Anitua et al. BMC Oral Health (2024) 24:1001
https://doi.org/10.1186/s12903-024-04675-y

BMC Oral Health

RESEARCH

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Assessing peri-implant bacterial community structure: the effect of microbiome sample collection method

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Abstract

Background Oral microbiota comprises polymicrobial communities shaped by mutualistic coevolution with the host, contributing to homeostasis and regulating immune function. Nevertheless, dysbiosis of oral bacterial communities is associated with a number of clinical symptoms that ranges from infections to oral cancer. Peri-implant diseases are biofilm-associated inflammatory conditions affecting the soft and hard tissues around dental implants. Characterization and identification of the biofilm community are essential for the understanding of the pathophysiology of such diseases. For that sampling methods should be representative of the biofilm communities Therefore, there is a need to know the effect of different sampling strategies on the biofilm characterization by next generation sequencing.

Methods With the aim of selecting an appropriate microbiome sampling procedure for periimplant biofilms, next generation sequencing was used for characterizing the bacterial communities obtained by three different sampling strategies two months after transepithelial abutment placement: adjacent periodontal crevicular fluid (ToCF), crevicular fluid from transepithelial abutment (TACF) and transepithelial abutment (TA).

Results Significant differences in multiple alpha diversity indices were detected at both the OTU and the genus level between different sampling procedures. Differentially abundant taxa were detected between sample collection strategies, including peri-implant health and disease related taxa. At the community level significant differences were also detected between TACF and TA and also between TA and ToCF. Moreover, differential network properties and association patterns were identified.

Conclusions The selection of sample collection strategy can significantly affect the community composition and structure.

Trial registration This research is part of a randomized clinical trial that was designed to assess the effect of transepithelial abutment surface on the biofilm formation. The trial was registered at Trial Registration ClinicalTrials.gov under the number NCT03554876.

Keywords Oral microbiota, Metagenomics, Transepithelial abutment, Crevicular fluid, Diversity

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Background

Peri-implant health is defined by the absence of erythema, redness, bleeding on probing, swelling and suppuration around dental implants. Despite high implant survival rates, different biological complications can affect osseointegrated implants, including peri-implant mucositis and peri-implantitis [58]. Peri-implantitis is an oral inflammatory process that affects the surrounding tissues of osseointegrated implants and results in the loss of supporting bone and destruction of soft tissues [99]. According to Daubert et al. [29] and Konstantinidis et al. [59], the prevalence of peri-implant disease range between 13 and 25%, leading to a significant increase of patient morbidity, economic burden and eventual implant loss [5, 32]. As is the case for periodontal diseases [67], the primary etiological factor in the development of peri-implant diseases is the biofilm, which is a complex synthropic microbial community consisting of adherent cells embedded within a matrix composed of extracellular polymeric substances [46]. However, some discrepancies have been identified in the biofilms from peri-implantitis and periodontitis sites [8, 79].

Approximately 700 species of *Prokaryota* have been identified in the oral cavity, predominantly ascribed to 12 phyla: *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Spirochaetes*, SR1, *Synergistetes*, *Saccharibacteria* (TM7) and *Gracilibacteria* [93]. Specifically, the mouth contains distinct niches with dynamic microbial communities, including saliva, gingiva, the hard and soft tissues of teeth, the tonsil, the gingival sulcus, the throat, the hard and soft palates and the buccal mucosa [31], and eventually dental implants [23]. These surfaces—whose differing chemistry, topography and stability provide different habitats for microorganisms—are colonized preferentially by different bacteria via surface-attachments, movements and complex interactions, resulting in spatial compositional variability [72]. Furthermore, oral bacterial communities exist in multiplex dynamic equilibrium states, with large and rapid changes in composition and activity in a temporal dimension in response to environmental conditions [44]. The microbial communities are in symbiosis with the host shaped by co-evolution, contributing to digestion and homeostasis, neurological signaling, regulating immune and endocrine functions, modifying metabolism and eliminating toxins [37, 41]. Nevertheless, under certain conditions, several pathogenic strains that are usually dominated by commensal bacteria can proliferate [84], and even some commensal bacteria can transit to a pathogenic lifestyle via complex changes involving gene expression patterns, the core genome and the pan-genome [70, 90, 115].

Several studies aiming to compare oral bacterial communities under health and disease conditions have been conducted [14, 38, 56], some of them focused on characterizing peri-implant and periodontal microbiota and its pathological changes [36]. The bacterial profile associated with peri-implant disease was reviewed by Pérez-Chaparro et al. [85], Rakic et al. [94], Sahrmann et al. [98], Butera et al. [15], Gazil et al. [43] and Rodríguez-Archilla and Palma-Casiano [96]. These authors revealed that the core peri-implantitis microbiome is enriched in periodontal-inflammation related taxa, including *Fusobacterium nucleatum*, *Parvimonas micra* and *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Prevotella nigrescens*, *Treponema denticola*, *Tannerella forsythia*, *Campylobacter rectus* and *Porphyromonas gingivalis*. According to most published research findings, oral bacterial communities from healthy implants, peri-implantitis and periodontitis sites show contrasting diversity and composition [28, 43, 105, 119]. However, conclusions regarding the role of individual taxa in oral pathogenesis are difficult to draw due to the presence of confounding factors, variability in experimental designs, interindividual variability and complex ecological communities. Furthermore, bacterial actions are often secondary to immunologic imbalance [4, 75].

Due to the irreversible nature of peri-implantitis, prognosis-based early therapeutic intervention is the best strategy to arrest the progression of the disease and prevent implant failure [114]. Thus, a wide range of early diagnosis methods have been suggested [19, 22, 95]. In this sense, molecular techniques—particularly metagenomic Next Generation Sequencing tests (mNGS)—are powerful approaches for the characterization of microbial communities in the oral cavity, and also for the detection and surveillance of obligate or facultative oral pathogens associated with peri-implantitis [50, 103]. Considering the high variability in microbiota composition between different oral microbial habitats at multiple scales, the selection of appropriate sampling methods is a key aspect of implant disease risk assessment [6]. Sample collection via direct removal of transepithelial abutment and bacterial DNA isolation from transepithelial abutment surface could be considered the most representative sampling strategy, but this is a destructive method and the replacement of the transepithelial abutment with another is required (thus making sampling harder, increasing the economic burden and preventing the possibility of long-term monitoring of the bacterial community in that particular transepithelial abutment).

Crevicular fluid collection via sterile paper points (either from an adjacent tooth or from the studied transepithelial abutment) was initially regarded as a promising approach, since it is nondestructive, fast and easy to carry out, and allows long-term sampling at different time points. The question is, could these alternative sampling strategies be considered representative of bacterial communities formed on transepithelial abutment surfaces? Are microbial communities of samples collected using these methods significantly different than those obtained by sampling directly transepithelial abutments? In what respect and to what level? Thus, the aim of this preliminary research was to select the most appropriate sampling approach to characterize bacterial biofilms from transepithelial abutment surfaces. For that, three sampling strategies were compared: crevicular fluid from an adjacent tooth (ToCF), crevicular fluid from transepithelial abutment (TACF) and transepithelial abutment (TA), with a particular focus on peri-implant or periodontal health and disease related taxa.

Methods

This research is part of a randomized clinical trial that was designed to assess the effect of transepithelial abutment surface on the biofilm formation. The trial was registered at Trial Registration ClinicalTrials.gov under the number NCT03554876. The study protocol and informed consent, in full accordance with the ethical principles of the Declaration of Helsinki of 1975, as revisited in 2000, were approved by the ethical committee of investigation with medicines of the Basque Country (FIBEA-06-EC/17/Multi-Im). This study constitutes a partial analysis of the results of this clinical trial aiming to assess the effect of sampling strategies on metagenomic outcomes. Additional information regarding the experimental design is provided as supplementary data (Supplementary Material 1). For the analysis of the effect of sampling strategy, transepithelial abutments with a machined surface were included. Patient selection for this randomized controlled clinical trial was based on the following criteria:

Inclusion criteria

- Patients with an age ≥ 18 years.
- Need for the placement of at least 3 dental implants.
- Complete mouth plaque index ≤ 20% and absence of active periodontal disease.
- Bleeding index ≤ 30%.
- Pocket probing depth at the adjacent teeth < 4 mm.
- No use of antibiotics in the last 6 months.
- Nonsmoker.
- Possibility of attending all the planned visits.
- Signing of informed consent.

