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SHORT-TERM EFFECTS OF HYALURONIC ACID ON THE SUBGINGIVAL MICROBIOME IN PERI-IMPLANTITIS. A RANDOMIZED CONTROLLED CLINICAL TRIAL

Authors: Ana Soriano-Lerma, MSc[*, †], Antonio Magán-Fernández DDS, MSc, PhD[‡], Juan Gijón, PhD[‡], Elena Sánchez-Fernández, MD, PhD[§], Miguel Soriano, PhD [, ¶], José A. García-Salcedo, PhD[†,], Francisco Mesa, MD, PhD[‡]

Affiliations:

[*] Department of Physiology (Faculty of Pharmacy, Campus Universitario de Cartuja), Institute of Nutrition and Food Technology "José Mataix", University of Granada, E-18071 Granada, Spain

[†] Microbiology Unit, Biosanitary Research Institute ibs.GRANADA, University Hospital Virgen de las Nieves, E-18014, Granada, Spain

[‡] Department of Periodontics, School of Dentistry, University of Granada, E-18071, Granada, Spain.

[§] Department of Oral Surgery and Implant Dentistry, School of Dentistry, University of Granada, E-18071, Granada, Spain.

 GENYO. Centre for Genomics and Oncological Research: Pfizer / University of Granada / Andalusian Regional Government, PTS Granada, E-18016, Granada, Spain

[¶] Center for Intensive Mediterranean Agrosystems and Agri-food Biotechnology (CIAMBITAL), University of Almeria, E-04001, Almería, Spain.

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Corresponding authors:

Antonio Magan-Fernandez, DDS, MSc., PhD.
Facultad de Odontología, Campus de Cartuja s/n,
Universidad de Granada. E-18071 GRANADA, Spain.
Tel +34-958240654 E-mail: amaganf@ugr.es

José A. García Salcedo, PhD
Microbiology Unit, Biosanitary Research Institute (ibs.GRANADA),
University Hospital Virgen de las Nieves, E-18014
GENYO. Centre for Genomics and Oncological Research: Pfizer/University of Granada/Andalusian Regional Government E-18014, GRANADA, Spain.
Tel +34-958175500 E-mail: jags@genyo.es

ABSTRACT

Background: The aim of our study was to evaluate the effects of a hyaluronic acid gel at 45 days on the microbiome of implants with peri-implantitis with at least one year of loading.

Methods: A randomized controlled trial was conducted in peri-implantitis patients. Swabs containing the samples were collected both at baseline and after 45 days of treatment. 16S rRNA sequencing techniques were used to investigate the effect of hyaluronic acid gel on the subgingival microbiome.

Results: 108 samples of 54 patients were analyzed at baseline and after follow-up at 45 days. Three strata with different microbial composition were obtained in the samples at baseline, representing three main microbial consortia associated with peri-implantitis. Stratum 1 did not show any difference for any variable after treatment with hyaluronic acid, whereas in stratum 2, *Streptococcus*, *Veillonella*, *Rothia* and *Granulicatella* did decrease ($p<0.05$). Similarly, *Prevotella* and *Campylobacter* ($p< 0.05$) decreased in stratum 3 after treatment with hyaluronic acid. Microbial diversity was found to be decreased in stratum 3 ($p<0.05$) after treatment with hyaluronic acid compared to the control group, in which an increase was found ($p<0.05$).

Conclusions: Hyaluronic acid reduced the relative abundance of peri-implantitis-related microorganisms, especially the early colonizing bacteria, suggesting a specific action during the first stages in the development of the disease. Hyaluronic acid did not alter relative abundances of non-oral genera. The use of hyaluronic acid in advanced stages of peri-implantitis resulted in a decrease in microbial alpha diversity, suggesting a protective action of the peri-implant site against bacteria colonization.

Keywords: Dental Implants; Peri-Implantitis; Hyaluronic Acid; Microbiota; Clinical Trial.

Introduction

Peri-implantitis is a pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant connective tissue and progressive loss of supporting bone. Peri-implantitis sites exhibit clinical signs of inflammation and increased probing depth, as well as radiographic bone loss, compared to baseline measurements [1]. Peri-implantitis microbial composition is characterized by aggressive and resistant species, and it is clearly distinct from periodontitis-related microbiome [2]. Although common periodontopathogenic bacterial species have been determined in both peri-implantitis and healthy sites [3], peri-implantitis has also been linked with opportunistic pathogens and a commensal-depleted microbiome [4]. This suggests that peri-implantitis may be mediated by a more heterogeneous biofilm compared to periodontitis and also requires less plaque accumulation than teeth [5]. It has been also shown how the peri-implant microbiome can be modulated and shifted into a more pathogenic one by external factors such as smoking or the titanium composition of the implant [6, 7].

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan, one of the most abundant in the extracellular matrix of periodontal tissues [8]. Its capacity to absorb water and increase its dry weight >50-fold gives the extracellular matrix a high degree of elasticity and tissue lubrication, favoring gas and molecule exchange and acting as barrier against macromolecules, viruses, and bacteria[9, 10]. Low-molecular-weight fragments play a role in signaling tissue damage and mobilizing immune cells, while high-molecular-weight (HMW) HA suppresses the immune response, preventing excessive inflammation [11].The topical treatment of non-keratinized sulcular epithelium was found to deliver high concentrations of pharmacological agents to periodontal tissue, gingiva, periodontal ligament, alveolar bone, and cementum [12]. To our knowledge, only one pilot study has evaluated the application of HA as a nebulizing spray. The study was performed only in 5 patients and after 15 days of follow-up, without considering criteria for the diagnosis of peri-implantitis and radiologic assessments [13].

However, submucosal microbiota of peri-implantitis lesions has not been extensively studied using culture-independent techniques, and the role of the peri-implant microbiome is not

completely known [1]. The identification of microbial clustering and colonization patterns could lead to the discovery of novel pathways disease progression [14]. The aim of our study was to evaluate the effects of an HMW-HA gel at 45 days on the microbiome of implants with peri-implantitis with at least one year of loading.

