* ATCC 33397, *Streptococcus constellatus* ATCC 27823, *Streptococcus gordonii* ATCC 10558, *Streptococcus mitis* ATCC 49456, *Streptococcus oralis* ATCC 35037, *Streptococcus sanguinis* ATCC 10556, *Streptococcus intermedius* ATCC 27335 and *Tannerella forsythia* ATCC 43037.

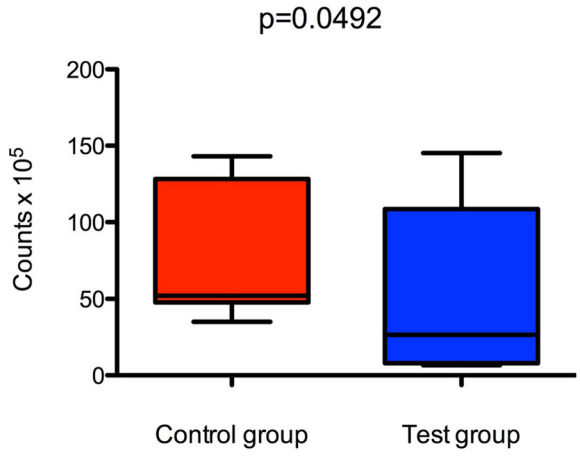

Figure 2. Box-plot of the total bacterial counts (x10^5) of 7-day biofilms formed on titanium surfaces of the Control group, without the electromagnetic healing cap being activated, and the Test group, with the electromagnetic healing cap activated on the first day and kept for the seven days of biofilm formation. The Mann-Whitney test was used to determine whether there were significant differences between the groups ($p > 0.05$).

Tryptone soy agar (TSA) with 5% sheep blood (Probac, São Paulo, Brazil) was used to grow the majority of the species under anaerobic conditions (85% nitrogen, 10% carbon dioxide, and 5% hydrogen). *E. nodatum* and *E. saburreum* were cultured on fastidious anaerobic agar (FAA) with 5% sheep blood (Laborclin, Pinhais, Brazil) whilst to culture *P. gingivalis*, TSA with yeast extract (YE) enriched with 1% hemin, 5% menadione, and 5% sheep blood was used. The medium containing TSA with YE enriched with

1% hemin, 5% menadione, 5% sheep blood and 1% N-acetylmuramic acid was used for *T. forsythia*. All species were allowed to grow on agar plates for 24 h and then transferred to glass tubes containing BHI culture medium (Becton Dickinson, Sparks, MD) supplemented with 1% hemin. The microorganisms included in the biofilm model and growing conditions are listed in Table 1.

After 24 h growth in the glass tubes, the optical density (OD) was adjusted for the inoculum to have about 10^8 cells ml^{-1} of each species. A dilution of individual cell suspensions was performed, and 100 µl aliquots containing 10^6 cells from each species were added to 11,700 µl of BHI broth complemented with 1% hemin and 5% sheep blood to obtain an inoculum of 15 ml .

All healing abutments connected into the dental implants were placed vertically in a 96-well plate to act as a substratum for biofilm formation (Figure 1). There were two groups of healing caps: (G1) a healing cap activated with a pulsed electromagnetic field (Test group), and (G2) the same healing cap without the pulsed electromagnetic field and not activated (Control group). Both groups were placed into a different 96-well plate to avoid electromagnetic interference between groups. A total of 150 µl of inoculum containing 10^4 cells of each species was added into each well with the MED/implant, and plates were incubated at 37 °C under anaerobic conditions. After incubation for 72 h, the culture medium was replaced with fresh BHI broth (supplemented with 1% hemin and 5% sheep blood) and maintained at 37 °C under