Exclusion criteria

- Has severe hematological disease.
- Has received or receiving in the last 30 days at least one of the following treatments: radiotherapy, chemotherapy, Immunosuppressive therapies, systemic corticoids, and anticoagulants.
- Presence of malignancy, hemangioma or angioma at the site where dental implants will be placed.
- Patients receiving bisphosphonates (oral or systemic).
- Presence of metabolic osseous disease.
- Presence of diseases that affect the oral mucosa.
- Presence of diabetes mellites.
- Severe parafunctional habits and/or temporomandibular joint disorders.
- Pregnancy or breast-feeding.
- Physical or mental disability to maintain good oral hygiene.
- Participating in other study.
- Other disabilities to participate in the study.

Clinical procedure

A total of 12 patients received professional oral hygiene and instructed how to maintain a good oral hygiene. After implant insertion, transepithelial abutments with machined surface were connected to the implants. Two months later, microbiome samples were acquired using different strategies. Sterile paper points size 30 (Maillefer, Ballaigues, Switzerland) were utilized to collect periodontal crevicular fluid from adjacent teeth (ToCF) and peri-implant crevicular fluid (TACF). These samples were collected from at least one adjacent healthy tooth and also from each previously connected transepithelial abutment with machined surface. After crevicular fluid collection, transepithelial abutments (TA) were removed for processing. The collected samples were stored at -80 °C until bacterial DNA isolation.

DNA extraction

Total microbial metagenomic DNA from each sample was extracted using the DNeasy PowerBiofilm DNA isolation kit (Qiagen, Germany) following the manufacturer's instructions. The strips were homogenized (1 cycle at 6400 rpm for 30 s) with a Precellys 24 Tissue Homogenizer (Bertin Technologies, France) and the implants were homogenized with an IKA MS 3 digital vortex (IKA, Germany) for 10 min at 2250 rpm. DNA quantification and quality control was performed using a Nanodrop

8000 (Thermo Fisher Scientific, MA, USA) and a Qubit fluorometer (Thermo Fisher Scientific, MA, USA). Extracted DNA was kept frozen at −30 °C until library preparation.

Library preparation and sequencing

16S rRNA library preparation workflow for MiSeq sequencing platform was performed as suggested by Illumina. The specific primers for the 16S rRNA gene (v3- v4 region) were selected from Klindworth et al. [57] and combined with Illumina adapter overhang nucleotide sequences to obtain a single amplicon of approximately ~ 460 bp. After PCR product purification, dual index barcodes and Illumina sequencing adapters were attached using the Nextera XT v2 index kit (Illumina, CA, USA) as a previous step to pooling, libraries were quantified by LabChip GX touch HT nucleic acid analyzer together with DNA 5 K/RNA/CZE chip (PerkinElmer, MA, USA) and diluted for an estimated sequencing depth of ~ 100.000 reads per sample. finally, pooled libraries were denatured with NaOH and diluted with hybridization buffer (library loading concentration = 6 pm) before MiSeq (llumina, CA, USA) sequencing. PhiX was included in each run to serve as an internal control for these low diversity libraries. Paired-end sequencing (2 × 300 bp) was performed using MiSeq v3 reagent kits (600 cycles) (Illumina, CA, USA).

Data processing

Secondary analysis was performed on BaseSpace using the 16S metagenomics application (Illumina, CA, USA). After assembling, full-length sequences from paired ends were referenced against the Illumina-curated version of Greengenes Consortium Database. The classification step is based on ClassifyReads, a high-performance implementation of the Ribosomal Database Project (RDP) Classifier [112].

Statistical analyses

Statistical analyses applied to explore the structure of bacterial communities with respect to their diversity, composition and bacterial association patterns across sampling methods and differential taxa abundance were performed in R [92]. Graphical data analysis was performed via *ggplot2* [113] and *fantaxtic* R packages for data visualization [110].

Alpha diversity

In order to summarize the structure of the observed bacterial communities, a set of common alpha diversity metrics in mNGS data were computed for each sample at both the species and the genus level: observed richness, Chao index [20, 21], Abundance Coverage Estimator (ACE) [25, 77], Shannon index [101], Simpson indices [54] and Fisher's alpha index [39] using *vegan* [78], *phyloseq* [73] and*PMCMRplus* [88] R packages. Generalized linear mixed-effects models were constructed and ANOVA tests were computed using *lme4* to analyze the effect of sampling method on alpha diversity indices [7]. Assumptions underlying parametric in parametric statistics were checked in model residuals through visual inspection (QQ Plots and density distributions) and also by significance tests (Shapiro-Wilk and Levene's test for assessing normality and homoscedasticity). Multiple comparisons were performed via Bonferroni corrected post hoc tests in the *multcomp* package [52].

Data normalization

Library size was standardized across samples using different normalization approaches available in *metagenomeSeq* [81] and *NetCoMi* [87] R packages: Total Sum Scaling – TSS [18], Cummulative Sum Scaling – CSS [82] and Centered Log-Ratio transformation – CLR [2].

Differential taxa abundance

Differential abundance testing was performed at different taxonomic ranks via differential expression analyses based on multivariate differential association computed by *MaAsLin2* R package [71].

Beta diversity

The effect of sampling method on beta diversity was assessed via Permutational Analyses of Variance (PERMANOVA) tests (1000 permutations) based on Aitchison distance for the community composition at both the OTU and the genus level [1]. For visual inspection, dissimilarity networks (Aitchison distance) were also constructed using *NetCoMi* [87] R package.

Association networks

After constructing microbial association networks based on SparCC (Sparse Correlations for Compositional data) correlation measure [40], differential network analyses were conducted using the discordant method [104]. At both the OTU and the genus levels, differential plot networks were constructed and compared using the *NetCoMi* R package. A sparsification threshold of 0.5 was used for comparing global network properties, centrality measures and hub taxa in the *NetCoMi* package via permutation tests using 1000 permutations. Adjusted Rand Index (ARI) and Graphlet Correlation Distance (GCD) measures were calculated to assess whether the clustering solutions are more or less similar than expected at random or distance similarities [91, 116]. As a measure of conditional dependence, SPIEC-EASI (Sparse InversE Covariance estimation for Ecological Association and

Statistical Inference) pipeline was also applied to non-transformed data. Association network properties were computed, and keystone taxa were selected from node degree (number of connections) and betweenness (node centrality) measures using *SpiecEasi* R package [65].

Results

The mean age of study participants was 56 years, ranging from 38 to 71 years, whereas the sex composition was 7 females (58%) and 5 males (42%). A total of 15 implants were placed in the following positions: 14 (1), 25 (1), 26 (1), 27 (1), 34 (1), 36 (2), 37 (2), 45 (1), 46 (2) and 47 (3). The number of dental implants in which transepithelial abutments with machined surface were placed ranged from one to two (only one transepithelial abutment with machined surface was placed in a total of nine patients, while a total of two transepithelial abutments with machined surface were placed in the remaining three patients).

As shown in Fig. 1, a significant effect of sampling method on most bacterial alpha diversity indices was detected, at both the OTU level (observed [$p = 0.00090$], Chao index [$p = 0.0026$] and Fisher's alpha index [$p = 2.4E^{-05}$]) and at the genus level (observed [$p = 0.019$], Chao index [$p = 0.047$], Fisher's alpha index [$p = 0.00038$] and Simpson's indices [$p = 0.014$]). After performing pairwise comparisons, significant differences in observed richness, Chao and Fisher's alpha indices were identified between ToCF/TACF and TA at both the OTU and the genus level, and in Simpson's indices at the genus level. No significant differences were detected in Shannon or Simpson's diversity indices at the OTU ($p = 0.81$ and $p = 0.20$, in each case) or in the Shannon index at the genus level ($p = 0.30$).

