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Evolution in the Peri-Implant Oral Microbiome and Their Relationship to Long-Term Marginal Bone Loss: A Randomized Clinical Study

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ABSTRACT

Objectives: To analyze the clinical, radiographic, and microbiological changes around implants with a multiphosphonate-treated surface, prosthetically loaded with two different protocols after 5 years of functional loading.

Material and Methods: A randomized clinical trial was designed to initiate prosthetic loading over single dental implants after 8 (control) or 4 weeks (test). Several variables were analyzed, including patients' level variables, intrasulcular biofilm, and marginal bone level at several time points, from 1 to 60 months after loading.

Results: A total of 23 patients attended the 5-year follow-up visit. No clinical variable changed over time, except mucosal thickness from dental impressions to prosthesis delivery. No significant radiographic differences were observed either over time or between groups. Microbiologically, there was a change in the microbiome from the constitution of the biological width to the final follow-up. Seven species changed significantly, with a significant increase in *Porphyromonas gingivalis* and *Tannerella forsythia* from 12 to 60 months and a decrease in the other species. However, changes in the relative abundance of species over time, whether increasing or decreasing, did not show a correlation with marginal bone loss.

Conclusion: Implants with a multiphosphonate-treated surface showed no differences in clinical and radiographic variables after 5 years of function, regardless of the prosthetic loading protocol used. From a microbiological point of view, although there was an evolution of the microbiome in the peri-implant sulcus towards Socransky's red circle pathogenic bacteria, no microorganism showed a significant correlation with the radiographic changes produced in the peri-implant bone over time.

Trial Registration: ClinicalTrials.gov identifier: NCT03059108

1 | Introduction

Faster osseointegration of dental implants is one of the main objectives of the implantology industry and science. In this sense, there has been a constant evolution and transition from the introduction of the very first polished metal surfaces to surfaces with different types and degrees of roughness and wettability (Padial-Molina et al. 2011), physically or chemically modified surfaces (Abrahamsson et al. 2023), and biomimetic surfaces (Sevilla et al. 2018; Cirera et al. 2020). More recently, there has been a trend to return to machined surfaces or implants with hybrid surfaces in order to modify microbiome adhesion and behavior after implant surface exposition to the oral media, if peri-implant bone resorption were to happen.

Implant's surface evolution is characterized by a duality of interests: achieving and maintaining osseointegration and, on the other hand, avoiding contamination if exposed to the oral environment.

At this time, no implant surface available in the market has shown a clear superiority over others in terms of osseointegration, although rough surfaces are more effective in promoting osseointegration. There are implant surfaces that, in humans, have been shown to be effective in promoting osseointegration in short healing periods, between 4 and 6 weeks of healing (Galindo-Moreno et al. 2012, 2017; Maiorana et al. 2015). It is very difficult to shorten this time frame because the osseointegration processes and biology take their minimum time for their own biological repair processes. Additionally, there is an interesting scientific debate in the community about the development of peri-implant diseases based on two important events: (1) the exposure of the surface to the oral environment and (2) the contamination of the surface by the microbiome. The general consensus is that peri-implant diseases are plaque-associated inflammatory diseases involving both soft and hard peri-implant tissues (Renvert et al. 2018).

However, although there are numerous studies describing, by different methodologies, the microbiome around implants in cross-sectional designs, there are few studies describing the evolution of the microbiome around dental implants followed in the short term (Siddiqi et al. 2016; Qiao et al. 2020; Jung and Lee 2023) or medium term (Costa et al. 2019; Lu et al. 2021; de Freitas et al. 2021) with the aim of analyzing how the presence of microorganisms around implants evolves over time in relation to the variation of the clinical variables themselves.

In 2021, we published the clinical and radiographic 1-year follow-up results around implants with a modified implant surface based on a permanently bonded multiphosphonate coating. Those implants were loaded by protocols initiated 4 or 8 weeks after implant placement and concluded that there were no significant differences in either bone or soft tissue variables in relation to the implant surface and the prosthetic loading time at which they were rehabilitated (Galindo-Moreno et al. 2021). Similarly, from the beginning of this clinical study, DNA samples were collected from the peri-implant sulcus to analyze the peri-implant microbiota of each implant and correlate it with the marginal bone loss and the exposure of the titanium to this peri-implant microbiota. We hypothesized that those implants whose rough titanium surface could be exposed to microbiological contamination would have a more abundant putative pathogenic microbiota than implants without any marginal bone loss, where the titanium would not be exposed to such contamination. Therefore, this study had two clear objectives. First, to analyze the possible clinical and radiological changes that occurred in these implants after 5 years of functional loading and, second, to determine the evolution of the composition of the peri-implant microbiota in relation to the radiographic marginal bone loss suffered by those implants after 5 years of clinical function.

2 | Material and Methods

2.1 | Ethical Considerations, Study Design, and Location

As described in the publication concerning the first year of follow-up (Galindo-Moreno et al. 2021), this clinical protocol was conducted in accordance with the World Medical Association Declaration of Helsinki, the Guideline for Clinical Investigation of Medical Devices for Humans—Guide to Good Clinical Practice (ISO 14155:2011) and the General Guidelines for Good Clinical Practice (2001/20/EC). Results are reported in accordance with the Consolidated Standards for Reporting Trials (CONSORT) statement (Data S1). Prior to the initiation of activities, the study was evaluated and approved by the Human Research Ethics Committee of the University of Granada (registration code 216/CEIH/2016).

A parallel-group randomized controlled clinical trial was performed to compare different loading protocols after the use of an internal conical prosthetic connection type of implant with a sandblasted and acid-etched surface (SLA type) treated with multiphosphonate (C1-B+ dental implants by MIS Implants Technologies Ltd., Israel). Although the original design of the study planned follow-up for only 1 year after prosthetic loading, it was subsequently considered convenient to perform an additional longer-term follow-up; so a subsequent evaluation was also performed 5 years later.

Patients were recruited for this study at the School of Dentistry of the University of Granada. After providing all the information regarding the study, objectives, procedures, responsibilities, expectations, and rights, the participants were asked to sign an informed consent before proceeding to the selection process. After inclusion in the study, study procedures were conducted at the School of Dentistry, University of Granada, while the samples collected were analyzed at the Biomedical Research Center of the University of Granada. All patients were included between February 2017 and January 2019.

2.2 | Sample Size

Until our first publication, there were no previous studies with similar aims to help establish an adequate sample size. Only one previous study with a longer loading protocol included 23 patients. That study described a trend of change in marginal bone

level after 1 year of function with no significant differences in the comparison with implants with untreated surface (Esposito et al. 2013). We therefore decided to initiate a pilot study with a larger initial sample size, set at a minimum of 30 patients.

2.3 | Study Population

As originally designed (Galindo-Moreno et al. 2021), the study population was selected based on the following criteria:

2.3.1 | Inclusion Criteria

Patients aged between 18 and 75 years with a missing tooth in the premolar or molar area of the maxilla or mandible with antagonistic and adjacent (mesial and distal) natural teeth.

2.3.2 | Exclusion Criteria

Patients with a need for bone grafting in the implant area, patients with any clinical and/or radiographic signs of active periodontal disease or other dental conditions, smokers (> 10 cigarettes/day), patients with diseases that could affect bone metabolism, such as uncontrolled type 1 or 2 diabetes (HgA1c > 8), known autoimmune or inflammatory disease, severe hematologic disorders, local or systemic infection that could compromise normal healing (e.g., extensive periapical pathology), hepatic or renal dysfunction/insufficiency, receiving cancer treatment or who had received cancer treatment within the 18 months prior to implant placement, a history of long-term oral bisphosphonate use (i.e., 10 years or more) or a history of intravenous bisphosphonate usage, a history of prolonged use (> 3 months) of antibiotics or medications known to alter inflammation and/or the immune system, patients with severe bone disease (e.g., Paget's disease of bone), pregnant women or nursing mothers, and patients who were unable or unwilling to follow the instructions related to the study procedures.

2.4 | Blinding

Randomization to study group was conducted after implant placement. Thus, although neither the patient nor the restorative dentist could be masked to the group assignment due to the loading protocol, both the surgical operator and other team members in charge of patient examination and follow-up, as well as the data analyst, were blinded to the group assignment.

2.5 | Randomization

Due to the small sample size, in order to avoid imbalance between groups in terms of the location of the missing tooth, gender, or bone type, randomization was performed by a minimization protocol (Saghaei and Saghaei 2011). A member of the clinic staff who was not involved in the clinical trial was in charge of performing the allocation.

2.6 | Clinical Procedures

The description of the clinical procedures is described elsewhere (Galindo-Moreno et al. 2021). Briefly, the clinical protocol consisted of the insertion of an implant in the designated position and by means of a single-stage protocol in order to minimize the waiting time for loading, especially relevant in the 4-week loading group. Group assignment, as mentioned, was performed after implant placement and tissue repositioning, in order to provide blindness to the surgical operator. Each implant was randomly assigned to one of two groups: Control (conventional loading protocol that started with dental impressions 8 weeks after implant placement) or Test (early loading protocol that started with dental impressions 4 weeks after implant placement). Dental impressions were sent to the prosthetic laboratory for the fabrication of a screw-retained metal-ceramic crown over a Ti-Base abutment (MIS Implant Technologies Ltd.) with 1.5 or 3 mm in height, depending on the thickness of the peri-implant mucosa. The crown was placed 2 weeks later, applying the company's recommended torque of 30 Ncm and making the necessary occlusal adjustments to avoid overloading.