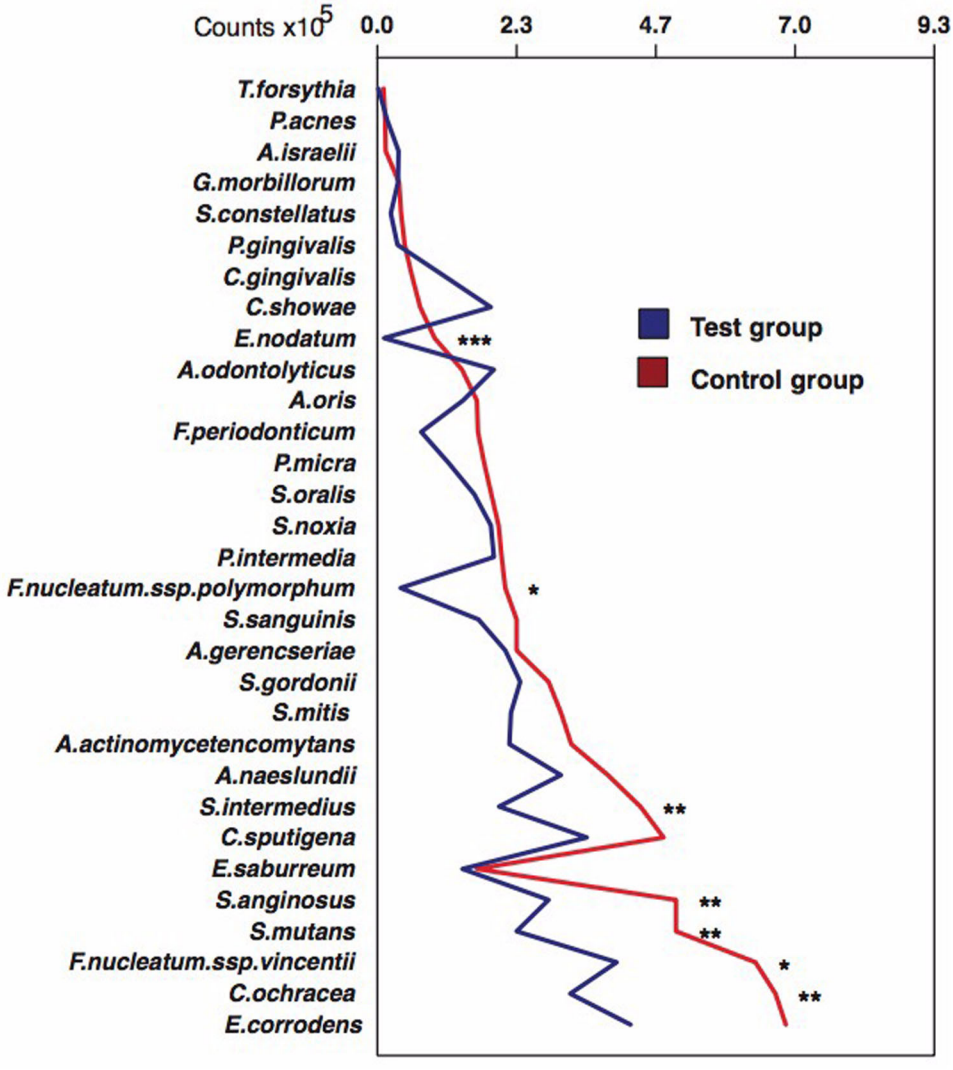

Figure 3. Mean bacterial counts (x10^5) of the biofilms formed on the titanium surfaces of the Control group, without the electromagnetic healing cap being activated, and the Test group, with the electromagnetic healing cap activated on the first day and kept for the seven days of biofilm formation. The data were analyzed using the Mann-Whitney test (* $p < 0.05$; ** $p < 0.01$, *$p < 0.001$).