According to Fig. 2, *Firmicutes* (39–53%), *Bacteroidota* (9.4–21%), *Actinobacteria* (6.7–8.9%), *Fusobacteria* (5.9–9%) and *Proteobacteria* (5.5–9.5%) are the most abundant phyla in the studied samples, with a higher proportion of *Firmicutes* in ToCF (53%) and TACF (52%), and more *Bacteroidota* (21%), *Fusobacteria* (9.0%) and *Synergistetes* (1.6%) in TA samples. Considering the class level, *Bacteroidia* (7.4–19%) and *Negativicutes* (7.3-13%) were two main taxa in all cases. Nevertheless, while the proportion *Actinomycetia* (8.3%) was higher in ToCF and that of *Bacilli* was higher in both ToCF and TACF (37%), the abundance of *Clostridia* was higher in TACF (7.6%) and TA samples (10%). The orders *Lactobacillales* (12-33%), *Bacteroidales* (7.5-19%), *Eubacteriales* (7.5-10%) and *Veillonellales* (5.4-11%) reached the highest abundances in all sample types, together with *Pasteurellales* (3.5%) in TA and *Actinomycetales* in ToCF (5.4%) and *Bacillales* in TACF (4.1%) and ToCF (5.3%). On the other hand, *Streptococcaceae* (8.4-22%), *Prevotellaceae* (4.5-12%), *Fusobacteriaceae* (3.9-8.0%) and *Veillonellaceae* (5.4-11%) were the most represented families in all sample types, along with *Selenomonadaceae* (2.5%) in ToCF, *Bifidobacteriaceae* (2.3%) and *Clostridiaceae* (3.0%) in TACF, *Enterococcaceae* and *Bacillaceae* in TACF (9.4% and 2.5% in each case) and ToCF (11% and 2.6% in each case), and *Peptostreptococcaceae* (6.9%), *Porphyromonadaceae* (6.1%), *Pasteurellaceae* (3.5%) and *Treponemaceae* (1.2%) in TA samples. At the genus level, *Streptococcus* (8.3-11%) and *Veillonella* (4.5-6.9%) were abundant genera in all sample types. Nevertheless, ToCF and TACF samples were richer in *Lactococcus* (9.0% and 14% in each case) and *Enterococcus* (11%), whereas *Prevotella* (11%), *Fusobacterium* (8.1%), *Porphyromonas* (5.8%) and *Peptostreptococcus* (4.9%) reached higher frequencies in TA samples.

As shown in Fig. 3, significant differences in taxa abundances between sampling methods were identified in 0.58% (10/1732) of the OTUs shared by ToCF and TA, and in 0.40% of the OTUs shared by TCFA and TA (7/1732). Significant changes were also detected after comparing TA and ToCF in 3.1% of the families (10/322), 4.1% of the orders (6/145), 8.7% of the classes (6/69) and 6.3% of the phyla (2/32); and TA and TACF in 0.60% (5/830) of the genera, 2.0% (6/306) of the families and in 1.4% (2/141) of the orders. Pairwise comparisons revealed significant changes in the frequency of some peri-implant or periodontal disease related taxa between different sampling methods. In TA samples, *Synergistetes*, *Slackia*, *Peptostreptococcus*, *Atopobium*, *Mogibacterium*, *Slackia exigua* or *Peptostreptococcus stomatis* showed increased abundance when compared to ToCF, whereas those of *Lactococcus*, *Enterococcus*, *Cellulomonas*, *Corynebacterium*, *Exiguobacterium*, *Bacillus*, *Microbacterium* and *Actinomyces naturae* were reduced. In contrast to TACF, TA samples also showed lower abundances of several taxa, including *Bacilli*, *Exiguobacterium*, *Enterococcus*, *Cellulomonas*, *Acinetobacter*, *Microbacterium*, *Paenibacillus*, *Lactococcus* and *Actinomyces naturae*.

Figure 4 shows compositional beta diversity biplot generated through Aitchison distance matrix considering taxa with at least 0.1% of sequencing reads. PERMANOVA analyses based on Aitchison distance showed that the effect of sampling method on bacterial community composition was statistically significant at both the OTU ($p = 0.0010$) and the genus level ($p = 0.0010$). Multilevel pairwise comparisons revealed significant differences between TA and ToCF (OTU level: $p = 0.0010$; genus level: $p = 0.0010$) and between TACF and TA (OTU level: $p = 0.0020$; genus level: $p = 0.0030$), but not between TACF and ToCF (OTU level: $p = 0.837$; genus level: $p = 0.797$).

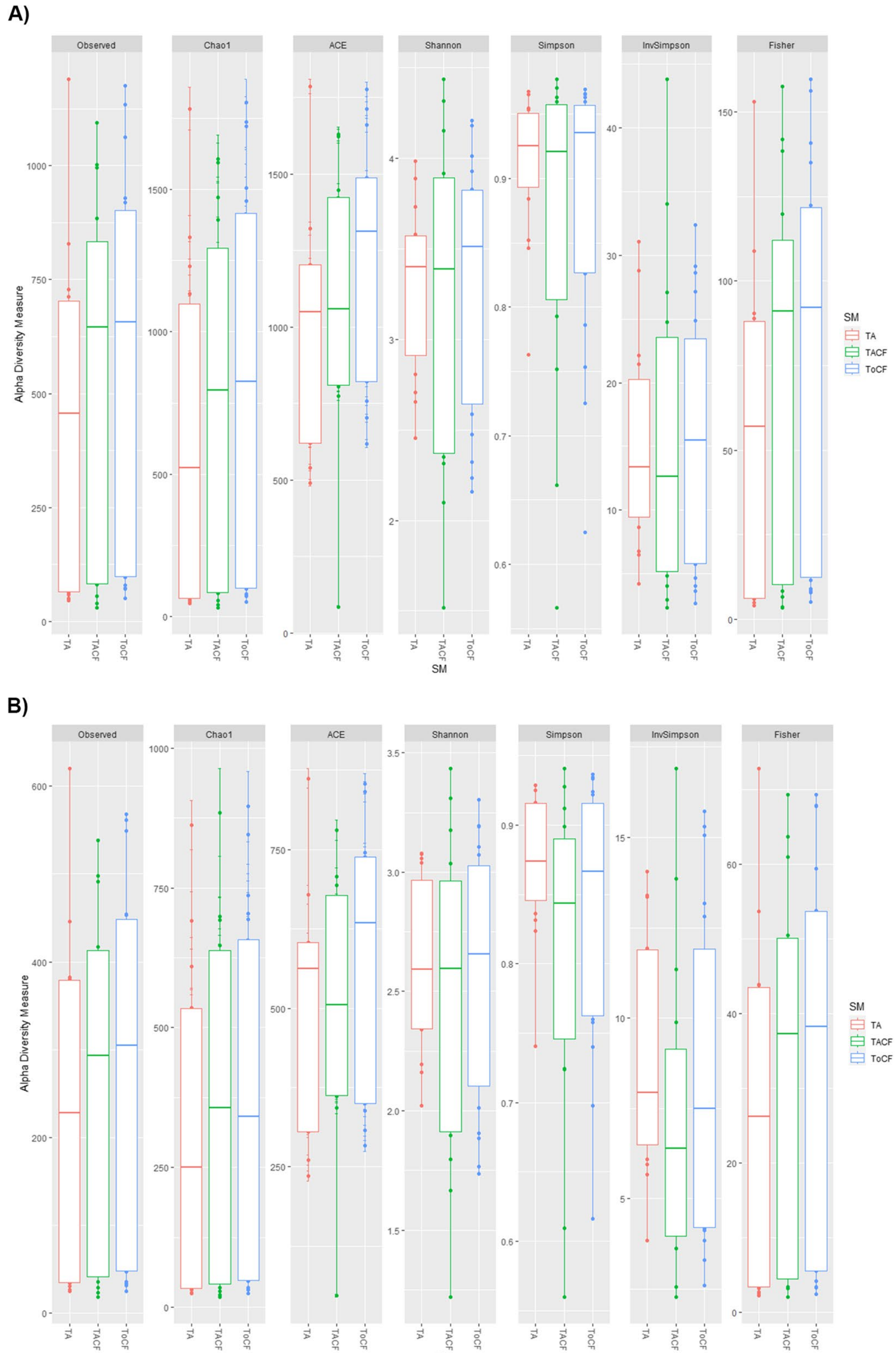

Fig. 1 Boxplot of alpha diversity indices reflecting taxa abundance and consistency in samples grouped by sampling method at: **A**) the OTU level, and **B**) the genus level