Material and Methods

Study design and patient recruitment

A double-blinded, controlled, randomized clinical trial was designed with three parallel groups, including 104 implants placed in 63 patients and diagnosed with peri-implantitis in a private oral surgery clinic in Granada, Spain. Diagnosis of peri-implantitis were according to the criteria of the Association of Dental Implantology (probing depth [PD] ≥ 4 mm, bleeding on probing, and radiological marginal bone loss >2 mm compared to baseline radiographs) [15]. All procedures performed in this study were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments. All patients who met inclusion criteria were asked to sign informed consent, and the study was approved by the Ethics Committee in Human Research of the University of Granada (Reference 589, 28/04/2011). The study protocol was registered at clinicaltrials.gov (NCT03157193; May 17th 2017). The study was designed in accordance with CONSORT guidelines [16].

Inclusion and exclusion criteria

Study inclusion criteria were: age >18 years, periodontally healthy or receiving periodontal supportive therapy and diagnosis of peri-implantitis in implants with at least 1 year of loading. Only single crowns and pillars of fixed partial dentures were included. Exclusion criteria were: consumption of antibiotics, either topical or systemic, rinses or anti-inflammatories in the previous four weeks, pregnancy or breastfeeding, the presence of cancer, and previous treatment for peri-implantitis. All implants were the same model [#], with conical design, 2.6 mm machined

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[#] Tapered Swiss Plus® (Zimmer Dental, Barcelona, Spain).

neck, inner connection, and microtextured surface, which were placed at bone crest level by the same surgeon following a one-stage surgery protocol (E.S.F.).

Experimental design and group allocation.

Patients were consecutively enrolled and randomly assigned to one of the following three groups by using a computer-assisted block randomization method until the sample size was reached. Sequentially numbered containers with the study products were provided by the manufacturer [**]. Container random allocation was performed using a randomization list balanced for the 3 groups created with a statistical software [††]. One examiner gathered all clinical data and samples (E.S.F.) and a different examiner managed random group allocation and gel applications, delivery and instructions to patients (A.M.F) in order to ensure double-blinding. Patients from test and control 1 groups were blinded in this process, but not the patient in the group control 2, since they did not receive any treatment. All patients received standard peri-implantitis treatment at the end of the study period.

Test group: These patients received, in the dental office, a single application by syringe of 0.8 % HMW-HA gel [‡‡] in the peri-implant pocket and around the implant, followed by application of the same gel (but at 0.2 %) by the patient at home, massaging the gingiva around the affected implant(s) three times/day for 45 days, always after tooth-brushing, followed by a 20-min period without eating or drinking.

Control group 1. The patients applied an exopolysaccharide gel [§§] three times/day for 45 days only at home, without the application in the dental office. This product is a viscous and insipid gel similar to the one used in the test group as excipient for the HMW-AH.

Control group 2. These patients received no topical application of any compound, either in the dental office or at home.

Sample collection and DNA extraction.

Subgingival plaque samples were obtained at baseline (t0) and after 45 days of treatment (t45) by Mombelli's method [17]. Two No. 30 paper points were obtained for each peri-implantitis site showing inflammatory signs, including gingival redness and swelling and immediately frozen at -80ºC until analysis. DNA isolation was as previously described [18], with some modifications. Briefly, swabs containing the sample were dissolved in 100 µL of lysis buffer (3% w/v sodium dodecyl sulphate in 50mM tris, 5mM EDTA, pH 8.0, 10µg/ml RNase A) at 68ºC for one hour, being the mixture recovered and transferred to a sterile bead beating tube, following from this point the subsequent procedure. Negative controls were included in all extraction batches to ensure the absence of contaminants. DNA quality and amount were determined using a spectrophotometer ‖.

High-throughput sequencing and bioinformatics analysis.

PCR amplification products of the V1-V3 variable regions of the 16S rRNA gene were obtained using fusion universal primers 27F (Illumina adaptors + 5'AGAGTTTGATCMTGGCTCAG3') and 533R (Illumina adaptors + 5'TTACCGCGGCKGCTGGCACG3'). Similarly, negative controls were included in each PCR batch to avoid the presence of contaminants. Amplicon multiplexing and sequencing was carried out with a dual indexing tag-tailed design using 8nt indices from the technique kit ¶¶. Paired-end sequencing of 16S PCR amplicon libraries was performed using the Illumina MiSeq instrument with v3 kit chemistry (300 + 300). Bioinformatics analysis and quality filtering were carried out using a specific software ## [19]. Chimeric reads were identified and excluded using Chimera UCHIME. Diversity was examined by operational taxonomic units (OTUs) at 3% dissimilarity and the distance-based greedy clustering algorithm (dgc), calculating the coverage, number of observed OTU, richness index Chao1, specific-diversity indexes (InvSimpson, Shannon) and evenness index Pielou.

‖ NanoDrop 2000 UV–Vis (ThermoFisher Scientific, Waltham, MA, USA).
¶¶ Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA).
Mothur v1.39.5 (University of Michigan Medical School, Ann Arbor, MI, USA).

Redundant, non-chimera FASTA files were taxonomically classified using RDP Bayesian classifier (trainset 14)[20]. Abundance was expressed as a percentage with respect to the total number of sequences in each sample. Genera with total abundance higher than 0.1% were considered for statistical analysis.

Statistical analysis

After checking the absence of normality in the variables with the Shapiro-Wilk test, statistical differences between t0 and t45 were assessed using non-parametric Wilcoxon test for paired samples implemented in a specific statistical package ***. Samples were stratified according to Euclidean distances-based cluster analysis using a different statistical package †††. Principal component analysis on pre-treatment samples was also implemented with the same software package. A *p*-value of 0.05 was considered as threshold for statistical significance. GPower 3.1 software (Universitat Kiel, Germany) was used to estimate the statistical power achieved with the sample. The effect of HMW-HA gel on the subgingival microbiome was estimated with an average statistical power of 0.8568 according to the detected effect size for each variable.