2.7 | Clinical and Radiographical Data

At each visit, clinical, radiographical, and microbiological data were collected. Clinically, at the time of implant placement, the following data were collected (measured in millimeters): (1) Mesiodistal distance between adjacent teeth; (2) Occlusal height; (3) Vestibular-lingual width of the ridge (before and after flap elevation); (4) Width of the keratinized mucosa, which was also recorded at the time of impressions and prosthesis delivery; (5) Vertical thickness of the peri-implant mucosa, which was also recorded at the time of impression. Additionally, healing index (Morelli et al. 2011), up to prosthesis placement, and Papilla Index (Jemt 1997), from prosthesis placement to final follow-up, were also registered. The presence or absence of erythema, edema, and suppuration in the peri-implant mucosa were recorded as indicators of inflammation. However, we did not measure bleeding on probing and probing pocket depth, as our protocol was designed not to remove the clinical crown at follow-up visits, in order to avoid altering the composition of the microbiota. We claim that in implant-supported single crowns, where there is a significant discrepancy between the diameter of the implant and that of the prosthetic crown, as well as differences in the emergence profile of each crown, depending on the surgical position and depth of each implant and the particular anatomy of each tooth to be restored, there is a risk of diagnostic confusion based on these clinical parameters. Therefore, we decided to focus our analysis on the correlation between microbiological and radiological variables, which we believe to be completely reliable and valid for the purpose of our study.

Radiographically, standardized periapical radiographic images of the area were obtained at the time of implant and prosthesis placement, and at 1, 3, 6, 12, and 60 months. Linear measurements of marginal bone and changes over time were measured by the same experienced operator (MP-M) using Image J software (NIH) taking as reference the cemento-enamel junction for

measurement on adjacent teeth, while the implant shoulder was used for the peri-implant marginal bone level measurements. During measurements, all images were internally calibrated with the implant diameter serving as a constant reference point, as this was always visible on the radiograph and not influenced by potential elongation issues. The data recorded at each time was (in mm): (1) Tooth-to-tooth distance; (2) Anterior tooth-to-implant and posterior tooth-to-implant distance; (3) Crown length; (4) Crown-implant ratio, by adding the crown length to the height of the Ti-Base and dividing the sum by the length of the implant; (5) Distance from the amelo-cement junction to the bone crest on the anterior and posterior teeth; (6) Implant marginal bone level (MBL) on the mesial and distal aspects, from which the average value was calculated; (7) Change in the mesial and distal (and average) marginal bone level from implant placement to the time of loading, and from loading to the 1-year and 5-year follow-up.

2.8 | Intrasulcular Biofilm Analysis

2.8.1 | Sample Collection

As indicated in the publication reporting the results of the 1-year follow-up (Galindo-Moreno et al. 2021), samples of intrasulcular plaque were collected at different times for microbiological analysis. We decided to analyze the samples collected after 1 month, 3 months, 12 months, and 5 years of crown placement. We selected those time points in order to allow the constitution of a stable microbiome after the changes that occur in the establishment of the biological width.

Sampling of intrasulcular biofilm was performed conventionally. First, excess saliva was removed with an ejector, and the area was isolated with cotton rolls. Next, after gently removing the excess fluids in the area with air, the biofilm sample was collected by inserting a sterilized paper point into the peri-implant sulcus for 30 s. Immediately afterwards, the paper point was transferred to an Eppendorf tube and frozen at −80°C until processing and analysis.

2.8.2 | DNA Extraction, Library Construction, and Quantification

DNA extraction from the paper points was performed following the instructions of the manufacturer of the DNA extraction kit, DNeasy Blood & Tissue Kit (QIAGEN). For the construction of the DNA library, we used the Illumina DNA PREP library kit (Illumina, San Diego, California, USA). Finally, the libraries were quantified using the Qubit dsDNA in QubitTM3 high sensitivity assay kit (ThermoFisher Scientific). Each sample library was normalized to 2 nM concentrations. The 650 pM library pool was pooled with 1% PhiX control (PhiX Control v3) and sequenced on an Illumina Next/Seq 2000 platform (Illumina) using a 300-cycle P1 flow cell.

2.8.3 | Microbiological Data Analysis

Since there were no significant differences in the parameters of marginal bone loss between the groups in the first year of follow-up, and because the implants used were similar in the two groups, we decided not only to evaluate possible microbiological differences between the two groups, but also to perform a comprehensive analysis of the microbiome across the entire sample of patients at the different times of follow-up. The raw data obtained from the Illumina sequencing were uploaded to the BaseSpace Sequence Hub platform (Illumina) where they are automatically sorted according to FASTQ sequences and subjected to quality control. Compositional/functional profiling and comparative analyses of microbiome data were performed with MicrobiomeAnalyst (Dhariwal et al. 2017; Chong et al. 2020; Lu et al. 2023). Data filtering for low abundance and low variance operational taxonomic units (OTUs) (based on the prevalence in 20% of samples and interquartile range or iqr set at 10%) was applied for all the relative abundance comparisons using different algorithms (metagenomeSeq, EdgeR, DESeq2, LDA, and LEfSe). The bacteria were identified based on Greengenes annotation. The data obtained were used for the generation of individualized Krona taxonomic hierarchy plots. Results were used for Alpha diversity analysis using Shannon diversity, *T* test on filtered data, and Beta Diversity (Principal Coordinate Analysis (PCoA) using Bray-Curtis index) plots converted them to relative abundance for generating plots in Microsoft Excel for MacOS and for comparative statistical analyses between times using Graphpad Prism 7 for MacOS.

2.9 | Statistical Analysis

For each type of variable, percentages, means, standard deviations, and standard error mean (SEM) were calculated, depending on whether the data were categorical or continuous. Categorical data were evaluated with the chi-squared test while, due to the sample size, continuous primary and secondary outcomes were analyzed using the nonparametric Wilcoxon rank sum test. A general linear model was used for over-time analyses with pairwise comparisons further evaluated by Tukey contrasts for radiographic data. R software (version 3.6.2) (The R Foundation for Statistical Computing, Vienna, Austria) was used for these analyses. Comparisons of relative abundance of the different microorganisms between times were performed by a nonparametric Kruskal–Wallis test for one factor (time) and Bonferroni adjustments for multiple pairwise comparisons, using IBM SPSS 28.0 for MacOS software (IBM Corporation). Pearson correlations were also established between relative abundance of some species and marginal bone loss. Statistical significance was set at p values < 0.05.

Graphical representation of some variables was performed with R (version 3.6.2) (The R Foundation for Statistical Computing), while others were represented using Microsoft Excel for Mac OS 16.35 and Graphpad Prism 7 for Mac OS.

3 | Results

For the current study, a total of 59 patients were evaluated for inclusion, of which 34 met the inclusion criteria, did not present any exclusion criteria, and decided to participate in the study. Inclusion, exclusion, allocation, and follow-up are summarized in Figure 1. As shown, a total of 23 patients attended the 5-year follow-up visit, 12 assigned to the test group and 11 to the control group. Since the microbiological analyses were only performed

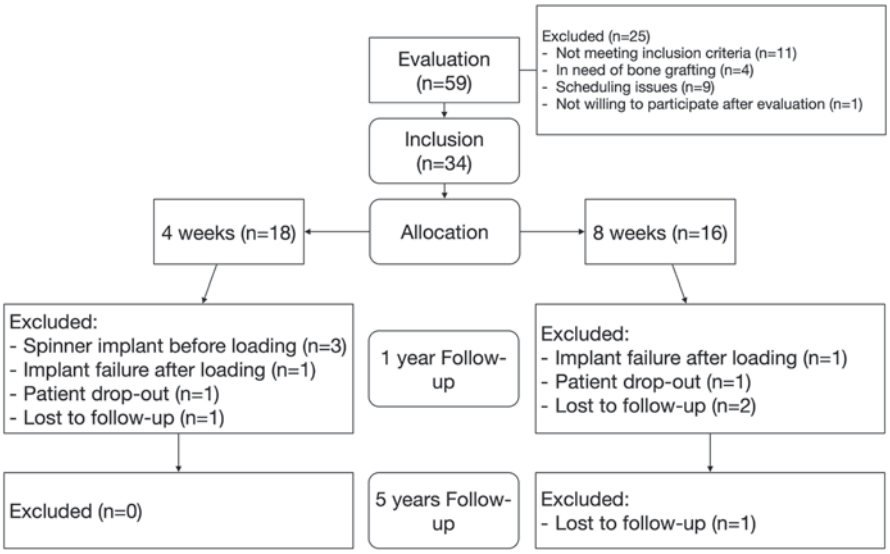

FIGURE 1 | CONSORT diagram of patients in the study.

on samples from patients who completed the 5-year follow-up, the clinical and radiographical data presented is only from those patients.

As previously reported, 1 year after prosthetic loading, we found no significant differences in any parameter associated with the marginal bone levels between the two groups (Galindo-Moreno et al. 2021). Due to this lack of significant differences between the two groups in the 5-year follow-up period, we conducted a group analysis and pooled sample analysis, as all patients received the same type of implant.

3.1 | Clinical Results After 5 Years

As shown in Table 1, clinical results can be summarized by indicating that there were no significant differences between groups in any variable, except for the thickness of the mucosa at the time of dental impressions and at the time of prosthesis delivery. At both time points, the mucosa was thinner in the test group ($p = 0.011$ and $p = 0.47$, respectively; Wilcoxon rank sum test). Regarding the papilla index, while most cases were classified as ideal after 12 months, a generalized decline was observed in almost every patient at the 60-month follow-up; still, most papillae were classified as 2 or 3 at that visit. No redness, edema, or suppuration was observed around any of the implants under evaluation.