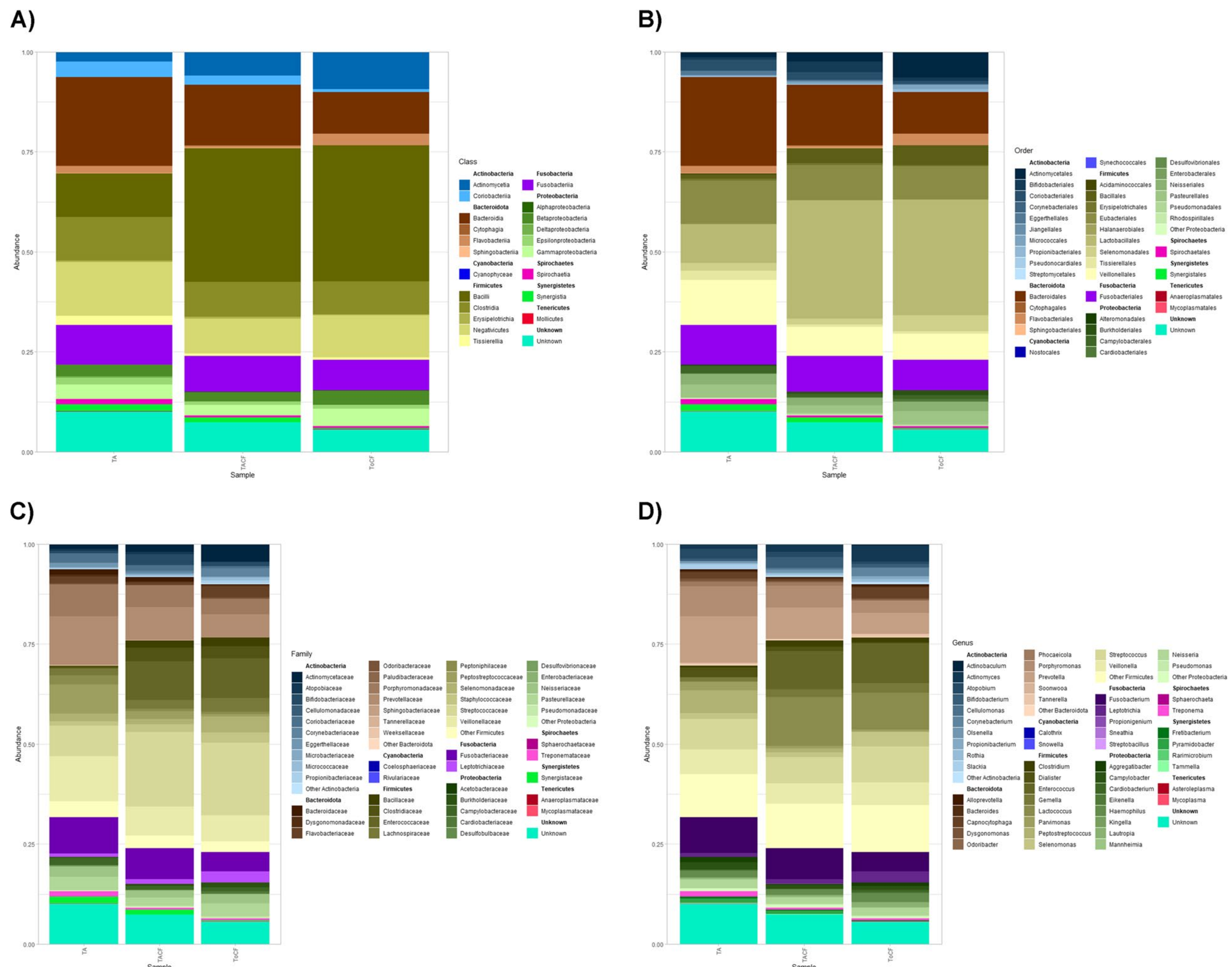

Fig. 2 Stacked bar graphs representing cumulative abundances of taxa that represent up to 10 most abundant taxa nested by *Phylum* (only top 10 phyla were plotted) and grouped by sampling method (TSS normalized data). Taxonomic ranks nested by *Phylum* were: **A**) the *Class* level, **B**) the *Order* level, **C**) the *Family* level, and **D**) the *Genus* level

Bacterial association networks of ToCF, TACF and TA communities constructed from SparCC correlations retaining taxa with at least 0.1% of sequencing reads are represented in Fig. 5, where nodes represent bacterial taxa and edges represent either co-presence (positive association) or mutual exclusion (negative association) relationships. At both the OTU and the genus levels, pairwise network comparisons revealed high clustering similarities in terms of ARI and GCD, and no significant changes in global network properties when comparing different sampling methods (Table 1). However, significant differences were detected in terms of hub taxa between ToCF and TA samples at the OTU level (Jaccard Index = 0.00, $p = 0.017$), with a higher influence of *Streptococcus* spp., *Veillonella atypica* and *Neisseria mucosa* in ToCF, and a particular importance of *Porphyromonas pasteri*, *Centipeda periodontii*, *Selenomonas sputigena*, *Veillonella tobetsuensis* or *Parvimonas micra* in TA samples (Table 2). Significant differences were also identified in betweenness centrality after comparing ToCF and TA sampling methods at the OTU level (Jaccard Index = 0.13, $p = 0.0033$). Nevertheless, no significant differences were observed after adjusting permutation *p*-values of the tests for differential centrality values for multiple testing. Further information on these measures is available in Supplementary Material 2.

After constructing SPIEC-EASI based ecological networks, the highest rated keystone OTUs attending to their node degree and betweenness were *Prevotella multisaccharivorax, Streptococcus dentapri, Peptococcus niger, Porphyromonas circumdentaria, Mycoplasma salivarium* and *Streptococcus gallinaceus* in ToCF, *Streptococcus oligofermentans, Actinomyces odontolyticus, Bacteroides heparinolyticus, Acidaminococcus*

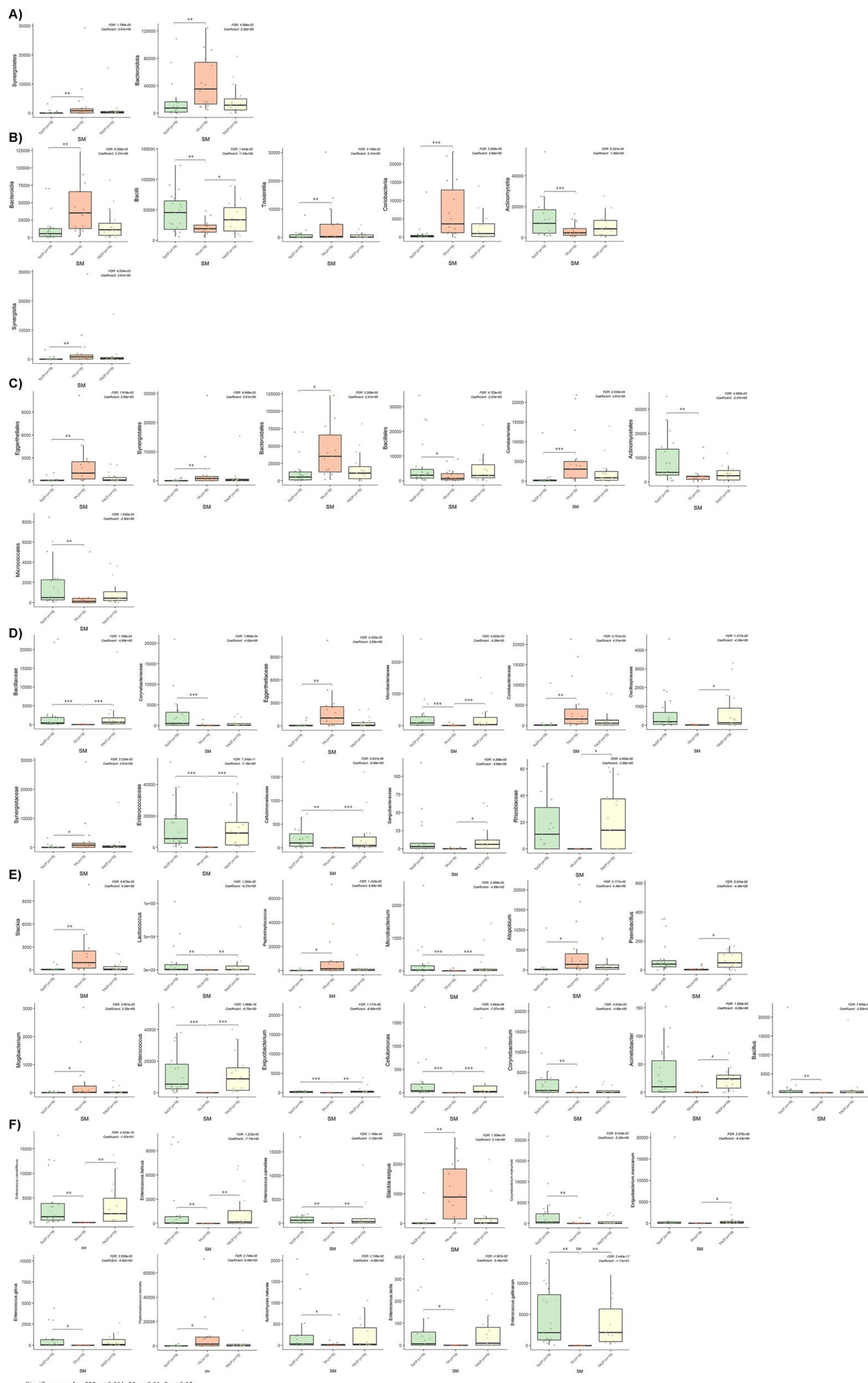

Fig. 3 Differentially abundant taxa between pairwise sampling method (SM) comparison identified through MaAsLin2 multivariate association testing: **A**) *Phylum* level, **B**) *Class* level, **C**) *Order* level, **D**) *Family* level, **E**) *Genus* level and **F**) OTU level