Results

A total of 104 implants placed on 63 patients were included in this study. No DNA amplification was obtained from 9 patients either at baseline, after follow-up or both. A total of 2 patients were lost to the follow-up (One patient from control 1 group, who forgot to use the placebo gel, and one patient from control group 2, who discontinued the study voluntarily without performing follow-up analyses).

A total 108 samples, one at baseline and one after 45 days, were analyzed in 54 patients. 38 samples were included in test group (19 patients), 34 in control group 1 (17 patients), and 36 in control group 2 (18 patients). A sociodemographic, clinical and implant-related variables

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*** SPSS v.20.0 (SPSS Inc., Chicago, IL, USA).
††† Statgraphics Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA).

descriptive are shown in Table 1. Analysis of the effect of HMW-HA on inflammation and clinical variables has been performed in a different study (in press) by our group.

Bacteria associated with peri-implantitis

The High-throughput sequencing of 16S rRNA gene amplicons resulted in a total number of 3.106.815 merged paired-end raw sequences. After quality-filtering, a final number of 2.150.443 sequences with an average length of 528 bp were obtained.

Considering pre-treatment and post-treatment samples, taxonomic analysis found a total of 27 phyla and 604 genera, 53 of which showed a relative abundance higher than 0.1%, being considered for subsequent analysis. The most abundant genera were *Fusobacterium, Prevotella, Porphyromonas, Ralstonia, Sphingomonas, Streptococcus, Treponema, Propionibacterium, Alloprevotella, Saccharibacteria_genera_incertae_sedis*, *Neisseria, Veillonella, Lactobacillus, Haemophilus, Staphylococcus, Campylobacter* and *Tannerella,* among others (Fig. 1). Euclidean distances-based clustering at genus level was performed on pre-treatment samples to identify stratifications in the population related to peri-implantitis. As a result, three strata with different microbial composition were obtained in the samples, representing three main microbial consortia associated with peri-implantitis in our study (Fig. 2). Stratum 1 was characterized by the presence of *Ralstonia* and *Sphingomonas* genera, accounting for approximately 80% of relative abundance in the samples of this strata, while *Streptococcus*, *Neisseria*, *Veillonella* and *Rothia* were especially abundant in stratum 2. Lastly, *Fusobacterium*, *Prevotella*, *Porphyromonas, Treponema, Campylobacter* and *Tannerella* were specifically enriched in stratum 3.

Principal component analysis was also performed on pre-treatment samples using the variables (genera) previously described and accounted for 43.4% of the bacterial variation considering the first two principal components (Fig. 3). Proximity within lines (variables) indicates correlation within those genera and association with the respective samples (dots). There is a clear distinction of the three defined strata, confirming the three main consortia involved in peri-implantitis. Samples were separated along the X axis, differentiating stratum 1 and 2 (on the

left) from stratum 3 (on the right), as well as along the Y axis, differentiating stratum 2 from the other two. The increase in the genera associated to stratum 3 (on the right in Fig 3) leads to a decrease in those associated to stratum 1and 2 (on the left in Fig 3) and vice versa. Similarly, stratum 2 is inversely correlated with the other two as they show opposite directions in the plot. Most of the samples were distinctly associated to a specific stratum except for some samples situated close to the coordinates origin without a clearly defined microbial composition.

Microbial diversity

Within-community alpha diversity was assessed both before (t0) and after 45 days of treatment (t45) in all experimental groups. To minimize sample size-induced bias between the datasets, we rarefied all samples by sub-sampling at 1975 sequences. The current surveying effort covered between 95% and 97% of the within-community alpha diversity in all samples (Table 2, Figure S1). Considering alpha-diversity indexes before treatment (t0), the lowest microbial diversity was found in stratum 1 as it showed the lowest values in InvSimpson, Shannon and Pielou indexes. However, stratum 2 and 3 were the most diverse strata since they showed similarly high values for Shannon and Pielou indexes. Stratum 2 showed the highest values for the number of detected OTU, Chao1 and InvSimpson indexes. Minor differences appeared in alpha-diversity as a consequence of treatment, except for stratum 3, in which a decrease in microbial diversity was noticed with lowering values in InvSimpson, Shannon, and Pielou indexes ($p<0.05$) after treatment with HMW-HA (Test group). Additionally, in the same stratum, an increase in bacterial diversity was shown in control group 2 as indicated by the rising value in InvSimpson index ($p<0.05$) and Shannon and Pielou indexes ($p<0.1$).

Antimicrobial effect of HMW-HA

Statistical differences between pre-treatment and post-treatment samples for each variable in all strata and groups were assessed with non-parametric Wilcoxon test for paired samples. Stratum 1 did not show any difference for any variable after treatment with HMW-HA, whereas in stratum 2, *Streptococcus* ($p<0.05$), *Veillonella* ($p<0.05$), *Rothia* ($p<0.05$) and *Granulicatella*

(p<0.05) did decrease (Table 3). Similarly, *Prevotella* (p< 0.05) and *Campylobacter* (p< 0.05) decreased in stratum 3 after treatment with HMW-HA (Table 3, Figure S2). Neither of these microorganisms varied in control 1 and control 2 groups in stratum 2 and 3, suggesting that these differences were due to the use of HMW-HA. The effect of the excipient and the non-treatment effect were mostly appreciated in stratum 3, which showed a significant increase in *Propionibacterium* (p<0.05), *Neisseria* (p<0.05), *Rothia* (p<0.05), *Pseudomonas* (p<0.05) and *Mycoplasma* (p<0.05), and a decrease in *Anaeroglobulus* (p<0.05) in the group administered with the excipient (control group 1). Additionally, a decrease in *Porphyromonas* (p<0.05) and an increase in *Atopobium* (p<0.05) and *Anaeroglobulus* (p<0.05) were found in the non-treated group (control group 2). No significant differences were recorded in excipient-treated and non-treated groups in stratum 1 and 2.

