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In situ substrate-formed biofilms using IDODS mimic supragingival tooth-formed biofilms

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ABSTRACT

This study aimed to compare the bacterial viability and diversity of a substrate-formed biofilm (SF-biofilm) *in situ* to a supragingival tooth-formed biofilm (TF-biofilm) in the same group of individuals. The impact of the device/disc position and toothbrushing during the formation of SF-biofilm was also assessed. Two tests were run. In test 1, 15 volunteers wore two hemi-splints carrying six discs of human enamel, glass, and hydroxyapatite for 2 days, and were instructed to not perform any oral hygiene measure. Biofilm samples were collected from the substrates and the contralateral tooth and were analysed using CLSM. In five volunteers, half of the biofilm present on the discs and their contralateral teeth were scraped and analysed using 16S pyrosequencing. In test 2, the microscopic analysis was repeated only on the SF-biofilm samples, and the volunteers were allowed to brush their teeth. Multivariate analyses revealed that the donors had a significant effect on the composition of the biofilm, confirming its subject-dependent character. The bacterial composition of the SF-biofilm was similar to the TF-biofilm, with significant differential abundance detected in very few taxa of low abundance. The toothbrushing during the formation of SF-biofilm was the only factor that conditioned the thickness or bacterial viability.

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Introduction

The creation of *in-vitro* biofilm models has contributed to significant advances in the study of biofilms, including the human dental biofilm [1]. Nevertheless, their known limitations have highlighted the need to develop *in-situ* oral biofilm models. These models include those based on an intraoral device that carries artificial substrates upon which the biofilm grows, allowing the analysis of an undisturbed supragingival biofilm. These *in-situ* models are required if we are to increase our knowledge of biofilm formation mechanisms and how to mitigate their contribution to oral diseases [2].

Several factors can affect the *in-situ* development of an artificial, substrate-formed biofilm (SF-biofilm) over an intraoral device, with the type of device being one of the main reasons. There are several device designs, which have different characteristics depending on the aim of the investigation. Buccal devices have been the most commonly utilised for *in-situ* supragingival biofilm analyses, with the intraoral device of overlaid disc-holding splints (IDODS) being the closest to the 'ideal' model, as it has more advantages than limitations [3].

Another important consideration is the type of substrate upon which the oral biofilm grows. Indeed, the artificial substrates discussed in the literature are composed of different materials (glass, hydroxyapatite, enamel, or titanium) and have been held in different positions inside the oral cavity [4–9]. None of these artificial substrates have demonstrated clear advantages over other substrate types. The position of the disc inside the intraoral device can also condition the characteristics of the SF-biofilm. This has been evaluated in various investigations [10,11], which reveal that the position of the substrate in the mouth has little impact on the development of the SF-biofilm.

Finally, the toothbrushing protocol followed by volunteers during SF-biofilm formation could also be a determining factor in terms of its characteristics. Although many authors have recommended that toothbrushing be performed without wearing the intraoral device and without the use of toothpaste [8,11], the influence of this oral hygiene approach has never been investigated in the literature.

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Supplementary data for this article should be accessed here

There are some studies in which 16S rRNA gene amplicon sequencing has been used to analyse the *in-situ* bacterial diversity of the SF-biofilm in several intraoral devices [9,12–14]. However, only one of them compared the diversity of the 24-h biofilm on titanium discs to that formed on the natural teeth [9].

No clear attempt has been made in the literature to standardise an *in-situ* biofilm model on a specific substrate and then study its characteristics and to compare it with a supragingival tooth-formed biofilm (TF-biofilm). Therefore, the aims of the present study were to: (1) use different materials to evaluate the bacterial viability and diversity of an *in situ,* 2-day SF-biofilm compared to a 2-day TF-biofilm in the same individuals and (2) examine the influence of several factors, such as the intraoral device/substrate position and the toothbrushing protocol, on the characteristics of the SF-biofilm. The following two hypotheses were tested: (1) the *in situ,* 2-day SF-biofilm formed on different materials has microbial characteristics that are similar to those formed on natural tooth surfaces and (2) different methodological factors, such as the device/substrate position and the toothbrushing protocol, significantly condition the microbial characteristics of an *in situ,* 2-day SF-biofilm formed on different materials.