No complications were reported between the 12- and 60-month follow-up except for the extraction of a second upper premolar adjacent to an implant in one patient due to a restorative complication after endodontic treatment.

3.2 | Radiographic Results After 5 Years

No statistically significant differences between groups were observed in any of the analyzed variables at any time point, except for crown length, which, as explained, was conditioned by the abutment height selected according to the thickness of the mucosa. All radiographical data is presented in Table 2.

Differences were observed when analyzing the evolution of the peri-implant marginal bone level over time. Besides the differences observed between each time point and the time of implant placement, reflecting the initial peri-implant bone remodeling, significant differences were also detected between the average mesio-distal MBL at the 60-month follow-up and the time of prosthesis delivery ($p = 0.012$; general linear model with Tukey contrasts) and the 3-month follow-up ($p = 0.033$; general linear model with Tukey contrasts). In summary, at 60 months, the peri-implant marginal bone was lower than at other time points, particularly in the test group in the mesial aspect. In any case, the average bone level was only around 0.5 mm below the implant platform (Figure 2). Among our population, only three patients showed a mean marginal bone loss between 1 and 1.5 mm at the 5 years of follow-up, although they were below 0.5 mm up to the 1-year visit. One implant showed more than 3 mm of loss, which we attributed to the traumatic extraction of an adjacent tooth. In addition, 8 implants exceeded the threshold of 0.5 mm of marginal bone loss in at least one of the mesial or distal aspects of the implant.

Specifically, the magnitude of bone level change between the time of implant placement and prosthesis delivery was −0.29 (0.39) mm in the control group and − 0.45 (0.42) mm in the test group ($p = 0.230$; Wilcoxon rank sum test); the value of all patients combined is −0.38 (0.41) mm. Similarly, the mesio-distal average level of change between the time of prosthesis delivery and the 12-month follow-up was −0.28 (0.24) mm, −0.49 (0.77) mm between the time of prosthesis delivery and the 60-month follow-up, and − 0.17 (0.87) mm between the 12- and 60-month follow-ups. In any case, no differences were observed between groups. No changes were observed in the adjacent teeth, except in the case where the posterior tooth was extracted, in which, obviously, no measurement was possible.

TABLE 1 | Description and comparison of clinical variables of patients followed up to 5 years.

	All patients $n = 23$ (100%)				Control group (8 weeks), $n = 11$ (47.83%)				Test group (4 weeks), $n = 12$ (52.17%)				p*
Age, mean (min, max) (years)	44 (30–58)				42 (30–58)				45 (35–53)				0.441
Gender, *n* (%)													
Female	10 (43.48)				6 (54.5)				4 (33.3)				0.305
Male	13 (56.52)				5 (45.5)				8 (66.7)				
Tobacco consumption, *n* (%)													
No	18 (78.26)				8 (72.7)				10 (83.3)				0.554
Low (< 5 cigarettes/day)	4 (17.39)				2 (18.2)				2 (16.7)				
Low (> 5, < 10 cigarettes/day)	1 (4.35)				1 (9.1)				0 (0.0)				
Mesio-distal distance (mm), mean (SD)	8.93 (2.02)				8.68 (1.23)				9.17 (2.59)				0.708
Occlusal height (mm), mean (SD)	6.95 (1.60)				7.09 (2.03)				6.81 (1.08)				0.689
Bucco-lingual width (mm), mean (SD)													
Before raising the flap	7.26 (1.68)				7.73 (2.00)				6.83 (1.27)				0.199
After raising the flap	6.83 (1.61)				7.27 (1.55)				6.42 (1.62)				0.200
Implant diameter, *n* (%)													
3.75 mm	15 (65.22)				8 (72.7)				7 (58.3)				0.469
4.20 mm	8 (34.78)				3 (27.3)				5 (41.7)				
Implant length, *n* (%)													
10.0 mm	11 (47.83)				6 (54.5)				5 (41.7)				0.537
11.5 mm	12 (52.17)				5 (45.5)				7 (58.3)				
Abutment height, *n* (%)													
1.50 mm	16 (69.57)				6 (54.5)				10 (83.3)				0.134
3.00 mm	7 (30.43)				5 (45.5)				2 (16.7)				
Peri-implant mucosa thickness (mm), mean (SD)													
Implant placement	2.72 (0.81)				2.77 (0.61)				2.67 (0.98)				0.661
Impressions	2.50 (0.66)				2.81 (0.75)				2.20 (0.40)				0.011*
Prosthesis delivery	2.65 (0.65)				2.91 (0.54)				2.42 (0.67)				0.047*
Papilla index (% within each visit) (MESIAL)**	0	1	2	3	0	1	2	3	0	1	2	3	
Prosthesis delivery	13.0	65.2	21.7	0.0	9.1	63.6	27.3	0.0	16.7	66.6	16.7	0.0	0.757

(Continues)

TABLE 1 | (Continued)

	All patients $n = 23$ (100%)				Control group (8 weeks), $n = 11$ (47.83%)				Test group (4 weeks), $n = 12$ (52.17%)				p*
1 week	4.86	14.3	71.4	9.5	0.0	9.1	90.9	0.0	10.0	20.0	50.0	20.0	0.175
1 month	0.0	9.1	77.3	13.6	0.0	0.0	90.0	10.0	0.0	16.7	66.6	16.7	0.328
3 months	0.0	54.6	27.3	18.2	0.0	27.3	27.3	45.4	0.0	9.1	27.3	63.6	0.513
6 months	0.0	4.8	23.8	71.4	0.0	10.0	40.0	50.0	0.0	0.0	9.1	90.9	0.109
12 months	0.0	0.0	36.8	63.2	0.0	0.0	44.4	55.6	0.0	0.0	30.0	70.0	0.515
60 months	4.4	26.1	39.1	30.4	0.0	18.2	45.4	36.4	8.3	33.3	33.3	25.1	0.598
Papilla index (% within each visit) (DISTAL)**	0	1	2	3	0	1	2	3	0	1	2	3	
Prosthesis delivery	13.0	56.5	30.4	0.0	9.1	54.5	36.4	0.0	16.7	58.3	25.0	0.0	0.775
1 week	0.0	9.5	81.0	9.5	0.0	9.1	90.9	0.0	0.0	10.0	70.0	20.0	0.288
1 month	0.0	9.1	77.3	13.6	0.0	0.0	90.0	10.0	0.0	16.7	66.6	16.7	0.328
3 months	0.0	13.6	31.8	54.5	0.0	18.2	36.4	45.4	0.0	9.1	27.3	63.6	0.667
6 months	0.0	0.0	47.6	52.4	0.0	0.0	60.0	40.0	0.0	0.0	36.4	63.6	0.279
12 months	0.0	0.0	21.1	79.0	0.0	0.0	33.3	66.7	0.0	0.0	10.0	90.0	0.213
60 months	8.7	34.8	39.1	17.4	9.1	27.3	45.4	18.2	8.3	41.7	33.3	16.7	0.904

*p value (control vs. test): nonparametric Wilcoxon rank sum test for continuous variables and chi-squared test for categorical variables.
**Papilla index was recorded as: 0 = No papilla, 1 = < 50% filling of the interproximal area, 2 = ≥ 50% filling of the interproximal area, 3 = Ideal papilla, and 4 = Overgrowth. There were no cases with papilla index higher than 3.

3.3 | Microbiological Results

After sample processing and quality checks, between 10 and 16 samples were suitable for analysis at the different time points. A small proportion of archaea (< 0.05%) belonging to the phyla Crenarchaeota and Euryarchaeota, and fungi (< 0.5%) belonging to the Ascomycota and Basidiomycota phyla were detected in all samples. However, no significant changes in the proportion of these phyla have been demonstrated among samples obtained at different times.

Shannon and Simpson indexes, ecological parameters of diversity, and Chao index and observed species, ecological parameters of richness, were evaluated. According to our data, no differences over time were observed in diversity parameters. In contrast, both the number of observed species and, thus, the Chao richness index decreased over time, with the comparison between 1 and 60 months being statistically significant (Figure 3A). The axonometric two-dimensional PCoA at the genus level showed significant differences only when comparing samples from 1 and 60 months (Figure 3B), showing altered oral microbiome populations.

By bacteria phyla, there were a total of 6 phyla with a relative abundance of over 1% at each time point (Figure 4A). Besides those, only Candidatus Saccharibacteria, with a relative abundance below 1%, changed significantly over time. Particularly, it significantly decreased from 1 to 60 months (Figure 4B).

Regarding the relative abundance at the level of genus, we found statistically significant differences over time in three of them. Particularly, *Porphyromonas* increased 12.3-fold from 1 to 60 months, *Tannerella* increased 3.5-fold from 1 to 12, and 5-fold from 1 to 60 months. In contrast, *Selenomonas* significantly decreased 87% from 1 to 60 months (Figure 5).

At the level of species, only 13 out of more than 200 showed a relative abundance higher than 1%. Over time, there were no significant changes. Of particular interest was *Fusobacterium nucleatum*, which was the most abundant species in all samples; it was higher during the first year but decreased at 5 years, although without statistically significant differences (Figure 6A). In addition, seven species showed at least one pairwise comparison between time points with statistically significant differences (Figure 6B); most of them showed a relative abundance below 1% at most time points. These species were *Campylobacter concisus*, *Capnocytophaga gingivalis*, *Fusobacterium pseudoperiodonticum*, *Porphyromonas gingivalis*, *Schaalia odontolytica*, *Selenomonas* sp. oral taxon 126, and *Tannerella forsythia*. Particularly, these species were different when comparing their abundance at 1 versus 60 months. Additionally, the relative abundance of *Schaalia odontolytica* decreased from 3 to 12 months, while *Tannerella forsythia* increased from 1 to 12 months.