Discussion

In the present study, we have evaluated the microbiome present in the implant site of patients suffering peri-implantitis and its evolution following the administration of three different treatments: HMW-HA (grupo test), gel excipient (control 1) and absence of treatment (control 2). We found three groups of patients with different microbiome in the peri-implant site, defining three strata in the population. We were able to identify the main taxa involved in the development of peri-implantitis as well as to determine the antibacterial effect of HMW-HA on patients suffering the disorder.

We found 12 genera present in diseased peri-implant sites and differently distributed in three consortia, all of them inversely correlated with the others (Fig 3). Most of the samples were associated to a specific stratum characterized by these consortia (Fig 3), suggesting that the described genera do not usually coexist. The majority of the samples were associated to stratum 3, characterized by the presence of *Fusobacterium*, *Prevotella*, *Porphyromonas, Treponema*, *Campylobacter* and *Tannerella* (Fig 2). Stratum 1 is characterized by the presence of *Ralstonia* and *Sphingomonas* (Fig 3), showing the lowest alpha-diversity before treatment (Table 2). Both

taxa are gram-negative genera commonly described as environmental bacteria and found in water supplies and surfaces, which suggests an exogenous cause of infection in our study. They are considered opportunistic pathogens and causative agents of nosocomial infections, including bacteremia, bacteriuria and respiratory disorders in the case of *Ralstonia* [21] and bacteremia, septic arthritis and osteomyelitis in the case of *Sphingomonas* [22]. *Ralstonia* has also been shown to colonize the upper gastrointestinal tract of HIV-positive patients in comparison to HIV-negative subjects, demonstrating a negative correlation with CD4+ T cell count [23]. *Sphingomonas* has been isolated from patients suffering periodontitis, being the most common non-oral gram-negative facultative rod involved in the disease [24]. Thus, this is the first study to date which suggests the presence of these non-oral bacteria as newly proposed peri-implantitis-associated taxa. Other previous studies have shown the possible role of other opportunistic species such as enteric rods and *Staphylococcus aureus* in peri-implantitis [25]. The rest of the genera found in the analysis have classically been described as members of the oral microbiome. Stratum 2 was enriched in genera belonging to purple and yellow subgingival microbial complexes (Fig 3) [26]. Both of them represent the early colonizers, gram-positive facultative bacteria that initially adhere to teeth surfaces forming a plaque biofilm, such as *Streptotoccus*, *Veillonella* [27], *Neisseria* and *Rothia* [28].These "early colonizers" provide the basis for the subsequent colonization of other obligate anaerobes or periodontopathogenic bacteria. Stratum 3 was characterized by orange and red complexes-associated genera (Fig 3), also known as "middle" and "late colonizers" respectively, which show a gram-negative, strict anaerobic profile as well as a strong relationship with periodontal diseases [29] but also have been identified in peri-implantitis [30, 31]. Both *Fusobacterium* and *Campylobacter* genera, belonging to the orange complex, harbour species associated with periodontal inflammation, thus making implants prone to infection by late colonizers [4]. Stratum 2 and 3 were the most diverse strata in our analysis before treatment (t0), since they included a higher number of associated genera (Table 2). The fraction of samples situated close to the coordinate origin show an intermediate microbial composition between early, middle and late colonizing bacteria, perhaps indicating a gradual transition from early to advanced stages in the development of peri-implantitis.

Thus, the obtained results in our study confirm that infection associated with peri-implantitis derives either from environmental bacterial, or from common oral bacteria that colonize the implant site.

The antimicrobial effect of HMW-HA was assessed comparing pre-treatment and post-treatment samples for each variable (genus) in the respective stratum. The greatest effect was found in the early colonizers consortium (stratum 2), since three out of four associated microorganisms (*Streptococcus, Veillonella* and *Rothia*) decreased after treatment with HMW-HA (Test group) compared to the use of gel excipient (control group 1) and the absence of treatment (control group 2) (Table 3). A mild effect was obtained in the middle colonizers (stratum 3), affecting only two genera (*Prevotella* and *Campylobacter*), whereas stratum 1 containing environmental bacteria showed the lowest effect, as no differences were found after treatment with HMW-HA (Table 3). These findings suggest that HMW-HA is especially effective during the first stages in the development of peri-implantitis, since it decreases early colonizing bacteria in the biofilm, and reduces their action as "bridge species", thus impairing the following colonization of orange and red complexes-associated pathogens. *Prevotella* and *Campylobacter* were also depleted in stratum 3 after treatment (Table 3), indicating that HMW-HA might affect some middle colonizing pathogens belonging to the orange complex. Additionally, in this same stratum 3, a general proliferation of microorganisms was noticed as a consequence of the administration of the excipient (control group 1), or the absence of treatment (control group 2), with the exception of *Anaeroglobublus* and *Porphyromonas*, which decreased in control group 1 and control group 2 respectively (Table 3). Lastly, stratum 1 containing environmental bacteria did not show any difference in any experimental group (Table 3), suggesting that infections associated with non-oral bacteria might be resistant to treatment with HMW-HA.

Microbial alpha-diversity was also affected by treatment with HMW-HA in our study. Differences between pre-treatment and post-treatment samples for total number of OTUs, Chao1, InvSimpson, Shannon and Pielou indexes were assessed in each stratum and treatment

group, finding only in stratum 3 a decrease in specific diversity indexes (InvSimpson and Shannon) and in evenness Pielou index after treatment with HMW-HA (Table 2). Consequently, an increase in InvSimpson index ($p<0.05$) and Shannon and Pielou indexes ($p<0.1$) were also noticed in the same stratum 3 as a result of the absence of treatment (control group 2) (Table 1). As described by Sanz-Martín et al. [4], diseased peri-implant sites presented a higher alpha diversity compared to healthy implants, which is in accordance with previous findings reviewed by Pokrowiecki et al. [29]. As dental plaque maturates, oral microbial communities become more complex and alpha diversity increases. However, local factors such as the implant surface topography have not shown any effects in terms of bacterial diversity in early colonization stage in previous studies [32]. HMW-HA might only influence stratum 3 in terms of diversity due to its advanced stage of development (higher diversity), contributing to implant health through the protection of the peri-implant site against microorganism colonization. Similarly, the absence of treatment in control group 2 led to an increase in alpha diversity that paralleled the enrichment in oral bacteria, possibly due to the progression of the infection in stratum 3. Therefore, the obtained results suggest that hyaluronic acid could be used during the first stages of peri-implantitis, even as a preventive measure, although further analysis need to be carried out to investigate these findings in detail.