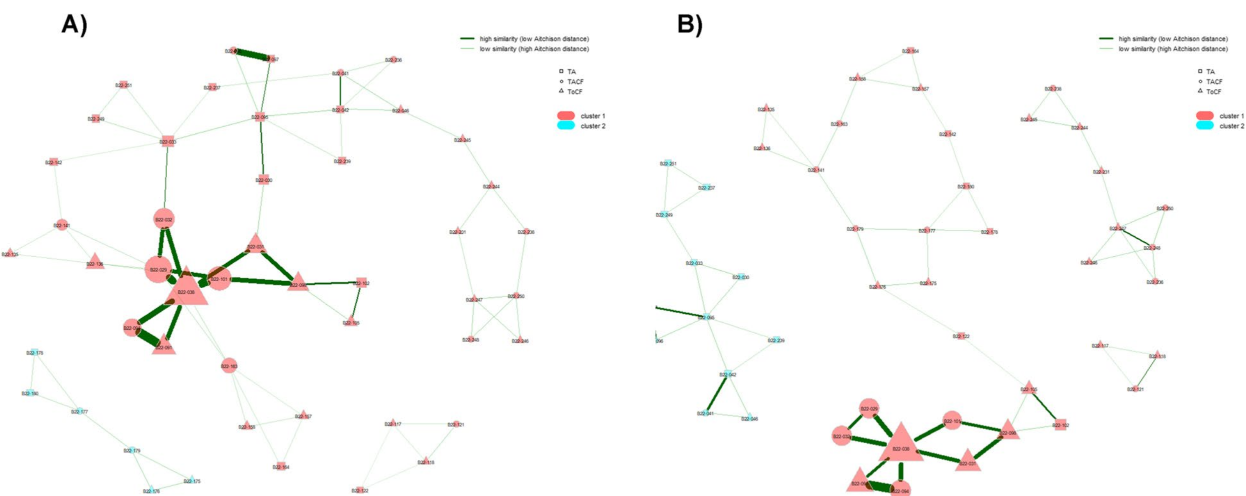

Fig. 4 Dissimilarity network based on Aitchison distance matrix representing the beta-diversity at: **A**) the OTU level, and **B**) the genus level retaining taxa with at least 0.1% of sequencing reads (zeros were replaced via multiplicative simple replacement and k-nearest neighbor was used as sparsification method)

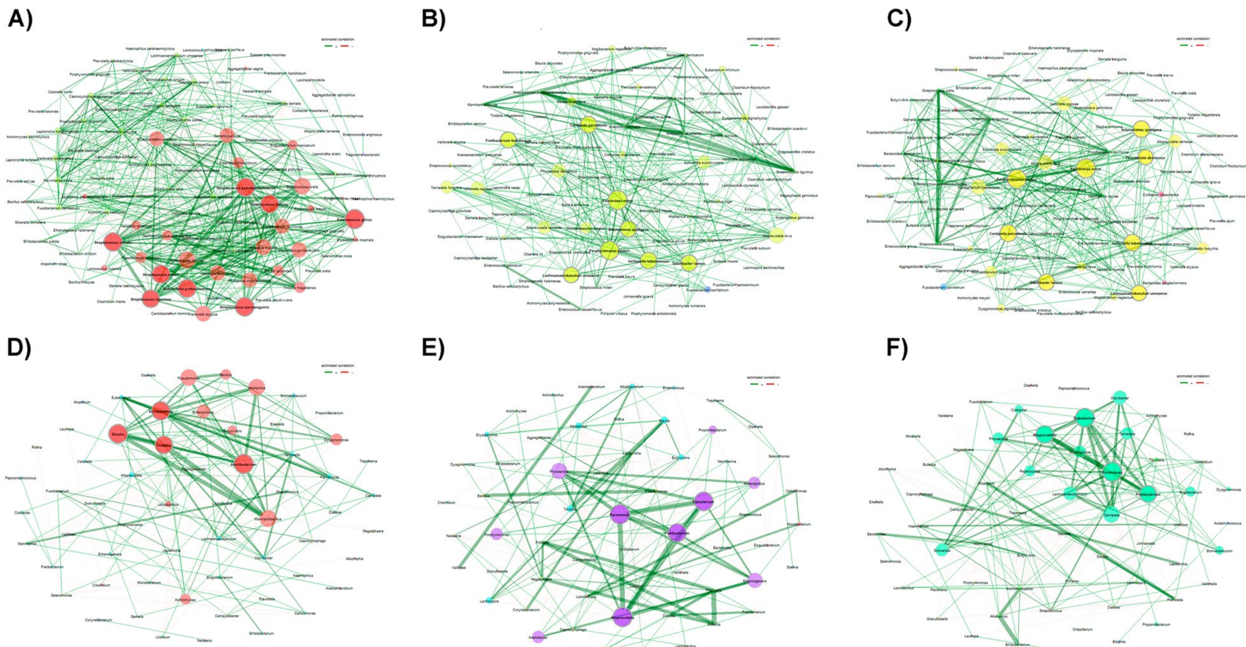

Fig. 5 Bacterial association networks from ToCF, TACF and TA at the OTU and genus levels constructed using SparCC correlation coefficients for compositional data retaining taxa with at least 0.1% of sequencing reads. For network sparsification, only edges corresponding to an absolute association greater or equal than 0.7 (OTU level) and 0.6 (*genus* level) were represented in order to improve network readability

intestini, *Moryella indoligenes* and *Leptotrichia wadei* in TACF, and *Rothia aeria*, *Streptococcus sobrinus*, *Moryella indoligenes*, *Actinobaculum massiliense*, *Prevotella paludivivens* and *Megasphaera sueciensis* in TA bacterial communities. At the genus level, keystone genera were *Butyrivibrio*, *Flavobacterium*, *Leptotrichia*, *Paracoccus*, *Pasteurella* and *Sphingomonas* in ToCF, *Haloactinobacterium, Porphyromonas, Sphingobacterium*, *Shuttleworthia*, *Moraxella* and *Propionibacterium* in TACF, and *Bacillus, Alloprevotella, Campylobacter, Pontibacillus, Eikenella* and *Parascardovia* in TA.

Differential network analyses (Fig. 6) revealed correlation changes between certain genera following pairwise comparisons. When comparing association patterns

Table 1 Results from testing global network metrics of the networks in Fig. 5 for group differences (1000 permutations). The computed measures for ToCF, TACF and TA, the absolute difference, and the *p*-value adjusted for multiple testing using the adaptative Benjamini-Hochberg method [11, 66] at both the OTU and the genus level are summarized

Whole network	**OTU level**					
Global network properties	**diff $_{ToCF-TACF}$**	***p*-value**	**diff $_{ToCF-TA}$**	***p*-value**	**diff $_{TACF-TA}$**	***p*-value**
Number of components	1.0	0.49	2.0	1.0	2.0	0.053
Clustering coefficient	0.027	0.60	0.021	1.0	0.0060	0.94
Modularity	0.059	0.29	0.0050	0.67	0.069	0.42
Positive edge percentage	1.7	0.51	0.22	1.0	5.9	0.092
Edge density	0.012	0.82	0.91	0.71	0.030	0.69
Natural connectivity	0.016	0.57	0.010	0.48	0.016	0.67
Adjusted Rand Index (ARI)	0.38	0.0	0.40	0.0	0.29	0.0
Graphlet Correlation Distance (GCD)	0.29	0.97	0.74	0.63	0.92	0.63
Whole network	**Genus level**					
Global network properties	**diff $_{ToCF-TACF}$**	***p*-value**	**diff $_{ToCF-TA}$**	***p*-value**	**diff $_{TACF-TA}$**	***p*-value**
Number of components	0.0	1.0	1.0	0.61	1.0	0.56
Clustering coefficient	0.0020	0.97	0.028	0.72	0.029	0.74
Modularity	0.0040	0.96	0.0080	0.94	0.014	0.92
Positive edge percentage	4.2	0.36	2.9	0.69	2.7	0.76
Edge density	0.0010	0.99	0.048	0.41	0.011	0.81
Natural connectivity	0.0030	0.87	0.023	0.11	0.0030	0.83
Adjusted Rand Index (ARI)	0.26	0.0	0.23	0.0	0.26	0.0
Graphlet Correlation Distance (GCD)	0.76	0.79	1.4	0.36	1.1	0.60