Regardless, the analysis of the potential correlations by the Pearson test between the relative abundance of each of these genera and species and the MBL at 60 months did not show statistical significance (Figure 7).

TABLE 2 | Description and comparison of radiographical variables of patients followed up to 5 years. All data is presented as mean (SD) in mm, except crown-to-implant ratio.

	All patients, $n = 23$ (100%)	Control group (8 weeks), $n = 11$ (47.83%)	Test group (4 weeks), $n = 12$ (52.17%)	p*
Distance dental implant-anterior tooth	2.45 (1.28)	2.07 (1.17)	2.79 (1.34)	0.314
Distance dental implant-posterior tooth	2.31 (1.18)	2.19 (1.13)	2.41 (1.28)	0.863
Crown length	8.91 (1.10)	8.38 (1.15)	9.39 (0.83)	0.044*
Crown-to-implant ratio	1.01 (0.13)	1.00 (0.17)	1.03 (0.08)	0.566
Anterior tooth MBL (DISTAL)				
Implant placement	2.49 (0.71)	2.25 (0.76)	2.69 (0.61)	0.152
Prosthesis delivery	2.65 (0.67)	2.42 (0.60)	2.86 (0.68)	0.132
3 months	2.61 (0.78)	2.35 (0.77)	2.92 (0.75)	0.295
6 months	2.83 (0.54)	2.69 (0.43)	2.93 (0.61)	0.310
12 months	2.92 (0.53)	2.86 (0.53)	2.97 (0.57)	0.798
60 months	3.02 (1.05)	2.97 (0.92)	3.08 (1.19)	0.880
Implant MBL (MESIAL)				
Implant placement	0.51 (0.44)	0.42 (0.28)	0.59 (0.55)	0.705
Prosthesis delivery	0.10 (0.37)	0.08 (0.43)	0.12 (0.32)	0.921
3 months	0.09 (0.18)	0.14 (0.17)	0.03 (0.18)	0.426
6 months	−0.06 (0.35)	−0.12 (0.45)	−0.01 (0.25)	0.944
12 months	−0.14 (0.47)	−0.26 (0.60)	−0.04 (0.32)	0.594
60 months	−0.31 (0.79)	−0.26 (0.61)	−0.55 (0.93)	0.518
Implant MBL (DISTAL)				
Implant placement	0.35 (0.29)	0.32 (0.31)	0.37 (0.29)	0.503
Prosthesis delivery	0.01 (0.34)	0.05 (0.18)	−0.02 (0.43)	0.553
3 months	0.01 (0.31)	0.09 (0.27)	−0.10 (0.35)	0.142
6 months	−0.22 (0.43)	−0.23 (0.39)	−0.22 (0.49)	0.778
12 months	−0.26 (0.41)	−0.25 (0.36)	−0.27 (0.47)	0.722
60 months	−0.39 (0.92)	−0.29 (0.54)	−0.48 (1.19)	0.644
Posterior tooth MBL (MESIAL)				
Implant placement	1.91 (0.87)	1.98 (1.23)	1.85 (0.45)	0.766
Prosthesis delivery	1.87 (0.82)	1.87 (1.16)	1.86 (0.50)	0.464
3 months	2.02 (0.92)	2.01 (1.10)	2.05 (0.66)	0.607
6 months	1.96 (0.82)	2.08 (1.04)	1.86 (0.63)	0.766
12 months	1.97 (0.74)	2.10 (0.97)	1.87 (0.54)	0.965
60 months	2.64 (0.90)	2.79 (1.03)	2.48 (0.76)	0.622
Average implant MBL				
Implant placement	0.43 (0.31)	0.37 (0.26)	0.48 (0.35)	0.512
Prosthesis delivery	0.06 (0.26)	0.06 (0.26)	0.05 (0.26)	0.872

(Continues)

TABLE 2 | (Continued)

	All patients, $n = 23$ (100%)	Control group (8 weeks), $n = 11$ (47.83%)	Test group (4 weeks), $n = 12$ (52.17%)	p*
3 months	0.05 (0.20)	0.11 (0.17)	−0.03 (0.22)	0.142
6 months	−0.14 (0.35)	−0.17 (0.39)	−0.12 (0.33)	0.549
12 months	−0.20 (0.40)	−0.26 (0.47)	−0.15 (0.35)	0.573
60 months	−0.40 (0.81)	−0.27 (0.50)	−0.52 (1.03)	0.976
MBL change from implant to prosthesis delivery				
Mesial	−0.43 (0.54)	−0.35 (0.57)	−0.49 (0.54)	0.656
Distal	−0.33 (0.35)	−0.22 (0.24)	−0.42 (0.41)	0.342
Average	−0.38 (0.41)	−0.29 (0.39)	−0.45 (0.42)	0.230
MBL change from prosthesis delivery to 12 months				
Mesial	−0.29 (0.32)	−0.33 (0.26)	−0.26 (0.36)	0.270
Distal	−0.27 (0.23)	−0.33 (0.29)	−0.24 (0.19)	0.962
Average	−0.28 (0.24)	−0.33 (0.22)	−0.25 (0.25)	0.364
MBL change from prosthesis delivery to 60 months				
Mesial	−0.55 (0.81)	−0.39 (0.47)	−0.68 (1.01)	0.872
Distal	−0.42 (0.86)	−0.39 (0.60)	−0.45 (1.05)	0.923
Average	−0.49 (0.77)	−0.39 (0.49)	−0.57 (0.95)	0.722
MBL change from 12 to 60 months				
Mesial	−0.09 (0.97)	0.11 (0.58)	−0.24 (1.21)	0.762
Distal	−0.25 (0.87)	0.13 (0.39)	−0.56 (1.04)	0.068
Average	−0.17 (0.87)	0.12 (0.42)	−0.40 (1.08)	0.515

*p-value (control vs. test): nonparametric Wilcoxon rank-sum test.

4 | Discussion

Our aim was to analyze clinical, radiological, and microbiological changes in a sample of implants after 5 years of follow up under different functional loading protocols in implants coated with a modified surface. This work is of special interest since, to our knowledge, there are no previous comparisons between changes in the microbiome and radiographical marginal bone loss around implants over such a long follow-up period (5 years). Although the sample of implants may be considered small, one finding is of extreme interest in our work: the microbiome of the peri-implant sulcus throughout the follow-up of the implants evolves towards putative pathogenic bacteria, mainly *Porphyromonas gingivalis* and *Tannerella forsythia*; in addition, although *Fusobacterium nucleatum* is the most abundant species, its massive presence does not condition necessarily the peri-implant pathology and its progression.

Clinically, it must be mentioned that all implants in the current study were considered successful with no complications nor significant changes in any of the variables under study up to the last follow-up, 60 months after the initial prosthetic loading. Similarly, from a radiographical point of view, we must consider most of the implants evaluated in the current study as successful after 5 years of function. One implant showed marginal bone loss > 3 mm, which we attributed to a traumatic extraction of the adjacent tooth. The Pisa Consensus Conference of Implant Success (Misch et al. 2008) determined that, among the success criteria for dental implants, a marginal bone loss of up to 2 mm 1 year after loading could be considered successful. Subsequent definitions (Berglundh et al. 2018) and clinical studies (Galindo-Moreno et al. 2015, 2022) have shifted to promote the idea that assumable marginal bone loss must be closer to 0 mm. This is so because early marginal bone loss is associated with mid- and long-term bone loss greater than the 2 mm limit mentioned earlier (Galindo-Moreno et al. 2015).

Regarding clinical parameters affecting the supracrestal soft tissues, we did not observe redness, edema, or suppuration around any of our implants. Although since 2023 the ID-COSM guidelines suggest reporting bleeding on probing (BoP) and probing pocket depth (PPD) measurements (Tonetti et al. 2023), no such data were collected in this study. Obviously, this work was initiated before the publication of these guidelines and also before the criteria for the definition of peri-implant diseases defined in the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions (Berglundh et al. 2018). The reason why numerous clinical measurements were taken