Although several classifications of peri-implant lesions have been previously published [33, 34], the objective of our study was to assess the effect of HMW-HA in the peri-implant microbiome, regardless of peri-implantitis severity. Further studies should aim to determine if peri-implantitis severity is associated with different microbiome profiles, and if there are differences in effect of therapy among peri-implantitis lesions of different severity according to these classifications.

HMW-HA reduced the relative abundance of peri-implantitis-related microorganisms, especially the early colonizing bacteria (*Streptococcus, Veillonella* and *Rothia*), suggesting a specific action during the first stages in the development of the disease. It also showed a mild action in middle colonizers (*Prevotella* and *Campylobacter).* However, it is ineffective once late colonizers have been established in the peri-implant site. Lastly, HMW-HA did not alter relative

abundances of non-oral genera *Ralstonia* and *Sphingomonas*. The use of HMW-HA in advanced stages of peri-implantitis resulted in a decrease in microbial alpha diversity suggesting a protective action of the peri-implant site against bacteria colonization.

Author contributions

A. Soriano-Lerma contributed to data acquisition, analysis, interpretation, drafted and critically revised the manuscript. A. Magán-Fernández contributed to design, data acquisition, analysis, interpretation, drafted and critically revised the manuscript. J. Gijón and E. Sánchez-Fernández, contributed to data acquisition and critically revised the manuscript. M. Soriano and J. A. Garcia-Salcedo contributed to data analysis drafted and critically revised the manuscript. F. Mesa contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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FIGURE LEGENDS

Figure 1. Genera distribution (percentage of relative abundance) considering all samples (pre and post-treatment).

Figure 2. Relative percentage of bacteria at genus level in three defined strata considering only the most abundant and differently distributed taxa in pre-treatment samples.

Figure 3. Principal component (PC) analysis. Biplots for the relative abundance of bacterial genera on pre-treatment samples considering taxa described in Fig 2. Samples are represented by dots and taxa are represented by lines. Red lines represent the coordinates axis.

Supplementary figure 1. Relative abundance (percentage) of bacteria at genus level before (t0) and after treatment (t45). (a) Antimicrobial effect of high-molecular-weight hyaluronic acid (HMW-HA) (Test group). (b) Effect of excipient (Control group 1). (c) Non-treatment effect (Control group 2).

Supplementary figure 2. Statistical differences between pre (t0) and post-treatment samples (t45) in microbial alpha diversity in each strata and group. (a) Richness index Chao1, (b) Specific-diversity index InvSimpson, (c) Specific-diversity index Shannon, (d) Evenness index Pielou. * $p<0.05$ **$p<0.1$.

TABLES

Table 1. Baseline description of patients (n=63) and implants (n=104).

Variable	Test	Control 1	Control 2
Patient's variables, n	21	21	21
Female, n (%)	14 (66.7)	12 (57.1)	13 (61.9)
Age, range (yrs.)	43-81	54-79	29-78
Age, mean±sd	60±9	64±6	58±12
# Implants, mean±sd	1.52±0.60	1.62±0.50	1.81±0.60
# Smokers, n (% >=10cig. /day)	1 (4.8)	3 (14.3)	3 (14.3)
# Cig. /day, mean±sd	0.5±2.2	4.5±11.5	3.1±6.7
# Brush/day, mean±sd	2.6±0.5	2.3±0.6	2.3±0.8
Diabetes, n (%)	1 (4.8)	2 (9.5)	2 (9.5)
Implant general variables, n	32	34	38
Months since loading, mean±sd	89±51	104±45	74±41
Unitary, n (%)	11 (34.4)	7 (20.6)	7 (18.4)
Prosthesis pillar, n (%)	21 (65.6)	27 (79.4)	31 (81.6)
Diameter 3.7 mm., n (%)	22 (68.8)	17 (50.0)	23 (60.5)
Diameter 4.1 mm., n (%)	9 (28.1)	16 (47.1)	9 (23.7)
Diameter 4.8 mm.), n (%)	1 (3.1)	1 (2.9)	6 (15.8)
Length 8 mm., n (%)	2 (6.3)	0 (0.0)	0 (0.0)
Length 10 mm., n (%)	16 (50.0)	14 (41.2)	20 (52.6)
Length 11 mm., n (%)	1 (3.1)	0 (0.0)	0 (0.0)
Length 12 mm., n (%)	13 (40.6)	18 (52.9)	15 (39.5)
Length 14 mm., n (%)	0 (0.0)	2 (5.9)	3 (7.9)
Upper Jaw, n (%)	18 (56.3)	28 (82.4)	32 (84.2)
Implant clinical variables			
BOP, n (%)	32 (100)	34 (100)	38 (100)
PD (mm.), mean±sd	4.81±0.96	5.47±1.26	4.84±0.89
CA loss (mm.), mean±sd	5.28±1.40	5.71±1.36	4.92±0.94
MBL (mm.), mean±sd	3.77±2.33	3.92±2.05	3.63±0.95

BOP: Bleeding on Probing, PD: Probing Depth, CA loss: Clinical Attachment Loss, MBL: Marginal Bone Level.

Table 2. Diversity indexes of 16S sequences from all experimental groups and strata, before (t0) and after treatment (t45).