Materials and methods

The present study was a randomised, observer-masked, cross-over study on the validation of a model for the *in-situ* development of a 2-day supragingival biofilm. The project received the approval of the Clinical Research Ethics Committee of Galicia 2014/008 and was registered with the number NCT02769260 (URL: https://clinicaltrials.gov/ct2/show/NCT02769260). All volunteers who agreed to participate in the study signed an informed consent form.

A volunteer recruitment procedure developed by our group for previous research was used to recruit 15 participants among dental students at the Faculty of Medicine and Dentistry at the Universidade de Santiago de Compostela (Spain). This procedure defines the inclusion and exclusion criteria applied [15]. The inclusion criteria were as follows: systemically healthy adult volunteers aged between 20 and 45 with a good periodontal health status (a minimum of 24 permanent teeth with no evidence of gingivitis or periodontitis – community periodontal index score = 0-) [16] and an absence of untreated caries at the start of the study). The following exclusion criteria were applied: smoker or former smoker; antibiotic treatment or the routine use of oral antiseptics in the previous 3 months; the presence of dental prostheses or orthodontic devices; and the presence of any systemic disease that could alter the production or composition of saliva. All volunteers were assessed by the same trained clinician to ensure that they met all the inclusion and exclusion criteria.

Experimental design

Two IDODS were designed for each participant using the steps established in a previous study [17]. In the present series, there was a modification in the position of one of the splints: one of them was located on the left or the right lower hemi-arch (randomly) and the other on the contralateral side of the mouth but in the upper arch. The two IDODS worn by the volunteers each held three discs (7.0 mm diameter and 2.0 mm thickness). These discs were composed of three different materials: human enamel, glass, and calcium hydroxyapatite. The enamel discs were prepared according to a previously described protocol [18]. The glass discs were produced and polished at 800 grit at the Institute of Ceramics of the Universidade de Santiago de Compostela. The calcium hydroxyapatite discs were acquired from Clarkson Chromatography Products (Williamsport, PA). All the discs were cleaned in a 3% aqueous solution of NaOCl for 10 min, rinsed in distilled water, and autoclaved before use.

In both IDODS (upper and lower), the discs were placed sequentially in the distal (between the first and second molar), medial (between the first molar and second premolar), and mesial (between the first premolar and canine) positions. Considering these three positions, three substrate combinations were established (combination 1: enamel, hydroxyapatite, glass; combination 2: glass, enamel, hydroxyapatite; and combination 3: hydroxyapatite, glass, enamel), with the intention being that each artificial substrate should occupy a different position (distal, medial, and mesial) in a number of participants. The volunteers were assigned to a substrate combination by an internet-based, balanced randomisation system [19], with all three combinations used by the same number of participants for the confocal microscopy analyses.

The IDODS were subjected to the following disinfection protocol before being given to the volunteers: immersion in 3% NaCl solution for 1 min in the ultrasonic cleaner, then 10 min in the ultrasonic cleaner in a 70% ethanol solution, and finally, 10 min in distilled water. The splints were stored in distilled water for 24 h the day before the start of the study for hydrating the materials [17] . All volunteers participated in the two different tests or experiments and were subjected to a professional hygiene protocol before starting each test. The two IDODS with the six different substrates (glass, hydroxyapatite, and enamel) were worn by the volunteers for 2 days to enable the growth of the SF-biofilm. Each volunteer wore the IDODS for 2-day periods between which a 2-week washout interval was established.

In test 1, where there was a no toothbrushing protocol, the volunteers were asked to refrain from any oral hygiene measure for the entire period. The IDODS could only be removed from the oral cavity during meals. During these short intervals, the splints were kept in an opaque container in humid conditions. In this test, samples of SF-biofilm and biofilms grown on tooth surfaces were collected from 15 volunteers and analysed by confocal laser microscopy and 16S rRNA gene amplicon sequencing (in five of the 15 volunteers).