Significance codes: ***: $p \leq 0.001$; **: $p \leq 0.01$; *: $p \leq 0.05$

from TACF and TA samples, the differential network analysis revealed significant correlation changes between a large number of genera, including *Clostridium* and *Bifidobacterium*, *Bifidobacterium* and *Dialister*, *Fusobacterium* and *Haemophilus or Actinomyces odontolyticus* and *Veillonella atypica* (inversely correlated in TACF and no correlated in TA), and *Peptostreptococcus and Haemophilus, Campylobacter and Oribacterium or Bifidobacterium* and *Porphyromonas* (no correlated in TACF and negatively correlated in TA). Pairwise comparisons between TA and ToCF sampling methods also showed strong positive correlations in ToCF samples that remained no significant in TA samples (*Fusobacterium* and *Selenomonas*, *Alkaliphilus* and *Dysgonomonas*, *Dysgonomonas* and *Actinomyces*, *Gemella* and *Haemophilus*, *Rothia* and *Streptococcus*, *Rothia* and *Parascardovia*, or *Haemophilus* and *Lautropia*) and viceversa (*Megasphaera* and *Prevotella*). Moreover, some negative correlations between taxa detected in ToCF samples were not identified in TA samples (*Treponema and Rothia,* and *Selenomonas* and *Rothia*) and viceversa (*Lautropia mirabilis* and *Haemophilus parainfluenciae, Actinomyces odontolyticus* and *Prevotella maculosa*, or *Atopobium parvulum* and *Streptococcus cristatus*). No significant differential correlations were observed between ToCF and TACF samples.

The effect of sampling strategy on the metagenomic profile of transepithelial abutments at different levels of analysis, including alpha diversity, beta diversity, differential taxa abundance and association network properties, was summarized in Table 3.

Discussion

When considering healthy peri-implant environments, bacterial colonization of the implant surface tends to be like healthy surrounding periodontal sites, with lower diversity [49]. However, it evolves toward the establishment of organized biofilms within the next two weeks [9]. Despite the fact that salivary pellicles adsorbed to implant surfaces promote the adhesion of microbes, its molecular features (chemical composition) and the immunological microenvironment determine the microbial colonization process [33]. In the present study, certain alpha diversity metrics (Observed taxa, Chao index, Fisher's alpha index and Simpson's indices) have revealed significant differences between crevicular fluid samples (ToCF and/or TACF) and transepithelial abutments (TA). Differential statistical significance between diversity metrics could be explained by the varying influence of richness, evenness and dominance in each index. Considering that TA samples, as opposed to crevicular fluid samples, account not only free-living bacteria around transepithelial abutments, but also most biofilm forming surface adhered bacteria, these results suggest that tooth surface could harbor a richer microbiota than TA surface. These results

Table 2 Jaccard index values (j) corresponding to Fig. 5 accounting the similarity of the sets of most central nodes (centrality value above the empirical 70% quantile) and also of the sets of hub taxa between pairs of networks (j is 0 if the sets are completely different and 1 for exactly equal sets)

OTU level									
Jaccard Index (similatity between sets of most central nodes)									
Centrality measures	$j_{ToCF\text{-}TACF}$	$P(J \le j)$	$P(J \ge j)$	$j_{ToCF\text{-}TA}$	$P(J \le j)$	$P(J \ge j)$	$j_{TACF\text{-}TA}$	$P(J \le j)$	$P(J \ge j)$
degree	0.53	0.99	0.019***	0.62	1.0	0.0030**	0.54	0.99	0.022*
betweenness centrality	0.24	0.12	0.93	0.13	0.0033**	1.0	0.27	0.26	0.85
closeness centrality	0.63	1.0	0.00069***	0.63	1.0	0.0015**	0.57	1.0	0.0082**
eigenvector centrality	0.68	1.0	0.000094***	0.38	0.76	0.37	0.52	0.99	0.031*
hub taxa	0.50	0.91	0.26	0.0	0.017*	1.0	1.0	1.0	0.0041**
Hub taxa									
Shared	*Enterococcus gilvus*, *Enterococcus italicus*, *Streptococcus infantis* and *Streptococcus pseudopneumoniae*			-			*Streptococcus infantis*, *Streptococcus oralis*, *Streptococcus pseudopneumoniae*, *Streptocococcus tigurinus* and *Fusobacterium naviforme*		
Not shared	*Neisseria mucosa*, *Streptococcus infantis*, *Streptococcus tigurinus*, *Actinomyces naturae* and *Blautia coccoides*			*Streptococcus infantis*, *Streptococcus pseudopneumoniae*, *Streptococcus tigurinus*, *Veillonella atypica*, *Neisseria mucosa*, *Porphyromonas pasteri*, *Centipeda periodontii*, *Selenomonas sputigena*, *Veillonella tobetsuensis* and *Parvimonas micra*			-		
Genus level									
Jaccard Index (similatity between sets of most central nodes)									
Centrality measures	$j_{ToCF\text{-}TACF}$	$P(J \le j)$	$P(J \ge j)$	$j_{ToCF\text{-}TA}$	$P(J \le j)$	$P(J \ge j)$	$j_{TACF\text{-}TA}$	$P(J \le j)$	$P(J \ge j)$
degree	0.22	0.23	0.90	0.22	0.23	0.90	0.17	0.10	0.97
betweenness centrality	0.27	0.36	0.79	0.20	0.15	0.94	0.20	0.15	0.94
closeness centrality	0.40	0.81	0.34	0.41	0.83	0.33	0.41	0.83	0.33
eigenvector centrality	0.22	0.17	0.92	0.50	0.95	0.13	0.50	0.95	0.13
hub taxa	0.0	0.088	1.00	0.50	0.89	0.41	0.50	0.89	0.41
Hub taxa									
Shared	-			*Parvimonas* and *Eubacterium*			*Parvimonas* and *Eubacterium*		
Not shared	*Alkalibacterium*, *Blautia*, *Erysipelothrix*, *Alloprevotella*, *Eubacterium* and *Parvimonas*			*Tannerella* and *Centipeda*			*Bulleidia* and *Alloprevotella*		