		Reads		Coverage (%)		Otu		Chao1		InvSimpson		Shannon		Pielou	
		t0	t45	t0	t45	t0	t45	t0	t45	t0	t45	t0	t45	t0	t45
Stratum 1 (n=12)	Test (n=4)	21544.5	6584.5	97	95	94.77	168.61	237.77	426.51	2.17	5.43	1.23	2.66	0.27	0.51
		38091	3761.75	1	4	55.73	47.73	190.37	453.15	4.72	1.49	1.74	0.32	0.35	0.09
	Control 1 (n=4)	21752	17046.5	96	97	123.13	102.10	297.75	336.07	2.04	3.44	1.51	2.06	0.31	0.45
		26273.5	23034.75	1	4	73.78	118.04	113.09	252.25	4.72	3.58	1.70	1.64	0.31	0.27
	Control 2 (n=4)	8939	10376	97	96	103.92	133.88	263.75	287.70	3.73	3.46	2.35	2.29	0.49	0.47
		13932.25	10606.5	1	2	50.23	49.01	95.90	215.85	3.05	5.25	0.84	0.88	0.18	0.14
Stratum 2 (n=14)	Test (n=6)	19997	8996.5	96	97	131.33	111.34	339.93	242.69	8.22	5.77	2.78	2.54	0.59	0.54
		23622.25	9770.25	2	1	39.27	47.54	152.97	73.64	9.34	6.85	0.79	0.79	0.13	0.13
	Control 1 (n=4)	8100	10922.5	96	95	143.16	182.16	358.20	408.95	7.93	8.25	2.87	3.09	0.59	0.61
		13066.75	22644.75	4	2	101.50	51.04	366.47	69.22	8.84	15.50	1.12	1.00	0.17	0.19
	Control 2 (n=4)	24509	17354.5	95	97	166.94	96.05	427.50	298.87	15.35	2.35	3.37	1.56	0.66	0.36
		39667.5	21540.5	4	3	147.24	96.34	329.47	263.20	20.80	5.72	2.29	2.01	0.39	0.36
Stratum 3 (n=28)	Test (n=9)	17142	16435	96	96	153.79	141.29	356.78	351.01	10.16*	4.99*	3.28*	2.51*	0.64*	0.52*
		21779.5	21359	2	3	51.75	113.79	145.45	179.78	7.15	3.70	0.56	1.13	0.07	0.16
	Control 1 (n=9)	14390	14320	97	96	115.30	142.59	287.53	313.48	6.42	6.20	2.75	2.87	0.58	0.58
		24140	13309.5	1	1	51.86	39.85	133.30	68.44	4.47	3.42	0.99	0.79	0.15	0.13
	Control 2 (n=10)	32652.5	21852	97	97	110.07	127.27	275.20	303.35	4.18*	5.90*	2.29**	2.51**	0.48**	0.53**
		44808.25	24719	2	2	49.79	67.15	168.46	81.12	2.49	6.41	0.88	1.10	0.15	0.18

All values expressed as median and interquartile range (IQR). *Differences between pre-treatment and post-treatment samples (p<0.05). ** Differences between pre-treatment and post-treatment samples (p<0.1). Statistical differences were assessed by non-parametric Wilcoxon test for paired samples.