In test 2, where there was a toothbrushing protocol, each volunteer wore the IDODS with the same substrate combination as applied in test 1 for the confocal microscopy analyses. These volunteers were allowed to perform oral hygiene measures but could not use any toothpaste or mouthwash. In this test, no biofilm samples were collected from the tooth surfaces, and SF-biofilm samples from 15 volunteers were analysed only by confocal laser microscopy (Figure 1). During the two tests, neither participant reported any incidents with the intraoral devices and discs.

Test 1 (without toothbrushing during biofilm formation)

The vestibular position of the disc marked the vestibular position of the contralateral tooth surface to be scraped, with the discs withdrawn sequentially from the distal to the mesial position starting with the upper splint.

- SF-biofilm and TF-biofilm collection for the confocal microscopy analyses and 16S rRNA gene amplicon sequencing

In 15 volunteers, the discs with the 2-day biofilm were submerged in 100 µL of the fluorescence solution LIVE/DEAD® BacLight™ (Live/Dead® BacLight™ Bacterial Viability Kit, Molecular Probes Inc. Leiden, the Netherlands) for 15 min. In all volunteers, a sterile curette was used to obtain a sample of the dental plaque formed on the distal vestibular surface of the tooth that was contralateral to the withdrawn disc. This plaque sample was also submerged in the fluorescence LIVE/DEAD® BacLight™ solution for 15 min.

In five of the 15 volunteers, the distal vestibular surface was scraped once with a sterile curette as soon as a disc was withdrawn from the splints. Each substrate always occupied the same position (distal for glass, medial for hydroxyapatite, mesial for enamel). A dental plaque sample from the mesial vestibular surface of the tooth that was contralateral to the withdrawn disc was obtained with another sterile curette. The harvested biofilm samples were suspended in 300 µL of a phosphate buffer and were kept frozen at −80°C until further pyrosequencing analyses were carried out.

Test 2 (with toothbrushing during biofilm formation)

- SF-biofilm collection for the confocal microscopy analyses

In 15 volunteers, the discs were submerged in 100 µL of the fluorescence LIVE/DEAD® BacLight™ solution for 15 min.

Analysis of biofilm samples using confocal microscopy

A single investigator, blinded to the study design, performed the microscopic observations using a Leica TCS SP2 confocal laser scanning microscope (CLSM, Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with an HCX APOL 63 x/0.9 water-immersion lens.

The protocol described by Quintas et al. [15] was followed to evaluate the different fields within the discs, with the thickness and bacterial viability analysed. In each field, the maximum biofilm thickness was divided into three layers: the outer layer (layer 1), the middle layer (layer 2), and the inner layer (layer 3).

The TF-biofilm samples were individually set on a slide and smoothly covered with a cover-slip. The field was analysed to examine the presence of bacterial accumulations. Four fields were selected as representative by a blinded observer. Their mean measures of bacterial viability represented the bacterial viability of the entire sample. The thickness and bacterial viability by layers were not evaluated because it was a non-structured biofilm.

Data were captured using the same settings in all cases, and certain parameters were established following previous investigations by our group [15]. The quantification of bacterial viability in the series of XY images was determined using a cytofluorographic analysis (Leica confocal software). In this analysis, the images of each fluorochrome were defined as 'channels' (SYTO 9 occupies the green channel and propidium iodide the red channel). For automatic computations, a Matlab toolbox called the Dentius Biofilm was developed, and its usability characteristics have already been described [15]. Determination of the mean viability percentage in each field required sections with a minimum biofilm area of 250 µm^2 (approximately 4,750 pixels). The mean viability percentage of the biofilm was calculated for the corresponding sample and each biofilm layer (in the case of the SF-biofilms).