Significance codes: ***: $p \le 0.001$; **: $p \le 0.01$; *: $p \le 0.05$

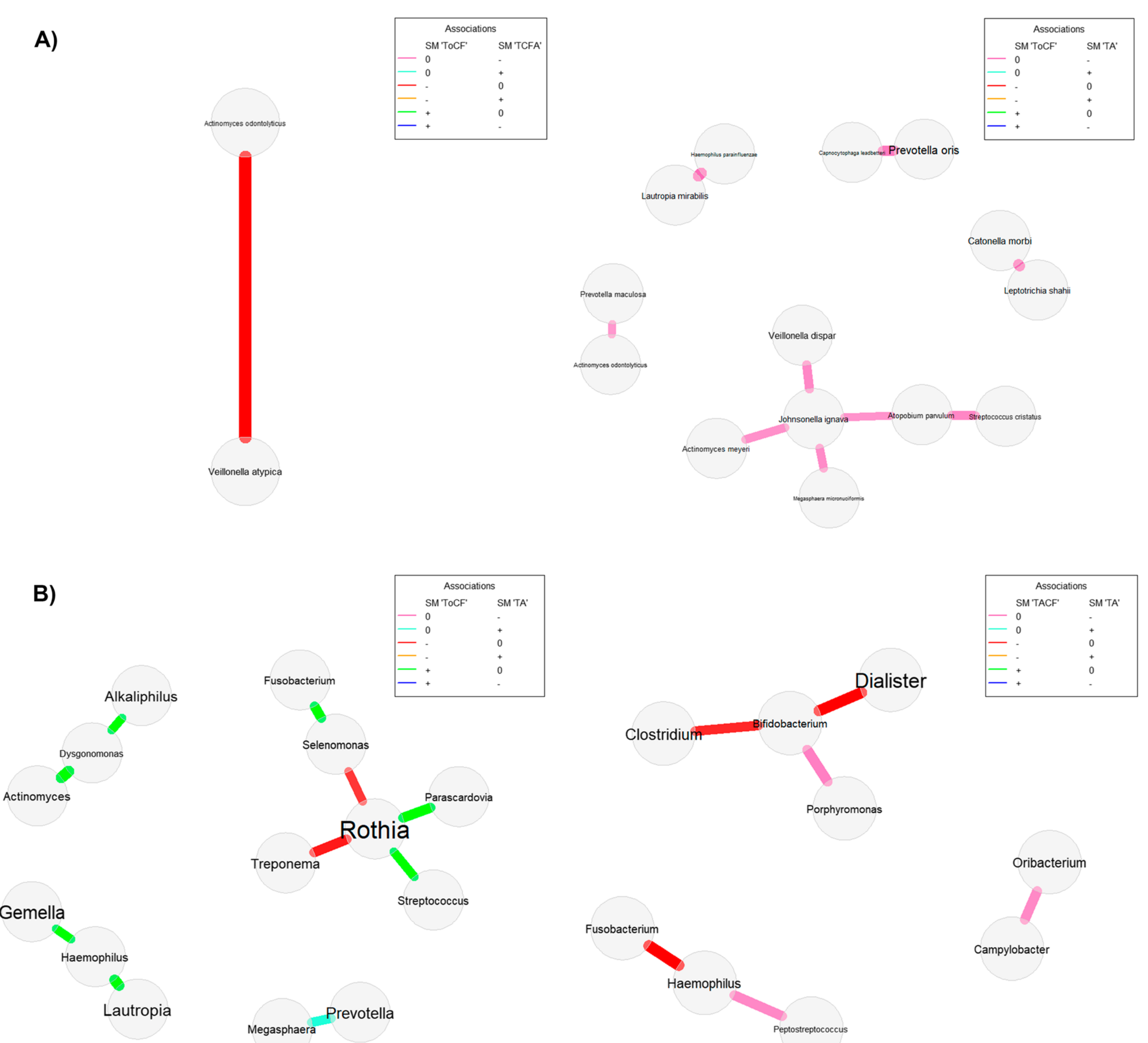

Fig. 6 Differential association networks based on Fig. 5: **A**) OTU level and **B**) *Genus* level

Table 3 Summary of the effect of sampling method on the description of the bacterial communities from transepithelial abutments

Component	Variable	Differences
Alpha diversity	Richness	Yes
	Diversity	Fisher's alpha (OTU and genus) and Simpson's indices (genus)
Beta diversity	Overall taxonomic composition	Yes
Differential abundance		Yes
Association network analysis	Global network properties	No
	Centrality measures	No
	Hub taxa	Yes
	Differential associations	Yes

– indicating a lower diversity in peri-implant microbiota—are in agreement with those of Dabdoub et al. [28] and Payne et al. [83]. Nevertheless, bacterial diversity in peri-implantitis sites is usually higher than that of healthy periodontal sites [106, 120], but lower than that of periodontitis environments [8].

According to the consulted bibliography, differentially abundant taxa related to healthy sites include mainly gram-positive cocci and non-motile bacilli, with higher frequencies of lactic acid bacteria (*Lactobacillales* and *Bifidobacterium*) [47], *Lactococcus* [15], *Haemophilus* [97], *Veillonella* [97, 102, 109], *Streptococcus* [97, 109], *Neisseria* [97], *Rothia* [97], *Prevotella* [42, 109], *Actinomyces* [64, 109]), *Leptotrichia* [15, 64], *Gemella* spp. [102], *Vibrio* [42], *Brevundimonas* [15, 118], *Pseudomonas* [118], *Oribacterium* spp., *Selenomonas* spp. and *Cardiobacterium* spp. [28], *Staphylococcus* [105], *Granulicatella adjacens*, *Veillonella dispar*, *Actinomyces meyeri* and *Streptococcus mitis* [27], *Propionibacterium acnes* [120], *Acinetobacter*, *Paracoccus* and *Moraxella* [45], *Dialister* [61], *Abiotrophia defectiva* [111], *Microbacterium* [108], *Corynebacterium* ([105]), *Novosphingobium capsulatum* [36], *Propionibacter*, *Lautropia*, *Chitinophagaceae, Brevundimonas nasdae, Delftia acidivorans*, *Rothia aeria*, *Anaerofilum pentosovorans*, *Anaerofilum agile*, *Pseudoramibacter alactolyticus* and *Porphyromonas* HMT-277/278 [15]. Moreover, Kroeger et al. [63] found higher abundances of *Lautropia mirabilis*, *Rhodobacteriaceae* or *Bergeyella* in shallow peri-implant pockets.

On the other hand, most published research findings confirm that peri-implantitis associated bacteria consist mainly of gram-negative motile anaerobic periopathogens and opportunistic bacteria, including higher abundancies of *Porphyromonas gingivalis* ([3, 10, 55, 68, 86, 97, 103, 107, 109, 117]), *Aggregatibacter actinomycetemcomitans* ([53, 68, 117]), *Prevotella intermedia* ([55, 68, 74, 109]), *Prevotella nigrescens* [68, 74], *Treponema denticola* ([17, 85, 103]), *Tannerella forsythia* ([3, 10, 74, 86, 103, 117]), *Fusobacterium nucleatum* ([3, 10, 55, 109]), *Parvimonas micra* [17, 62, 109], *Eubacterium* [64, 109], *Butyrivibrio* [28, 64], *Filifactor alocis* [97, 109], *Pseudoramibacter* [60], *Desulfobulbus* [16, 34], *Streptococcus* ([27, 55, 109]), *Exiguobacterium* [24], *Streptococcus mutans* and *Peptococcus* [28, 64]. Koyanagi et al. [62] reported that *Chloroflexi*, *Tenericutes*, *Synergistetes*, *Peptostreptococcus stomatis* and *Solobacterium moorei* were detected only in peri-implantitis sites.

Peri-implantitis has been also associated with higher relative frequencies of *Eikenella corrodens* [17], *Streptococcus intermedius*, *Streptococcus mitis*, *Haemophilus influenzae* and *Treponema socranskii* [86], *Campyblobacter gracilis*, *Dialister invisus*, *Eubacterium infirmum* and *Mitsuokella* (da Silva et al. 2013), *Campylobacter rectus* [17, 26], *Fusobacterium* [64, 74], *Slackia exigua*, *Parascardovia denticolens* and *Centipeda periodontii* [109], *Mycoplasma* and *Treponema* [64], *Streptococcus non-mutans* [28], *Neisseria*, *Kingella*, *Enterococcus*, *Fretibacterium* and *Bacillus* [55], *Propionibacterium*, *Paludibacter*, *Staphylococcus*, *Filifactor* and *Mogibacterium* [105], *Treponema maltophilum* [97], *Olsenella* and *Sphingomonas* [76], *Veillonella* [30, 64], *Treponema* [63, 117], *Prevotella tannerae* [35], *Actinomyces* [28], *Fretibacterium fastidiosum* [97], *Pseudoramibacter alactolyticus* [28, 62], *Campylobacter* [64] and *Staphylococcus aureus* [86], *Stenotrophomonas*, *Leuconostoc*, *Faecalibacterium prausnitzii*, *Haemophilus parainfluenzae*, *Prevotella copri*, *Bacteroides vulgatus* and *Bacteroides stercoris* [80]. Furthermore, the following taxa are also strongly associated with subgingival plaque of periodontitis: *Alloprevotella*, *Phocaeicola*, *Johnsonella* and *Mycoplasma* [24].

Oral micro-habitats, including teeth, transepithelial abutments and crevicular fluid, provide unique biological niches that harbors specific bacterial communities. Compositional variation in oral microbiota related to sampling origin has been highlighted by multiple studies, including crevicular fluid around dental prostheses that were fabricated by various biomaterials and fabrication techniques [13, 48], supra and subgingival biofilm [103, 117] and different peri-implantitis lesions [63, 89]. Moreover, dental intervention-related perturbations lead to significant environmental changes at the microscale level, including surface topology, chemistry and immunological response, resulting in dysbiosis and structural disruption of the oral microbiota. Compositional variation was higher between bacterial communities from transepithelial abutments (TA) and periodontal crevicular fluid (ToCF). In this sense, a significant reduction in certain peri-implant or periodontal health related taxa was observed in TA when compared to ToCF (*Bacilli*, *Actinomycetia* and *Lactococcus*), while several of those related with oral dysbiosis showed increased relative frequencies (*Synergistetes*, *Peptostreptococcus*, *Slackia, Atopobium* and *Mogibacterium*).

Considering higher taxonomic ranks, higher abundances of certain taxa after implant insertion (*Bacteroidota*, *Synergistetes* and *Coriobacteriaceae*) and lower frequencies of *Actinomycetia*, *Bacillaceae, Cellulomonadaceae* and *Enterococcaceae* were identified in TA samples. In this context, Heyman et al. [51] reported analogous population trends for *Bacteroidota* and *Coriobacteriaceae* after implant placement using a murine model. On the other hand, certain peri-implant and periodontal disease related taxa, including *Mogibacterium*, *Slackia*, *Peptostreptococcus*, *Atopobium*, *Slackia exigua*

and *Peptostreptococcus stomatis* showed increased abundances in transepithelial abutments. Similar results were obtained after comparing crevicular fluid from transepithelial abutments (TACF) with transepithelial abutments (TA), with lower frequencies of several opportunistic commensal enterococci, *Cellulomonas, Lactococcus* and *Actinomyces naturae* in TA samples. When compared with crevicular fluid samples extracted transepithelial abutments, TA were also depleted in other taxa, including *Bacilli*, *Paenibacillus*, *Exiguobacterium*, *Microbacterium* and *Corynebacterium*. These results do not agree with those of Schaumann et al. [100], who concluded that peri-implant and periodontal tissues share a similar biofilm composition at the genus level.