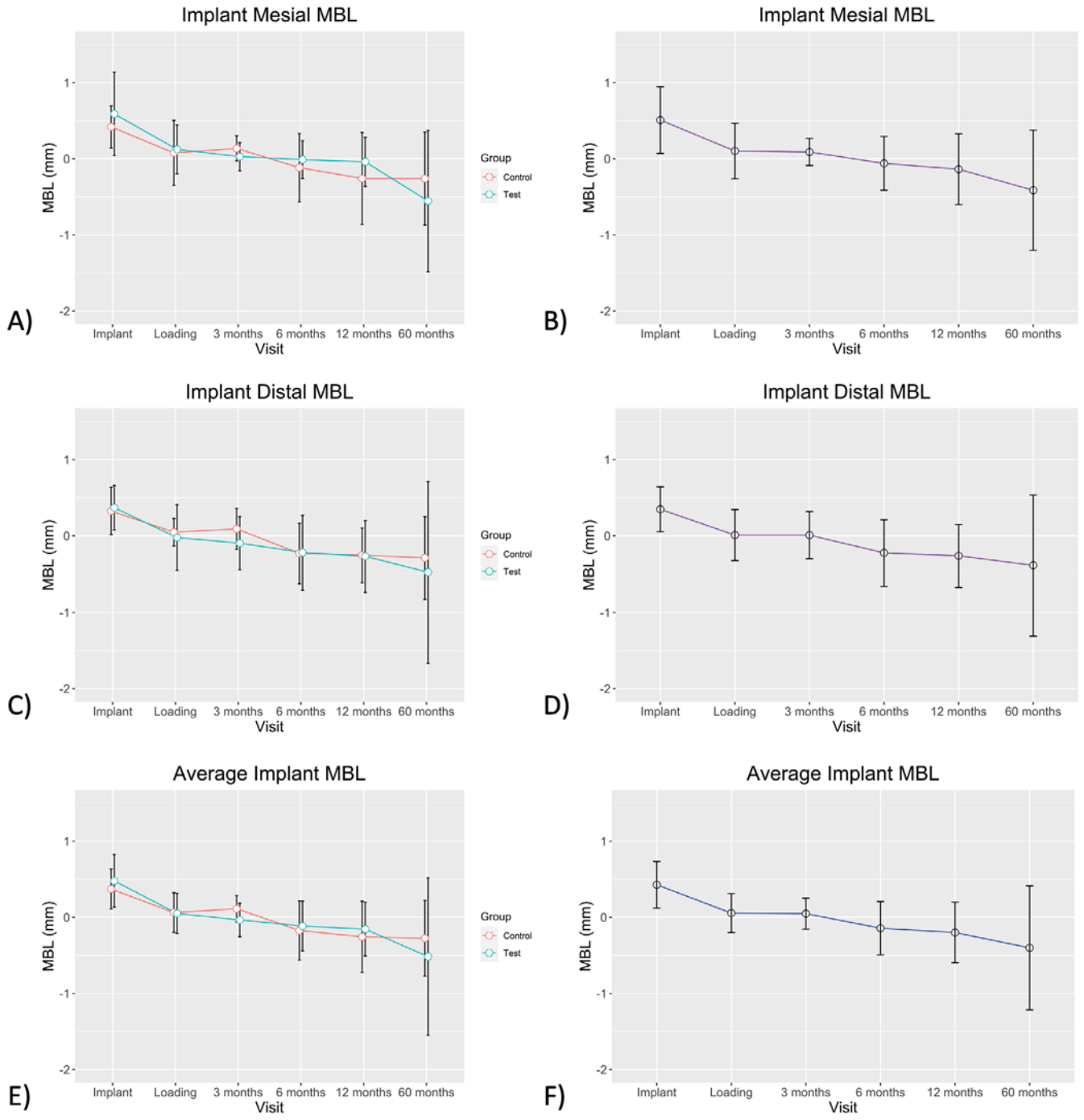

FIGURE 2 | Graphical representation of marginal bone level: (A, B) mesial, (C, D) distal and (E, F) average by (A, C, E) groups and (B, D, F) combined.

but not these two parameters is that we believe that probing implant-supported single crowns without removal of the prosthesis leads to a high degree of diagnostic confusion around healthy implants, overestimating peri-implant pathology, something long discussed (Atassi 2002). Firstly, iatrogenic bleeding on probing is highly probable (Dukka et al. 2021), both because of the discrepancy between implant diameter and clinical crown diameter at the sulcus level, and also because of the different emergence profiles of each prosthetic crown, different at each location, which makes it extremely difficult to probe around the implant to the limits of the peri-implant sulcus. Secondly, in implants without bone affectation, the probing depth hardly reaches the limits of the peri-implant sulcus, underestimating its value for the same related reasons; thus, this value could be argued as being absolutely arbitrary. To correctly probe an implant, it is necessary to remove the prosthetic crown, which, in our case, would clearly modify the composition of the peri-implant microbiota after each follow-up, invalidating this study. Even more, although we understand the importance of probing around implants, neither BOP nor PPD have shown ideal sensitivity and specificity to indicate marginal bone loss around implants. Studies published by authors of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions have recently demonstrated the low predictive

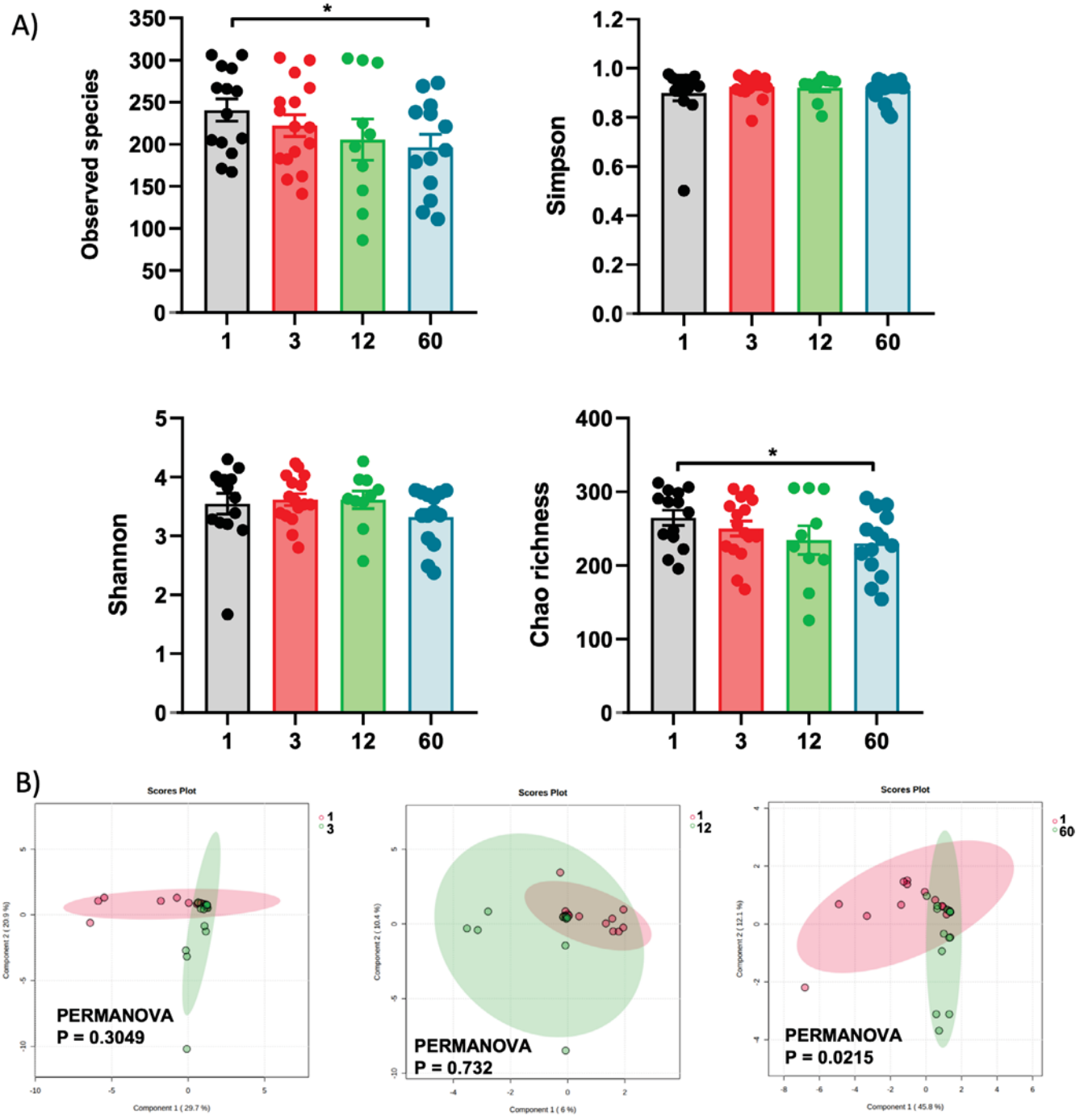

FIGURE 3 | Time evolution of microecological parameters of alpha and beta diversity. (A) Ecological parameters of richness, and diversity. (B) Principal Coordinate Analysis (PCoA) in the oral microbiota from all time samples collection. Values are expressed as mean ± SEM ($n = 10–16$). *$p < 0.05$ compared with samples collected at 1 month.

value of these clinical measurements (Romandini et al. 2020a,b; 2021; Berglundh et al. 2021). Moreover, the literature is robust regarding this aspect: BOP is not related to MBL around implants in the long term, according to the longest longitudinal studies published on this topic in European populations (Roos-Jansåker et al. 2006; Dierens et al. 2013; French et al. 2016; Renvert et al. 2018; Doornewaard et al. 2018; Ramanauskaite et al. 2024). Consequently, to us, the most effective method to detect peri-implant MBL is radiographical analysis, as we conducted, but not probing.