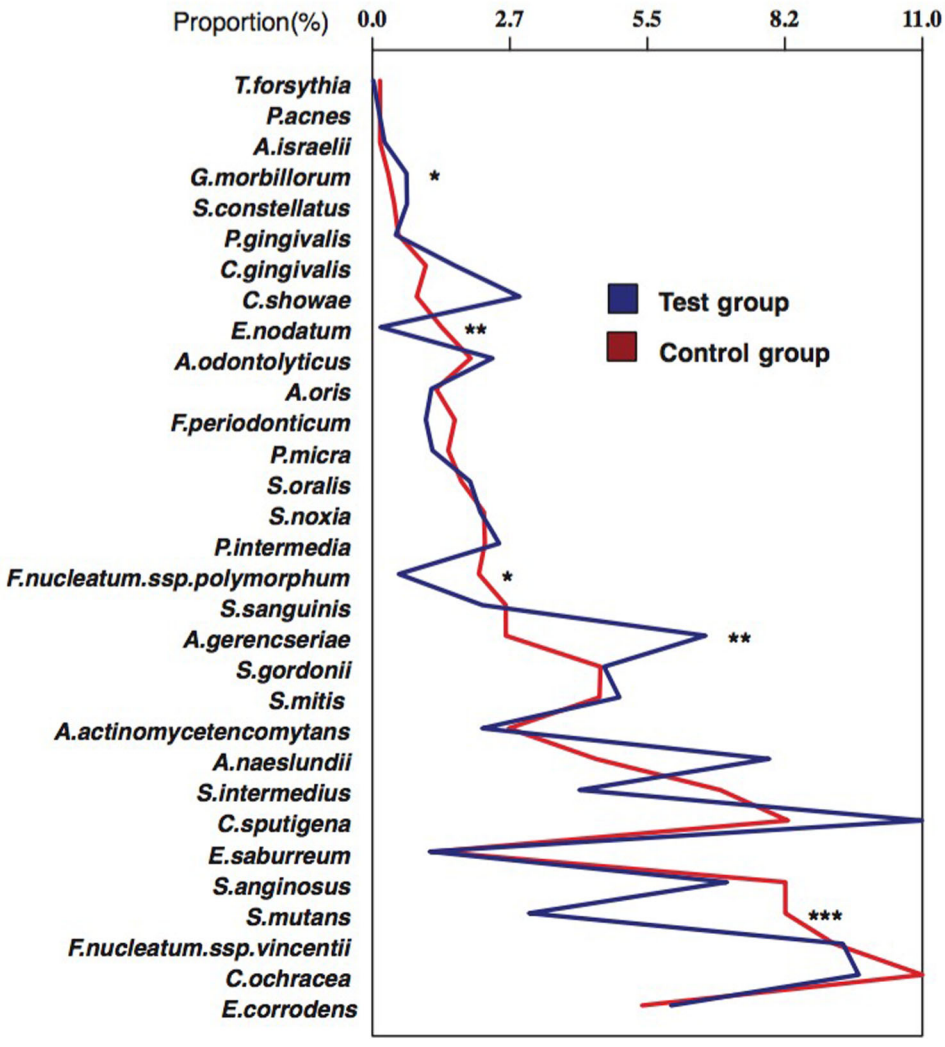

Figure 4. Mean proportions of the bacterial species formed on the titanium surfaces of the Control group, without the electromagnetic healing cap activated, and the Test group, with the electromagnetic healing cap activated on the first day and kept for the seven days of biofilm formation. The data were analyzed using the Mann-Whitney test (* $p < 0.05$; ** $p < 0.01$, *$p < 0.001$).

anaerobic conditions for seven days of biofilm formation. The healing caps, activated and non-activated, were kept during these seven days. Three independent experiments were performed (Miranda et al. 2019; Pingueiro et al. 2019; Miranda et al. 2020).

DNA-DNA hybridization (checkboard DNA-DNA)

Ten 7-day biofilm coated MED/dental implants of each of the groups were transferred to Eppendorf tubes containing 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7, 6]), and then 100 µl of 0.5 M NaOH were added. The tubes containing the implants and the final solution were boiled for 10 min and the solution neutralized with the addition of 0.8 ml of ammonium 5 M. The samples were analyzed individually for the presence and quantity of the 31 bacterial species, using a DNA-DNA hybridization technique. Briefly, upon lysis of the samples, the DNA was plated onto a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After DNA attachment to the membrane, it was placed in a Miniblotter 45 (Immunetics). Digoxigenin labeled with DNA probes of the entire genome for the subgingival species used were hybridized to individual lanes of Miniblotter 45. After hybridization, the membranes were washed, and DNA probes were detected using a specific antibody to digoxigenin conjugated to phosphatase alkaline. The signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL), and the results were obtained using Typhoon Trio Plus (Molecular Dynamics, Sunnyvale, CA). Two lanes in each race contained the standards with $10^5$and 10^6 cells of each species. Signals obtained with the Typhoon Trio were converted to absolute counts, by comparison with the patterns on the same membrane. Failure to detect a signal was recorded as zero. The values obtained after

the experimental period for the G1 group were compared with the values of the control group G2 (Socransky et al. 1994; Miranda et al. 2019; Pingueiro et al. 2019; Miranda et al. 2020).

Statistical analysis

For microbiological analyses, mean counts ($x10^5$), the percentage of the total DNA probe counts, and total counts of the specific bacterial species within biofilms were determined initially in each sample. The significance of differences between groups was sought using the Mann-Whitney U test. The microbiological analyses were adjusted for multiple comparisons (Socransky et al. 1991).

Results

Figure 2shows total counts of the bacterial species formed on the surfaces of two healing cap groups. Significant difference was observed between groups. The mean total bacterial counts were lower in the test group in comparison with the control group ($p = 0.0492$)

Figure 3 shows the mean counts (x 10^5) of the 31 species evaluated in the biofilms formed in the two groups. Seven species differed significantly between groups; *E. nodatum, F. nucleatum* ssp. nucleatum, *S. intermedius, S. anginosus, S. mutans, F. nucleatum* ssp. vicentii *and C. ochracea* were elevated in the control group *($p < 0.05$).* In the test group, four bacterial species had higher mean levels when compared with the control group, *A. israelli, C. gingivalis, C. showae, and A. odontolyticus.* However, no significant differences were observed between the groups ($p > 0.05$).

The mean percentage of DNA probe counts of the 31 individual species evaluated in the biofilm model are presented in Figure 4. The proportions of two bacterial species were significantly higher in the Test group (*G. morbillorum and A. gerencseriae*) while 3 bacterial species in the control group (*E. nodatum, F. nucleatum* ssp. nucleatum *and S. mutans*).