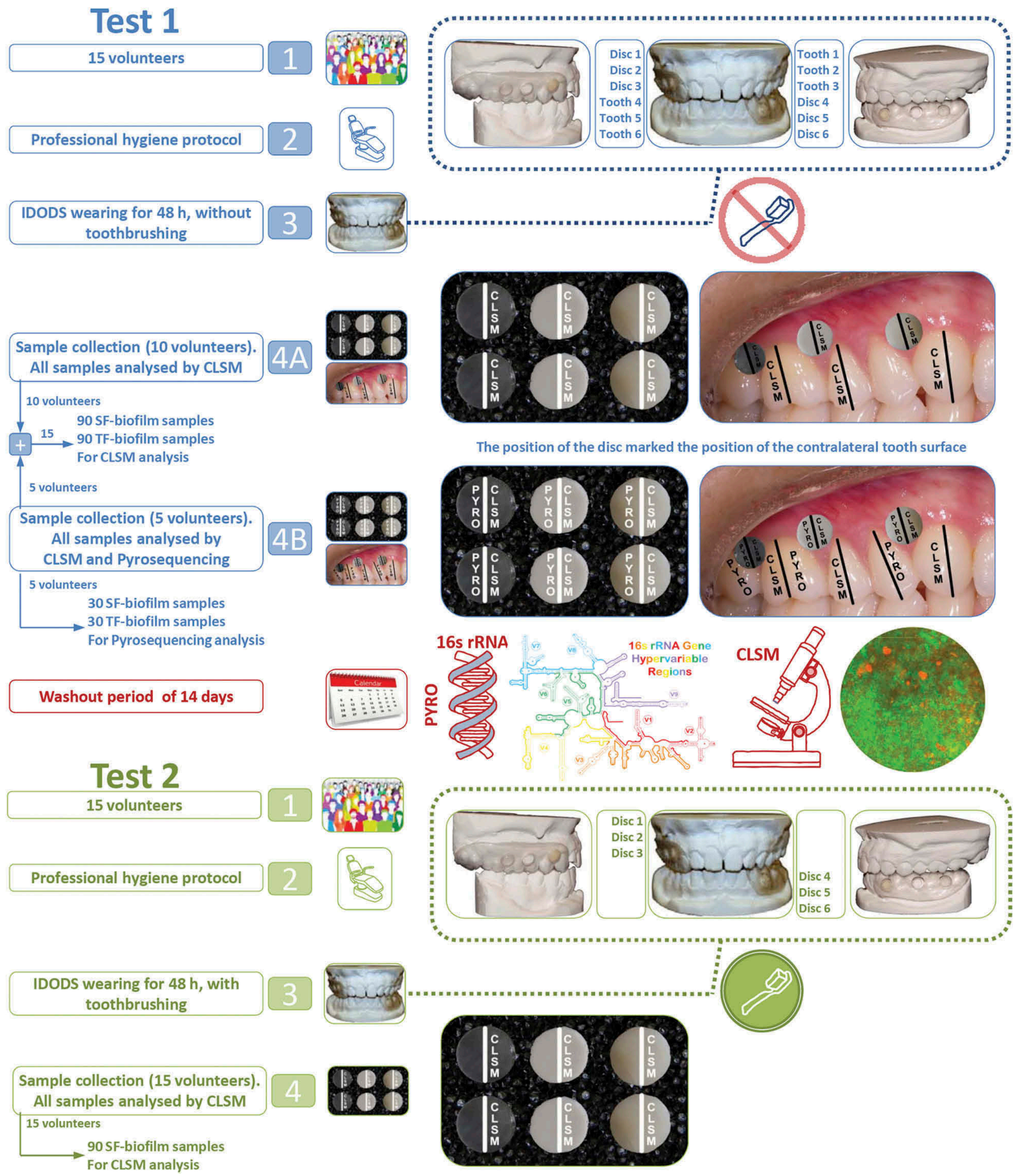

Figure 1. Methodological protocol of the study.