Unfortunately, the comparison between our results and those available in the literature is complicated because of the absence of long-term follow-up of similarly designed studies. Most of the available studies focus on early loading of multiple implants. Regardless, to mention some with similar results after a similar follow-up time and loading protocols, Arghami and co-workers' series, using an implant coated with hydroxyapatite, found, after 7 years of follow-up, marginal bone loss of 0.511 ± 0.094 mm (Arghami et al. 2021). Using the same type of implant used in the current study but without the surface coating, Saglanmak found

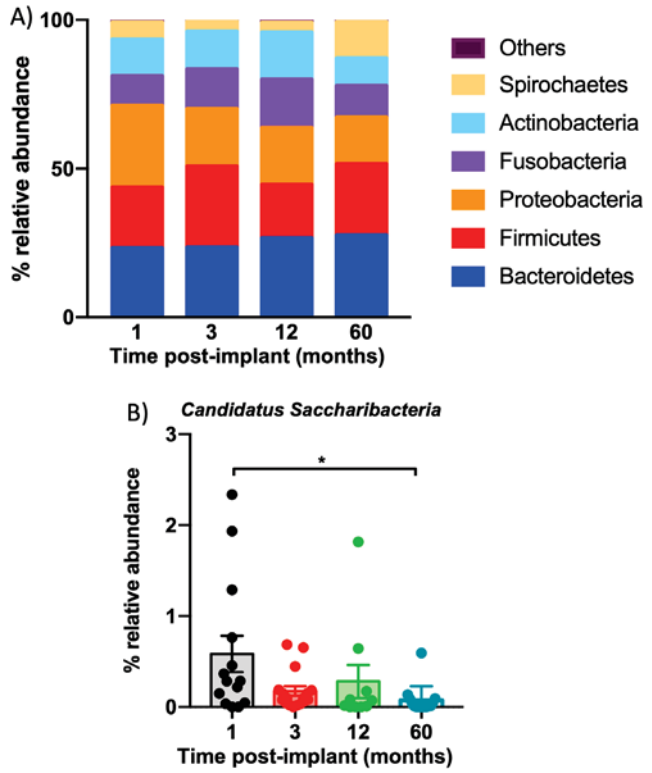

FIGURE 4 | Post-implant time evolution of oral microbiota at phylum level. (A) Representation of the relative abundance at the level of phylum per time point. (B) Representation of the relative abundance of Candidatus Saccharibacteria over time. Values are expressed as mean ± SEM ($n = 10–16$). *$p < 0.05$ compared with samples collected at 1 month.

higher bone loss after 5 years (Saglanmak et al. 2021). However, they combined data from single and multiple implants. In addition, their loading protocol was slightly different from ours. In this case, we should keep in mind the importance of transmucosal abutments as a key modifier of peri-implant bone maintenance or resorption, as originally described by Galindo-Moreno and co-workers in 2014 (Galindo-Moreno et al. 2014) and later confirmed by other studies. In single unit implants, the height of the Ti-base type abutments does not play such a role as the crown and the abutment behave as a single piece; in this case, the height of the surrounding mucosa may have an influence on the peri-implant bone remodeling. Regardless, Pommer and co-workers conducted a meta-analysis, including 29 studies and 965 implants, and indicated that results regarding peri-implant bone remodeling around single unit implants are not influenced in the long term by the loading protocol (Pommer et al. 2021).

Due to current peri-implant disease concept that defines peri-implant pathology as a plaque-associated disease (Berglundh et al. 2018), knowing the evolution of the peri-implant microbiome is essential to understand when and why peri-implant diseases might be initiated and perpetuated. It is important to highlight that even having perfectly established treatment protocols for these entities, the scientific literature has yet to provide conclusive evidence regarding the unequivocal link of any pathogenic agent with the development of these diseases (Herrera et al. 2023). And this is so clearly evident because with the current technologies almost every microbiological agent can be isolated in any clinical situation, from peri-implant health to peri-implantitis. Baas-Becking and Beijerinck rightly said: "Everything is everywhere, but the environment selects" (de Wit and Bouvier 2006).

This study reports a small proportion of archaea (<0.05%) belonging to the phyla Crenarchaeota and Euryarchaeota, and fungi (<0.5%) belonging to Ascomycota and Basidiomycota phyla, in all samples. However, these micro-organisms' abundance did not change significantly over time. Faveri and coworkers reported that the archaea *Methanobrevibacter oralis* (Euryarchaeota phylum) was the most prevalent phylotype in their sample of implants, representing 92% of the clones in healthy implants and 95.3% in implants with peri-implantitis. *Methanobacterium congolense/curvum* (Methanobacteriota phylum) was detected only in one of their healthy implants and three diseased implants, indicating that although archaea can be detected both around healthy or diseased implants, the presence of these microorganisms increases in the presence of peri-implantitis (Faveri et al. 2011). Similarly, Aleksandrowicz and coworkers described, by 16S rRNA gene-based polymerase chain reaction assay, that archaea, mainly *Methanobrevibacter oralis*, were detected in 10% of samples from diseased peri-implants, but not from healthy implants, highlighting that archaea are present only in diseased but not healthy implants. Thus, they concluded that archaea may have a potential role in peri-implantitis (Aleksandrowicz et al. 2020). Obviously, we cannot support these conclusions, since in our implant sample the presence of archaea is quite marginal; it is presented in all our healthy samples and does not change over time.

Regarding fungi, Chen and workers reported recently that among the subgingival fungal diversity, Basidiomycota was the dominant fungal species (Chen et al. 2023), similarly to our results. A recent meta-analysis has described that *Candida* (Ascomycota phylum) is usually present both in diseased and healthy implants, although their presence is higher in peri-implantitis. Chen's research also describes that the concentration of *Candida* in the peri-implant sulcus can increase over time according to how many dental implants are in the oral cavity. Its role can be associated with other periodontopathogens, such as *Veillonella parvula*, *Tannerella forsythia*, *Porphyromonas gingivalis*, or *Parvimonas micra* (de Lafuente-Ibáñez Mendoza et al. 2021). However, in our sample, Ascomycota phylum did not change over the time around dental implants.

Regarding bacteria, our results revealed that Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Actinobacteria, and Spirochaetes were the phyla that had the greatest weight of the total microbiological population, belonging to them more than 95% of all bacteria detected at any of the times analyzed. Only the phylum Candidatus Saccharibacteria decreased significantly in relative abundance over time from the first follow-up to 60 months. Our study's microbiome composition is similar to most studies. For example, Yu and coworkers showed that their population's microbiome was mostly composed of Firmicutes (40.33%) (Yu et al. 2019). Sun and coworkers also reported the prevalence of 8 phyla in a sample of healthy and diseased implants, pre- and post-treatment, resulting in Firmicutes, Bacteroidetes, Fusobacteria, Synergistetes, and Spirochaetes being expressed in priority in the pre-treatment group, decreasing in the post-treatment group (Sun et al. 2022). In our population, although

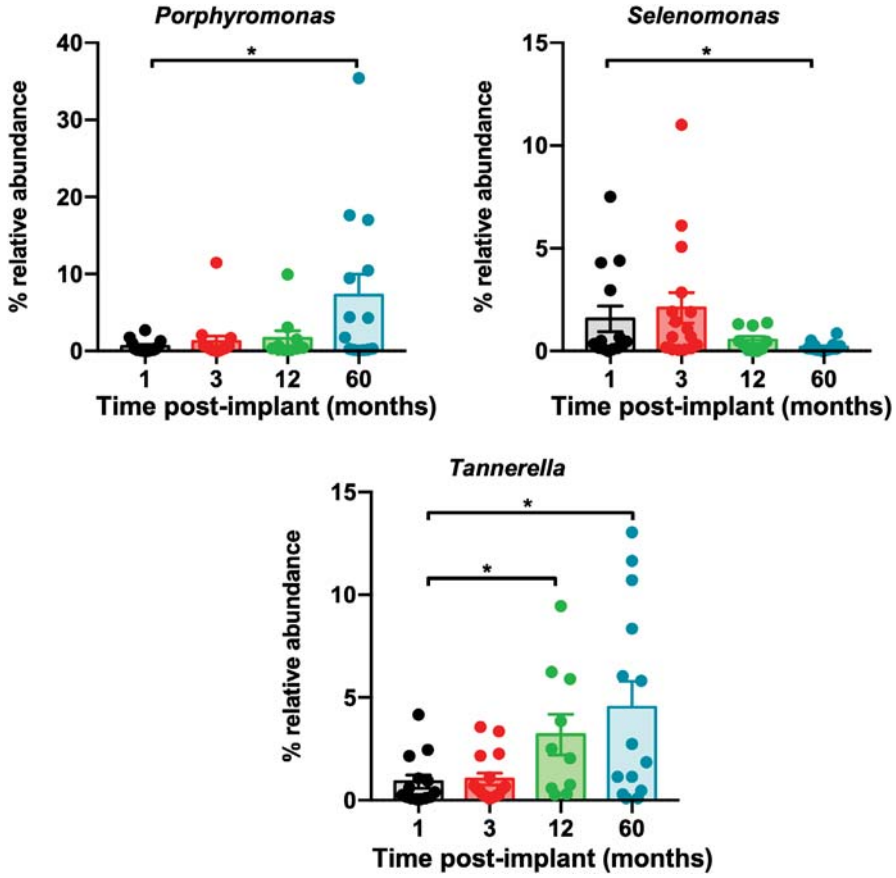

FIGURE 5 | Post-implant time evolution of oral microbiota at the genus level. Representation of the relative abundance over time of genera Porphyromonas, Tannerella, and Selenomonas. Values are expressed as mean ± SEM ($n = 10–16$). *$p < 0.05$ compared with samples collected at 1 month.

a significant decrease of Candidatus Saccharibacteria, a non-significant pattern of decrease of Proteobacteria, and an increase of Bacteroidetes, Firmicutes, and Spirochaetes were observed over time, no such significant differences were observed, unlike the aforementioned studies. This could be due to the fact that most of our implants did not reach pathological marginal bone loss in their evolution. Since the basic etiopathology principles of periodontitis have been applied equally to peri-implantitis, that is, peri-implantitis is an endogenous, polymicrobial, opportunistic infection (Charalampakis and Belibasakis 2015), Candidatus Saccharibacteria should have increased, as this family of microbes is increased in periodontal disease (Pérez-Chaparro et al. 2014). Under the name Candidatus Saccharibacteria, there are no studies in the literature that associate these bacteria with implants. However, under the name TM7 (former name), there are very few analyses linking these ultra-small bacteria to the development of peri-implant pathology. Different studies have reported the presence of TM7 in periodontitis but not around healthy or diseased implants from the same patients (Sousa et al. 2017; Yu et al. 2019). In contrast, although in small relative abundance at all follow-up times, our study has detected the presence of Candidatus Saccharibacteria around healthy implants, although decreasing over time. In this sense, Chipashvili and coworkers have described that TM7 has the ability to proliferate in nutrient-rich inflammatory environments (Chipashvili et al. 2021), using such nutrients, but it is thought not to play a contributing role in the inflammatory response itself (Hajishengallis 2014).