Discussion

The present *in vitro* study showed that PEMF released by MED shaped as healing abutments had an impact on bacterial colonization around the dental implants. According to these data, the use of PEMF promoted a lower level of bacterial colonization, including a lower level of colonization of species that are important for initial biofilm colonization, such as *S. intermedius, S. anginosus, S. mutans, F. nucleatum* ssp. vicentii and *F. nucleatum* ssp. nucleatum. It could be speculated that exposure to PEMF at 10-59 Hz impacted the bacterial cellular cycle inducing a difference in transmembrane potential, resulting in an electrical breakdown and local changes in the layer, resulting in the destruction of transmembrane gradients and a loss of viability (Ulmer et al. 2002).

The effect of low-frequency electromagnetic fields on biological systems has been studied over the years and could be of great benefit to outcomes for essential purposes. Some studies have shown that low-frequency electromagnetic fields have the capacity to promote alterations in the bacterial membrane (Oncul et al. 2016) in addition to being capable of promoting enhanced peri-implant bone healing (Barak et al. 2016; Nayak et al. 2020). Thus, this strategy can be added to the biological concept of dental implant procedures, not only to improve bone healing but also to modify the microenvironment in the peri-implant sulci in diseased dental implants.

PEMF effects were unique; the growth rates of seven of the 31 species (*E. nodatum, F. nucleatum* ssp. nucleatum, *S. intermedius, S. anginosus, S. mutans, F. nucleatum* ssp. vicentii *and C. ochracea*) were significantly reduced, while four species (*A. israelli, C. gingivalis, C. showae, and A. odontolyticus*) showed increased rates although not statiscally significant. Taken together, it could be suggested that there was an important difference between these species, which contributed to their specific responses to the PEMF. Indeed, *E. nodatum, S. intermedius, S. anginosus, S. mutans, A. israelli and A. odontolyticus* are Gram-positive species, whereas both *F. nucleatum*, *C. ochracea, C. gingivalis, C. showae* are Gram-negative. In addition, *C. ochracea* and *C. gingivalis, C. showae* also reacted differently to the PEMF, suggesting that membrane morphology and composition could not be the only factor. It is far more likely that these fields interact with the dental biofilm on multiple levels simultaneously changing the climax community of this specific environment. However, it must be pointed out that the present study evaluated the impact of PEMF relative to a single parameter. Different electromagnetic fields could increase not only the number and duration of the pulse but also the frequency. These factors could induce irreversible electroporation mechanisms of microorganism inactivation, such as electrolysis and release of several free radicals, which could result in killing bacteria, alone or associated with electric fields (Rubin et al. 2019).

Few studies have been focused on the microbial effect of the PEMF, and as far as the authors are

aware, this is the first study that tested the effect of an electromagnetic field on a complex polymicrobial dental biofilm (Huwiler et al. 2012; Oncul et al. 2016). Oncul et al. (2016) reported a slight decrease in the growth of *S. aureus* and *E. coli* mono-species biofilm. The authors suggested that the pulsed electromagnetic field could promote damage to the bacterial membrane and therefore make an essential biological change in their metabolic status. The same profile of results was also reported by Bayir et al. (2015). These results were in agreement with these data which found some influence of the PEMF on bacterial colonization, at least in this *in vitro* biofilm model.

The concept of MED was introduced by Barak et al. (2016) using a pre-clinical model. They found a 56% higher trabecular bone fraction associated with an enhanced number of trabeculae and connectivity density in the PEMF group when compared with the control group. Therefore, these results suggest that in combination with the hard tissue healing findings described by Barak et al. (2016), there could be a benefit in the type of subgingival colonization around the dental implant. Since biofilm formation on oral implants is capable of causing inflammation in peri-implant tissues, which endangers the long-term success of implant supported restorations, the possibility of having a device that can act as a bio-modifier of the subgingival microbial profile and promote a healthy microbiota around dental implants would have wide clinical application.

Despite these promising results, this study had some limitations. This was an *in vitro* study and a clinical study with a large number of subjects should be conducted in order to confirm these results and check the influence of PEMF on the subgingival colonization around dental implants. However, considering that some relevant conclusions could be drawn in spite of these limitations, the results may serve as a basis for future studies. PEMF clearly had some impact on bacterial colonization even in a multicomplex biofilm model. Therefore, this *in vitro* analysis should be interpreted with caution, and further clinical studies should be conducted to confirm the clinical application of the PEMF healing abutment.

Conclusions

Within the limitations of the present study, MED-generated PEMF may have an antimicrobial effect on bacterial species and can be considered as a new treatment modality to control bacterial colonization around dental implants.

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Disclosure statement

Oleg Dolkart (Consultant), Elad Yakobson (employee, COO), and Shlomo Barak (founder) work at Magdent Company. The other authors declare no competing interests.

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