Analysis of biofilm samples using 16S rRNA gene amplicon sequencing

DNA was separately extracted from each biofilm sample using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). The manufacturer's instructions were followed, with the addition of a lysozyme treatment (5 mg ml^{-1} at 37 °C for 30 min) [20]. PCR amplification of the 16S rRNA gene was performed with the high-fidelity ABGene DNA polymerase (Thermo Scientific, Empson, Surrey, UK). The universal bacterial primers were used for the V1-V2-V3 hypervariable regions of the 16S rRNA genes, 8F-27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 514R-533R (5′-TTACCGCGGCKGCTGGCACG-3′). An annealing temperature of 52°C and 20 amplification cycles were applied to minimise the PCR amplification bias. The primers used for the sequencing have been described previously [21]. Two PCRs were performed per sample to amplify the 16S rRNA genes and introduce adaptor sequences and sample-specific barcode oligonucleotide tags into the DNA. In five samples, a

PCR product could not be obtained and a nested-PCR was performed. Following Benítez-Páez et al. [22], the PCR product in these samples was purified and used as a template for a secondary PCR in which the primers were shifted three bp towards the 3′ end, and included the pyrosequencing adaptors A and B and the eight bp 'barcode' specific to each sample. The 500 bp PCR products were purified using the Nucleofast PCR purification kit (Macherey-Nagel, Düren, Germany) and further cleaned with AMPure XP beads (Roche, Basel, Switzerland) before pyrosequencing. The PCR products were pyrosequenced from the forward primer end only using a GS-FLX sequencer (Roche) with Titanium chemistry at the Centre for Advanced Research in Oral Health, CSISP-FISABIO, Valencia, Spain. One-eighth of a plate was used for each pool of 30 samples, which were amplified with a different forward primer containing a unique eight-bp 'barcode' [23].

A 16S data-processing pipeline was established using the Mothur software package [24], including the high-quality trimming approach with slight modifications. This pipeline was applied in the Human Microbiome Project [25]. The sequences were separated using the sample-specific 'barcodes'. A 40-bp sliding window was used, and the sequence was trimmed when the average quality score dropped below 30. The sequence was also trimmed if any sequence had an ambiguous base call, a homopolymer longer than 8 bp, one or more mismatches to the barcode, or more than four mismatches to the primer. Only reads longer than 200 bp were considered. Unique sequences were then aligned to the appropriate SILVA-based reference alignment [26]. Chimeras were identified using a Mothur-based implementation of the chimera.slayer program. A total of 6.8% of reads were filtered out as potential chimeras. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity and were assigned a taxonomic identity using the mothur-based implementation of the human oral microbiome database (HOMD) [27]. Each read was taxonomically assigned down to the species level using a 90% minimum confidence threshold. A phylogenetic tree was generated from a unique sequences fasta file using the Megan software, resulting in a Newick tree format [28].

Statistical analyses

Statistical analyses were conducted using the R software [29]. The variable 'biofilm sample' was the unit of analysis. Of the 15 volunteers whose 2-day biofilm samples were subjected to CLSM analyses, five were randomly selected for the 16S rRNA gene amplicon sequencing. In test 1, 180 biofilm samples (90 from the SF-biofilm and 90 from the TF-biofilm) were analysed using CLSM and 60 biofilm samples (30 from the SF-biofilm and 30 from the TF-biofilm) using 16S rRNA gene amplicon sequencing. In test 2, 90 biofilm samples were analysed by CLSM, with all of them obtained from the artificial substrates.

In the CLSM analysis, the data on thickness and bacterial viability in the biofilm samples were expressed as the mean and standard deviation as well as the median and interquartile range. The type of distribution of the quantitative variables was determined using the Shapiro–Wilk's test, with a non-normal distribution for most variables. The Friedman test and the Wilcoxon signed-rank test (for pairwise comparisons) were used for different analyses: a comparison of the inter-biofilm results (SF-biofilm vs TF-biofilm); a comparison of the intra-SF-biofilm results taking into account the material of the artificial substrate and the IDODS/disc position (including differentiating between the three biofilm layers); and a comparison of the inter-test results (test 1 SF-biofilm without toothbrushing vs test 2 SF-biofilm with toothbrushing, including differentiating between the three biofilm layers). In the Benjamini–Hochberg's correction for multiple comparisons [30], a Q parameter of 0.1 was established, which corresponds to a false discovery rate (FDR) of <10%. Measurements were statistically significant if the adjusted p-value was < 0.05.