Table 3. Percentage of relative abundances for bacterial genera associated with peri-implantitis and those varying between pre (t0) and post-treatment (t45) samples in all experimental group and strata.
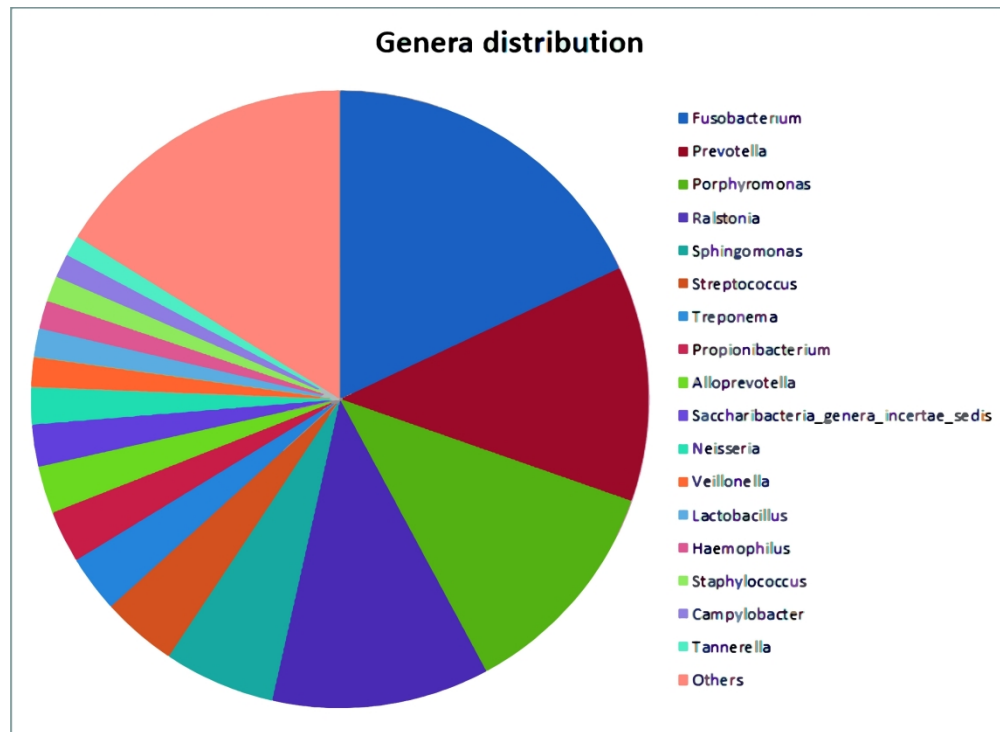
		Stratum 1 (n=12)						Stratum 2 (n=14)						Stratum 3 (n=28)					
		Test (n=4)		Control 1 (n=4)		Control 2 (n=4)		Test (n=6)		Control 1 (n=4)		Control 2 (n=4)		Test (n=9)		Control 1 (n=9)		Control 2 (n=10)	
		t0	t45	t0	t45	t0	t45	t0	t45	t0	t45	t0	t45	t0	t45	t0	t45	t0	t45
Fusobacterium	Median	0.2	4.7	1.6	26.1	1.2	5.9	5.4	21.5	15.2	14.0	4.6	5.2	20.6	8.7	31.5	18.6	20.9	20.0
	IQR	28.7	37.8	4.9	58.1	2.0	25.0	20.7	38.3	16.1	25.2	12.0	20.9	30.0	35.2	21.6	37.5	36.9	17.6
Prevotella	Median	0.8	1.3	0.6	5.0	3.8	2.5	7.1	6.3	9.3	9.8	11.9	7.0	14.3*	5.7*	9.5	19.8	14.6	17.5
	IQR	21.4	9.0	2.5	6.0	4.5	5.3	13.3	15.5	7.0	6.5	9.4	38.7	12.8	11.1	19.7	20.4	21.7	23.1
Porphyromonas	Median	0.1	0.9	0.6	0.1	1.3	2.2	2.5	1.8	2.1	2.8	21.7	8.1	12.4	1.6	5.0	3.0	38.1*	14.8*
	IQR	0.5	10.3	0.9	1.2	4.0	3.0	7.0	18.2	8.1	7.9	61.1	42.9	31.0	32.7	26.0	20.2	42.9	28.7
Ralstonia	Median	62.9	11.5	71.2	19.8	29.5	27.8	1.2	0.6	2.9	3.6	1.0	1.9	0.2	0.2	0.1	0.1	1.0	0.3
	IQR	68.7	36.5	49.5	75.8	46.2	55.5	25.4	29.1	15.0	21.6	2.4	58.3	1.6	28.6	0.2	1.0	2.5	5.5
Sphingomonas	Median	5.7	13.3	11.8	1.9	34.9	5.1	0.8	4.0	1.7	1.5	0.3	0.3	0.2	0.2	0.1	0.6	0.2	0.1
	IQR	31.1	29.8	9.1	26.8	32.7	10.3	6.4	23.1	1.5	43.9	1.2	4.3	8.7	9.0	0.8	1.5	0.2	1.6
Streptococcus	Median	0.4	1.5	1.0	0.3	0.6	4.5	26.5*	1.3*	6.2	5.0	6.3	1.4	0.9	0.6	0.4	0.4	0.1	0.5
	IQR	1.3	0.3	1.0	3.7	1.0	11.7	19.4	1.4	17.0	11.0	26.9	3.3	10.0	1.9	2.2	3.4	0.4	2.0
Treponema	Median	0.0	0.1	0.2	2.3	0.8	0.7	0.4	2.3	0.9	2.0	1.9	1.1	4.2	3.0	4.0	2.8	5.0	3.0
	IQR	0.2	0.8	0.1	4.9	1.0	1.4	1.8	4.1	1.2	2.3	2.7	2.9	5.0	4.5	4.1	4.9	7.7	4.3
Propionibacterium	Median	2.1	4.0	3.3	1.1	2.3	2.4	0.5	2.8	1.2	2.0	0.1	0.1	0.1	0.1	0.0*	0.3*	0.1	0.1
	IQR	1.8	10.9	5.6	2.6	1.9	4.7	1.2	8.7	3.5	3.1	0.3	0.8	4.1	3.2	0.2	0.9	0.1	0.4
Neisseria	Median	0.0	0.1	0.2	0.0	0.4	0.2	3.8	1.0	0.3	0.7	0.4	0.1	1.0	0.1	0.0*	0.1*	0.1	0.1
	IQR	0.2	0.4	18.7	1.1	1.2	0.3	18.3	3.4	16.5	1.6	10.1	1.7	1.9	1.3	0.3	1.2	0.3	0.2
Veillonella	Median	0.0	0.9	0.2	0.0	0.3	0.8	4.4*	0.1*	1.8	2.6	1.4	0.3	0.4	0.2	0.2	0.2	0.0	0.2
	IQR	0.3	1.3	0.3	0.0	7.3	2.4	9.9	0.4	29.8	5.0	9.4	1.9	1.4	1.1	0.4	1.0	0.0	0.3
Campylobacter	Median	0.0	0.4	0.1	4.1	0.5	0.4	0.3	0.9	1.1	1.1	0.7	0.1	1.1*	0.7*	0.2	0.8	0.8	0.6

	IQR	1.0	1.5	0.3	14.3	1.7	1.2	1.0	2.0	2.0	1.2	1.8	0.9	2.3	1.3	1.2	1.3	1.9	0.9
Tannerella	Median	0.0	0.4	0.1	0.0	0.1	0.3	0.0	0.4	1.1	0.3	0.2	0.1	0.3	0.6	0.7	0.3	1.9	1.5
	IQR	0.0	0.5	0.1	1.1	1.6	0.3	0.3	3.2	3.0	0.6	1.2	0.7	1.4	2.1	1.5	1.1	2.6	3.0
Rothia	Median	0.0	0.0	0.1	0.1	0.0	2.2	1.7*	0.1*	0.3	0.3	0.0	0.0	0.1	0.0	0.0*	0.1*	0.0	0.0
	IQR	0.2	0.1	0.2	0.6	0.1	8.6	3.7	1.6	1.5	0.4	0.4	0.0	0.2	0.1	0.1	0.4	0.0	0.0
Pseudomonas	Median	0.1	0.6	0.1	0.1	0.8	0.5	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.01*	0.04*	0.0	0.0
	IQR	0.6	3.4	0.2	1.0	1.9	0.9	1.0	0.3	0.1	0.3	0.0	0.1	0.5	0.2	0.1	0.2	0.0	0.2
Mycoplasma	Median	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0*	0.2*	0.2	0.3
	IQR	0.0	0.0	0.0	0.0	0.3	0.5	0.2	0.6	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.7	1.0	0.4
Atopobium	Median	0.0	0.0	0.0	0.0	0.0	0.5	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0*	0.02*
	IQR	0.1	0.1	0.0	0.0	0.2	0.8	0.3	0.1	2.8	4.2	0.2	0.1	0.2	0.1	0.3	0.1	0.0	0.4
Anaeroglobulus	Median	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1*	0.03*	0.0*	0.02*
	IQR	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.5	0.0	2.5	0.3	0.3	0.6	2.8	0.2	0.0	0.4
Granulicatella	Median	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	IQR	0.1	0.0	0.1	0.0	0.0	0.1	1.6*	0.1*	0.2	0.1	0.4	0.2	0.0	0.0	0.0	0.1	0.0	0.0