At the genus level, our study found a significant decrease in the genus *Selenomonas*, but mainly from 3 to 60 months, alongside a notable increase in the genera *Porphyromonas* and *Tannerella*, especially at the final follow-up. Clearly, there is a selective shift in the microbiome toward Socranski's Red Complex bacteria, the most abundant bacteria in deep periodontal pockets. However, it is interesting to note that Li and collaborators described that the abundance of 14 bacterial genera in peri-implant mucositis was similar to that of healthy implants in less-inflamed mucositis lesions, whereas it was similar to that of peri-implantitis in samples with severe mucosal inflammation. In any case, in that study, all these genera were present in all conditions analyzed (Li et al. 2023). Thus, understanding the pathology as a dysbiosis phenomenon, it could be suggested that the early colonizers could influence future shifts in the community. Some studies have pointed out that the early appearance of genera such as *Actinomyces*, *Bacteroidetes*, *Pseudomonas*, or *Peptostreptococcaceae* could serve as pioneers for a pathological peri-implant microbial community, providing not only nutrition but a suitable pH and ideal anaerobic environment for pathogenic bacteria, significantly associating with pathogenic genera of *Porphyromonas*, *Tannerella*, *Treponema*, and *Prevotella* (Zheng et al. 2015). Lu et al. (2021), in a two-year follow-up study evaluating peri-implant mucositis in patients with a previous history of periodontitis, found quite similar results, establishing that the genera *Porphyromonas*, *Prevotella*, *Tannerella*, and *Treponema* increased dramatically in the first 2 years after

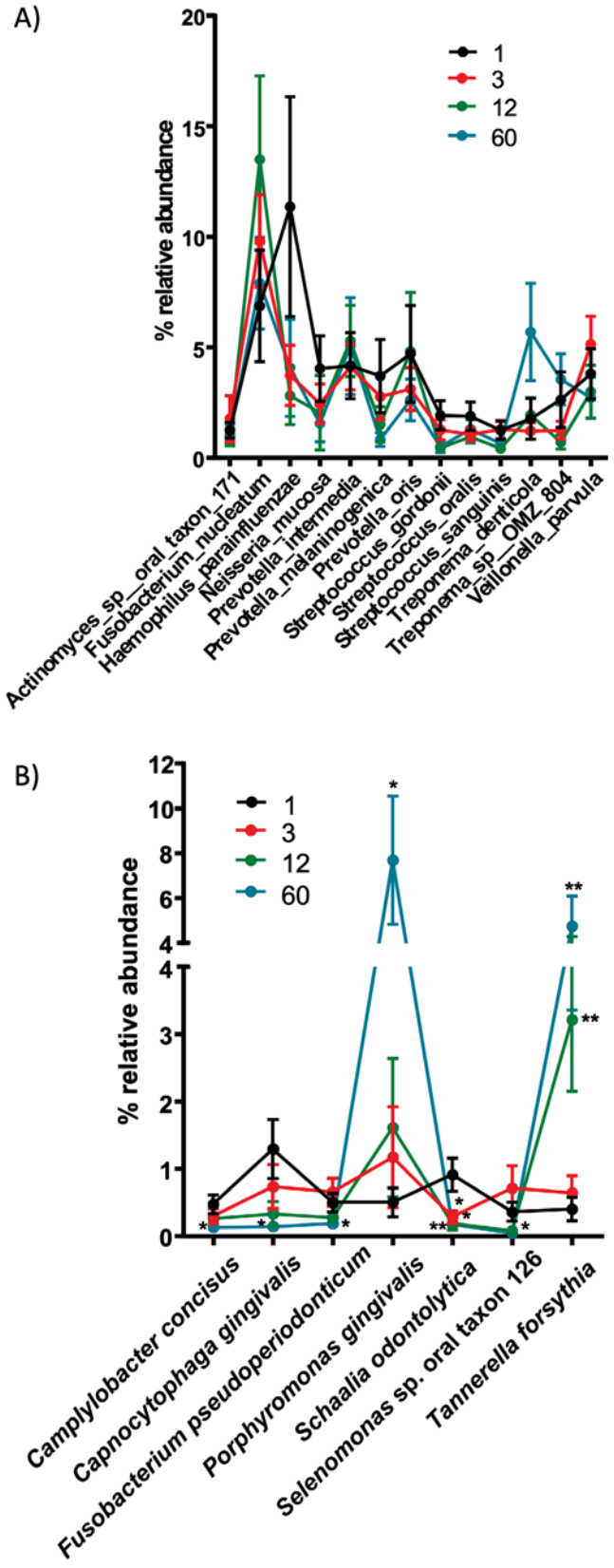

FIGURE 6 | Representation of the relative abundance over time of species with a relative abundance > 1%, without (A) or at least one statistically significant difference (B) in the pairwise comparison of time points. Values are expressed as mean ± SEM ($n = 10–16$). *$p < 0.05$ and **$p < 0.01$ compared with samples collected at 1 month.

restoration, considering such bacteria as biomarkers of peri-implant mucositis.

Obviously, in our patients, at the species level, these microbiological changes have a similar translation, where seven species in particular showed an evolutionary change, so that mainly *Capnocytophaga gingivalis* and *Schaalia odontolytica*, which are more abundant at the first follow-up, decreased significantly from the formation of the new peri-implant sulcus to 5 years of evolution, but also *Campylobacter concisus*, *Fusobacterium pseudoperiodonticum*, and *Selenomonas* sp. oral taxon 126, which decreased significantly over the time of follow-up. In contrast, *Tannerella forsythia* increased significantly from the first year of follow-up, and *Porphyromonas gingivalis* became significantly more abundant in the last follow-up period, as most of the studies suggest.

It seems obvious that the microbiome changed towards a more putative pathogenic spectrum over time in a significant way. This change could lead to thinking that bacteria classically associated with periodontal pathology are the ones that promote the development of the change in the peri-implant tissues. However, a differential and unique aspect of our contribution is that this study analyzes the follow-up of radiographic marginal bone loss in relation to the microbiome of the peri-implant sulcus over 5 years, with the aim of analyzing this correlation. From this point of view, it is very relevant that despite the significant increase in the abundance of *Porphyromonas gingivalis* and *Tannerella forsythia* in the peri-implant sulcus of our population over time, no significant correlation was found between the peri-implant microbiome and the progression of radiological marginal bone loss. When the presence of microorganisms in the peri-implant sulcus of the implants with radiologic marginal bone loss > 0.5 mm in any of their interproximal aspects was analyzed, there was a trend towards an increase of both *Bacteroidota* and *Schaalia* bacteria and a decrease of *Selenomonas* genera. In fact, from the detailed analysis of the microbiome of each of our patients, those patients with the highest relative abundance of *Porphyromonas gingivalis* were not those who showed marginal bone loss > 0.5 mm; moreover, the patients who showed higher levels of marginal bone loss showed especially higher levels of *Fusobacterium nucleatum* and *Prevotella oris* very early in the onset of bacterial colonization, but not *Porphyromonas gingivalis* at the end of the study. Classic (Van Assche et al. 2011) or current studies (Lu et al. 2021) comparing the microbial composition between healthy implants and those with peri-implant mucositis find significantly more *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, and *Treponema denticola* in pathology, suggesting that these bacteria may be key players in peri-implant inflammation. However, it is interesting to note that this association could not be evidenced in our patients in the short or in the long term, as no correlation was found between microbial and clinical or radiographical data.

There are few studies in the literature analyzing the evolution of the microbiome in the same sample of implants. Lu and collaborators related that commensal genera, including *Actinomyces* and *Streptococcus*, decreased from T1 (1 month) to 2 years of