Dental intervention-related perturbations lead to significant environmental changes at the microscale level, including surface topology and chemistry, resulting in dysbiosis and structural disruption of the oral microbiota. Abundance-based associations also revealed differential correlation across sampling methods, particularly when TA was compared to ToCF. More specifically, differential associations affecting mainly peri-implant health and peri-implantitis related taxa (*Bifidobacterium*, *Haemophilus*, *Campylobacter*, *Peptostreptococcus*, *Porphyromonas*, *Treponema*, *Selenomonas*, *Fusobacterium, Rothia* and *Prevotella*) were detected after performing pairwise comparisons. Moreover, significant variations regarding hub OTUs were identified between ToCF and TA. These findings suggest that both peri-implant and crevicular fluid bacterial communities are characterized by a particular distinctive associational footprint as opposed to that of the adjacent teeth or transepithelial abutments and also that environmental gradients and complex ecological interactions contribute to variations in ecosystem structure and function.

According to the results found in the present study, the abundances of most peri-implantitis or periodontal disease related taxa were higher in TA samples, whereas those of peri-implant health associated bacteria were higher in ToCF or TACF. Thus, current data suggest that implant micro-habitats are usually characterized by a dysbisotic shift, enriched in *Synergistetes, Bacteroidota*, *Atopobium*, *Slackia*, *Peptostreptococcus*, *Mogibacterium* and *Slackia exigua*, and depleted in *Bacillus*, *Paenibacillus*, *Enterococcus*, *Lactococcus*, *Microbacterium*, *Corynebacterium* and *Actinomyces naturae*. Differential abundance patterns between crevicular fluid from transepithelial abutments and transepithelial abutments, not detected when comparing crevicular fluid samples from different origins, reflect the particularities of specific oral niches [69]. Furthermore, sampling method was a significant source of bacterial community dissimilarity. In this sense, significant differences were identified at the community level between TA and ToCF and also between TACF and TA, but not between ToCF and TACF. With respect to network properties, sampling procedures did not significantly differ in terms of global network properties or centrality measures. However, variations in hub OTUs were identified between ToCF and TA. By contrast, TACF and TA shared the most relevant hub taxa at both the OTU and the genus level.

As stated by Berry and Widder [12], hub taxa are not necessarily keystones in the microbial community, but ecologically relevant hub taxa are likely to be. Thus, different keystone taxa were also identified in bacterial communities from different sampling origins. Considering node degree and betweenness, *Streptococcus oligofermentans*, *Actinomyces odontolyticus*, *Bacteroides heparinolyticus*, *Acidaminococcus intestini*, *Moryella indoligenes*, *Leptotrichia wadei*, *Haloactinobacterium, Porphyromonas, Sphingobacterium*, *Shuttleworthia*, *Moraxella* and *Propionibacterium* have a pivotal role in TACF samples, whereas *Rothia aeria*, *Streptococcus sobrinus*, *Moryella indoligenes*, *Actinobaculum massiliense*, *Prevotella paludivivens*, *Megasphaera sueciensis, Bacillus, Alloprevotella, Campylobacter, Pontibacillus, Eikenella* and *Parascardovia* showed a higher influence in TA samples. On the other hand, *Prevotella multisaccharivorax, Streptococcus dentapri, Peptococcus niger, Porphyromonas circumdentaria, Mycoplasma salivarium, Streptococcus gallinaceus*, *Butyrivibrio*, *Flavobacterium*, *Leptotrichia*, *Paracoccus*, *Pasteurella* and *Sphingomonas* were identified as drivers of microbiome structure and functioning in ToCF samples. With the exception of *Flavobacterium* (ToCF), *Sphingobacterium* (TACF), *Rothia aeria* (TA), *Moraxella* (TACF), *Sphingomonas* (ToCF) and *Rothia mucilaginosa* (TA), most keystone taxa were obligate (eg. *Bacteroides heparinolyticus, Moryella indoligenes*, *Acidaminococcus intestine*, *Peptococcus niger*, *Megasphaera sueciensis, Shuttleworthia*, *Campylobacter, Butyrivibrio, Actinomyces, Propionibacterium*) or facultative anaerobes (eg. *Streptococcus* spp., *Parascardovia*, *Alloprevotella, Bacillus, Paracoccus, Leptotrichia, Eikenella*, *Porphyromonas*, *Pasteurella*, *Mycoplasma salivarium* or *Actinobaculum massiliense*).

Conclusions

According to these findings, it should be highlighted that bacterial community assessment via mNGS could be considered a promising strategy for peri-implant and periodontal health surveillance and early peri-implant disease diagnosis. Despite the fact that, regardless of sampling approach, association network properties and centrality measures from transepithelial bacterial communities were similar, significant differences were detected in terms of hub taxa, alpha and beta diversity

and individual taxa abundance, including some peri-implant and periodontal health related taxa. In most aspects, crevicular fluid samples (ToCF and TACF) are not representative of bacterial communities developed on transepithelial surfaces. As a result, it can be concluded that the choice of sampling strategy can deeply affect the results of oral microbiota profiling, with a particular emphasis on peri-implant health related bacterial taxa.

Abbreviations

CLR	Centered Log-Ratio transformation
CSS	Cummulative Sum Scaling
mNGS	Metagenomics Next Generation Sequencing
OTU	Operational Taxonomic Unit
SM	Sampling method
TA	Transepithelial abutment
TACF	Crevicular fluid from transepithelial abutment

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12903-024-04675-y.

Supplementary Material 1. FIBEA-06-EC/17/Multi-Im clinical trial experimental design.

Supplementary Material 2. Results summary from testing centrality measures of the networks in Figure 5 for group differencesat: A) the OTU level, and B) the genus level. The absolute differences between sampling procedures and the *p*-values are shown. The *p*-values were adjusted for multiple testing using the adaptive Benjamini-Hochberg method [11], where the portion of true null hypothesis is determined according to Langaas et al. [66]. Only the 10 taxa with the highest absolute group difference are included in the summary table. All measures were normalized to [0,1].

Acknowledgements

The authors thank for technical and human support provided by SGIker (UPV/EHU/ ERDF, EU).

Authors' contributions

Eduardo Anitua and Mohammad Hamdan Alkhraisat designed the study; Eduardo Anitua, Alia Murias-Freijo and Ricardo Tejero performed the clinical procedures and collected/managed the samples; Roberto Tierno analyzed the data and prepared the manuscript, and Eduardo Anitua and Mohammad Hamdan Alkhraisat revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Availability of data and materials

Sequence Read Archive (SRA) data corresponding to the National Center for Biotechnology Information (NCBI) BioProject PRJNA1138960 are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1138960.

Declarations

Ethics approval and consent to participate

This research is part of a randomized, parallel-group, evaluators-blinded, and controlled randomized clinical trial that was designed to assess the effect of transepithelial abutment surface on the biofilm formation. The trial was registered at Trial Registration ClinicalTrials.gov under the number NCT03554876. All participants provided a written informed consent to participate. The study protocol and informed consent, in full accordance with the ethical principles of the Declaration of Helsinki of 1975, as revised in 2013, were approved by the ethical committee of investigation with medicines of the Basque Country (FIBEA-06-EC/17/Multi-Im).

Consent for publication

Not applicable.

Competing interests

Dr. Anitua reports other from BTI Biotechnology Institute, during the conduct of the study. Other from BTI Biotechnology Institute, outside the submitted work. In addition, Dr. Anitua has a patent US8123524B2 issued to BTI Biotechnology Institute. Dr. Murias-Friejo has nothing to disclose. Dr. Tierno reports personal fees from BTI Biotechnology Institute, during the conduct of the study. Personal fees from BTI Biotechnology Institute, outside the submitted work. Dr. Tejero reports personal fees from BTI Biotechnology Institute, during the conduct of the study. Personal fees from BTI Biotechnology Institute, outside the submitted work. Dr. Alkhraisat reports personal fees from BTI Biotechnology Institute, during the conduct of the study. Personal fees from BTI Biotechnology Institute, outside the submitted work.

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Received: 29 June 2023 Accepted: 25 July 2024
Published online: 26 August 2024

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