All values expressed as median and interquartile range (IQR). *Differences between pre-treatment and post-treatment samples (p<0.05). Statistical differences were assessed by non-parametric Wilcoxon test for paired samples.

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Figure 1. Genera distribution (percentage of relative abundance) considering all samples (pre and post-treatment).
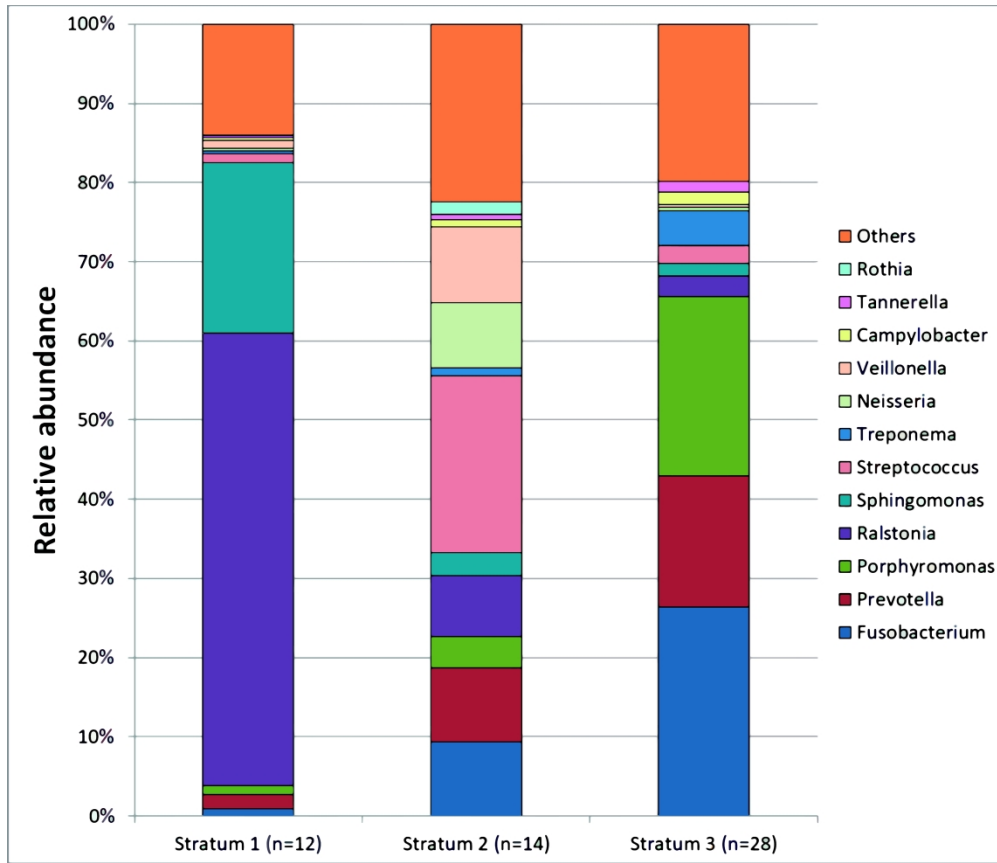
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Figure 2. Relative percentage of bacteria at genus level in three defined strata considering only the most abundant and differently distributed taxa in pre-treatment samples.
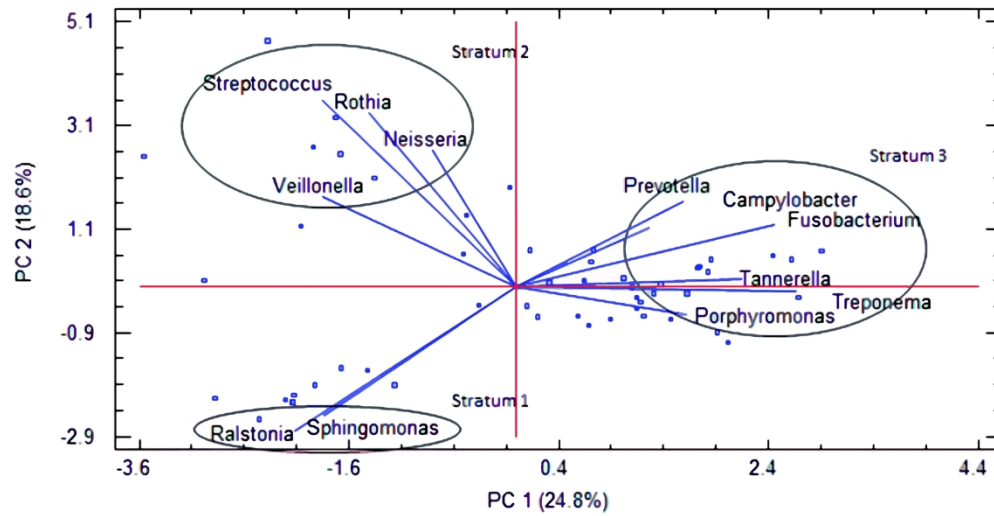
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Figure 3. Principal component (PC) analysis. Biplots for the relative abundance of bacterial genera on pre-treatment samples considering taxa described in Fig 2. Samples are represented by dots and taxa are represented by lines. Red lines represent the coordinates axis.

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