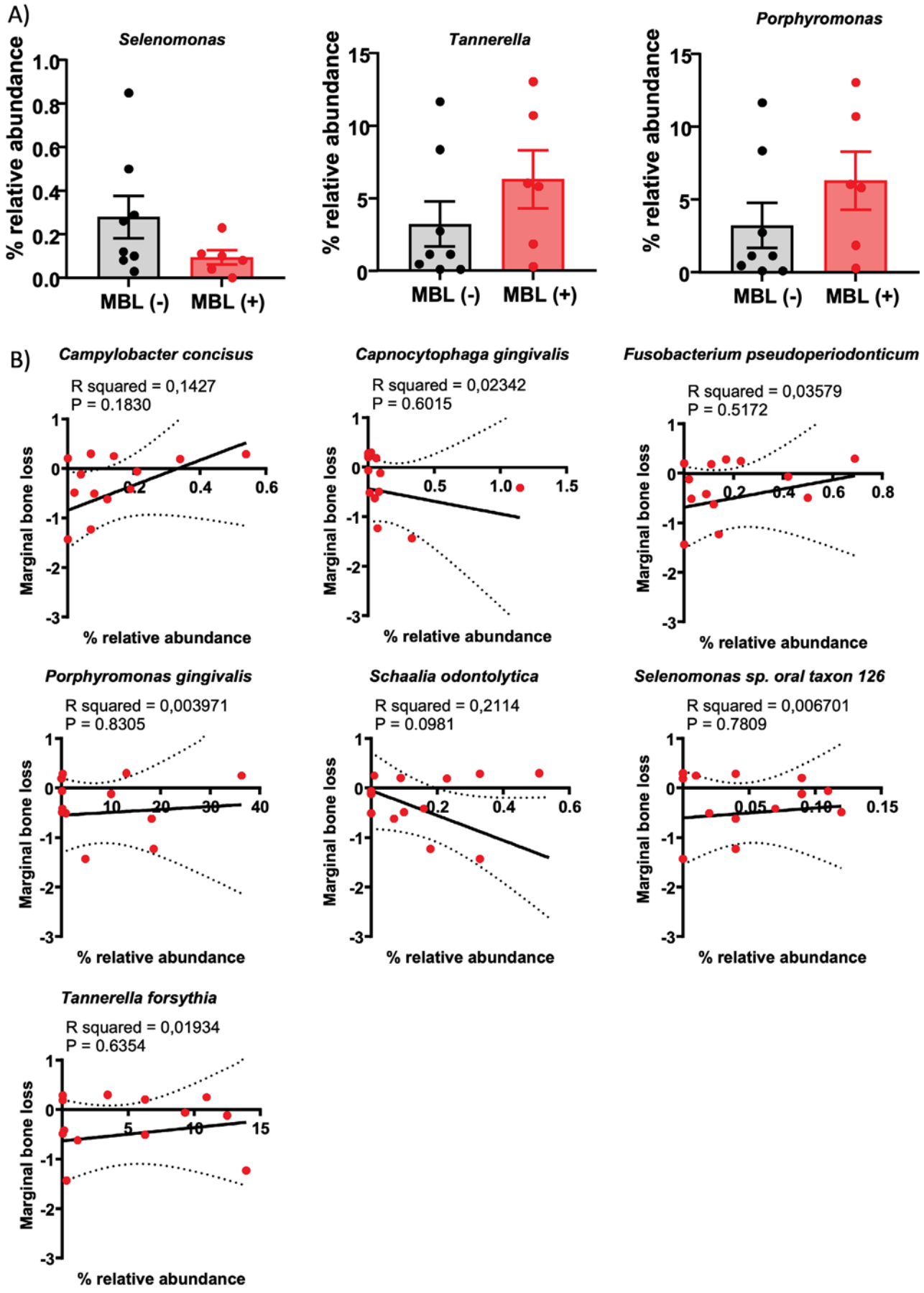

FIGURE 7 | Legend on next page.

FIGURE 7 | Analysis of microbiome at different marginal bone levels around implants after 5 years of function. No statistically significant correlation was found between them, either at the genus or species level. (A) Representation of the relative abundance of genus depending on the cut-off value of MBL. Genus represented are those with at least one statistically significant difference in the pairwise comparison of time points. MBL (+) represents implants with radiographic marginal bone loss higher than 0.5 mm in either the mesial or distal sides of the implant. No significant differences were observed. (B) Correlation analysis performed by Pearson test between marginal bone loss and % of relative abundance of bacterial species which significantly changed after 60 months. No significant correlations were observed.

follow-up, showing themselves as antagonistic to the development of these pathogenic genera in the submucosal community. Importantly, microbial richness measured through the Chao1 index did not show significant differences between the three follow-up times, indicating that the number of operational taxonomic units (OTUs) did not change significantly, but microbial diversity, as measured by the Shannon index, increased dramatically from T1 to T3 (Lu et al. 2021).

Siddiqi and coworkers analyzed the colonization by three potentially pathogenic organisms (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Staphilococcus aureus*) of both titanium and zirconium implants 6 months after implantation in edentulous patients, using qRT-PCR techniques. Their results provided relative levels of the bacteria studied below the lower limit of quantification in all the samples analyzed (lingual and peri-implant samples), concluding that the organisms analyzed clearly did not emerge at detectable levels 6 months after implant placement, regardless of the type of implant material used (Siddiqi et al. 2016). Although their results are consistent with other studies, also unable to detect such bacteria in completely edentulous patients (Kocar et al. 2010), we understand that this may be due to the sensitivity of the microbiological detection techniques used. Devides and Franco also failed to detect the presence of *Porphyromonas gingivalis* in edentulous patients before implant placement, but nevertheless, this species was present in 46.7% and 53.3% of patients 4 and 6 months after implant placement, respectively, describing that bacterial colonization of peri-implant sulci is both time-and oral ecosystem-dependent (Devides and Franco 2006).

de Oliveira Silva and co-workers conducted a 3-year follow-up study of the supra- and subgingival microbiological profile of single-tooth implants rehabilitated with either zirconia or titanium abutments. Their findings indicated that the mean marginal bone loss around dental implants after 3 years was superior to our mean marginal bone loss for titanium abutments, reporting 0.99 ± 0.41 mm for implants restored with titanium abutments and 0.76 ± 0.21 mm for implants rehabilitated with zirconia (de Oliveira Silva et al. 2020). However, probing depth in titanium-rehabilitated implants increased significantly during the first year of loading ($p < 0.05$), whereas it remained unchanged for zirconia restorations. Bleeding on probing increased with time in both materials investigated ($p < 0.05$) with no statistical relationship between bleeding on probing and probing depth. Microbiological data from that analysis, published in two different manuscripts (de Oliveira Silva et al. 2020; de Freitas et al. 2021), confirmed that, as expected, microbiological counts of subgingival and supragingival samples were significantly lower at baseline (T0) in each of the groups. Therefore, significant differences were found when comparing the microbial genome counts of the different sites and substrates after 3 years in function. The lowest microbial diversity in the subgingival samples was obviously observed at the beginning of the study. Of the 37 species that the authors aimed to analyze, only five colonized the peri-implant sulci of titanium rehabilitated (*Treponema denticola*, *Streptoccocus sanguinis*, *Streptoccocus gordonii*, *Streptoccocus sobrinus*, and *Veillonella parvula*) and zirconia rehabilitated (*Porphyromonas gingivalis*, *Streptoccocus mitis*, *Aggregatibacter actinomycetemcomitans* serotype a., *Veillonella parvula*, and *Peptostreptoccocus anaerobius*) at baseline. However, after 1 year of loading, microbiological diversity was higher, and all investigated species colonized the biofilms. Moreover, the species detected were found in greater numbers as the analysis points evolved over the years. In contrast to these previous studies, in our study, the Chao richness index decreased over time, in contrast to Lu and co-workers, who reported that microbial richness measured through the Chao1 index did not show significant differences between the three follow-up times (Lu et al. 2021). In our study, the number of species also decreased significantly after 5 years of evolution, but the microbial alpha-diversity kept constant during the time, in opposition to de Oliveira Silva and co-workers, who reported the lowest diversity at the beginning of the study (de Oliveira Silva et al. 2020; de Freitas et al. 2021).

Finally, it is also highly important to consider other theories that propose, in summary, that rather than "who they are", we should consider "what they are doing" (Takahashi 2015). In fact, studies by Dabdoud et al. in 2016 concluded that specific genes but not species of bacteria were stronger in the potential to differentiate between health and disease (Dabdoub et al. 2016). These conclusions are the result of using highly potent biochemical methods and advanced computation. Advances in this area are continuous.

The present study has some limitations. Firstly, the sample size was small, although a power analysis was performed on the basis of previous studies that guaranteed statistical significance. Likewise, the clinical follow-up of all patients could not be uniform in each analysis period due to personal reasons of some patients. The microbiological study was carried out with respect to the total DNA of the samples taken from the peri-implant sulci, which entailed a greater purification of the sample, since DNA from origins other than bacterial was obtained. This also meant having to withdraw some samples that did not guarantee the number of reads necessary for an adequate analysis. In addition, more advanced computational methods could be applied in order to deepen the understanding of the samples under evaluation.

5 | Conclusion

In conclusion, in the present study, implants with multiphosphonate-treated surfaces, loaded under two different functional protocols, showed no differences in clinical and radiographic variables after 5 years of function. From a microbiological point of view, although there was an evolution of the microbiome of the peri-implant sulcus towards Socransky's red circle putative pathogenic bacteria, no microorganism showed significant correlation with the radiographic changes produced in the peri-implant bone over time. Future research with larger populations should ensure that it is possible to discern whether peri-implant bone changes are a consequence of the pathogenic action of specific microorganisms, or whether, on the contrary, the presence of specific microorganisms in specific biological environments is a consequence of the adaptation of these microorganisms to the changes that gradually occur in the tissue.

Author Contributions

Pablo Galindo-Moreno: conceptualization, methodology, investigation, resources, writing – original draft, writing – review and editing, supervision, project administration, funding acquisition. **Lourdes Gutierrez-Garrido:** investigation, data curation, writing – review and editing. **Juan Duarte:** formal analysis, resources, data curation, writing – original draft, writing – review and editing, visualization. **Iñaki Robles-Vera:** formal analysis, writing – review and editing. **Natividad Martin-Morales:** investigation, writing – review and editing. **Francisco O'Valle:** data curation, visualization, writing – review and editing. **Allinson Olaechea:** investigation, writing – review and editing, writing – original draft. **Ana Belén Carrillo-Galvez:** investigation, writing – review and editing. **Miguel Padial-Molina:** conceptualization, methodology, validation, formal analysis, resources, data curation, writing – review and editing, visualization, project administration.

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Ethics Statement

All procedures performed in studies involving data from human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The protocol was previously approved by the Ethics Committee on Human Research from the University of Granada (216/CEIH/2016).

Consent

Written informed consent for this randomized clinical study was obtained from each patient before any study procedures were initiated.

Conflicts of Interest

Pablo Galindo-Moreno lectures regularly for MIS Implants Technologies Ltd. and Dentsply Sirona, among other companies, although there is no commercial interest or royalties on any products mentioned in this manuscript. The other authors declare no conflicts of interest. None of the funders have influenced the reporting of the outcomes in any way.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.