The statistical analysis of the 16S rRNA sequencing data was performed according to the protocol recently proposed by McMurdie and Holmes [31], using implementations in R such as the Phyloseq and DESeq2 packages [32,33]. Previously, an independent filter based on the OTUs with an abundance of ≤2 counts and present in <3 biofilm samples (5%) was excluded from the statistical analysis [34].

Several bacterial diversity parameters were calculated at the OTU level in the Phyloseq package [32]. The Chao1 Index and the Abundance-based Coverage Estimator (ACE) were used as estimators of taxa richness [35,36]. Both Shannon and Simpson indexes take into account the abundance and evenness of the taxa present [37,38]. Non-parametric tests (the Friedman test and Wilcoxon signed-rank test) were used in the alpha-diversity estimators for the different comparisons. Measurements were statistically significant if the adjusted p-value using the Benjamini-Hochberg correction [30] was <0.05.

To analyse the global structure of the different types of biofilm, a multivariate analysis based on the principal coordinates analysis (PCoA) was performed with Fast UniFrac using a weighted algorithm [39,40]. Using the Adonis function in the vegan library based on 9,999 permutations, a permutational multivariate analysis of variance (Permanova) was applied to assess the influence of the variables 'type of biofilm substrate' and 'type of patient' on the composition of biofilms [41].

To analyse the bacterial composition of the different types of biofilm, a univariate analysis based on the differential abundance of counts at the genus and OTU levels was performed using DESeq2 [33]. Calculating the differential abundance and the modelled uncertainty using a negative binomial model and normalisation was achieved using variance-stabilising transformations. The results were expressed in 'Log2foldchange', which is the mean of the difference of the abundance of counts in a logarithmic base. Hence, for example, a Log2foldchange = 0 is $2^0 = 1$, which means the equal abundance of a given OTU between two classes; a Log2foldchange = 1 is $2^1 = 2$, which means a double abundance of the first class compared to the second class; and a Log2foldchange = 2 is $2^2 = 4$, which means there is four times more abundance. The Wald tests with the Benjamini–Hochberg correction [30] were applied to compare the different types of biofilm and to obtain the adjusted p-value (Q parameter = 0.1, FDR < 10%). Measurements of differential abundance were statistically significant if the adjusted p-value was < 0.05 (-log10 adjusted p-value = 1.3). The graphics were performed in the ggplot package [42].

Results

- Factors affecting the bacterial viability and thickness of the SF- and TF-biofilms

The mean viability of the SF-biofilm, both with and without toothbrushing, was higher than that of the TF-biofilm (74.20 ± 14.34% and 70.40 ± 13.39% vs 57.20 ± 16.47%; adjusted p-value < 0.001 for both comparisons) (Figure 2).

In test 1 (without toothbrushing), the mean SF-biofilm thickness was 22.47 ± 4.36 µm; in test 2 (with toothbrushing), this value was 19.51 ± 4.65 µm. In the intra-test comparisons, the type of substrate and the intraoral device/substrate position did not affect the thickness or viability of the SF-biofilm. It was only in test 1 that significant differences in the mean viability by layers of the SF-biofilm were detected between the distal and medial positions of the discs for layer 3 (64.75 ± 19.31% vs 77.78 ± 14.39%, adjusted p-value = 0.033) (Table 1). In the inter-test comparisons, the mean SF-biofilm thickness was significantly higher in test 1 than test 2 (adjusted p-value < 0.001). These differences were mainly in the medial positions of the substrate (test 1 = 23.16 ± 4.68 µm vs test 2 = 18.92 ± 4.61 µm, adjusted p-value = 0.007). The mean viability of the SF-biofilm was significantly higher in test 1 than test 2 (74.20 ± 14.34% vs 70.40 ± 13.39%, adjusted p-value < 0.046). These differences were mainly in the medial and mesial positions of the substrate and the deeper layers of the biofilm (medial position layer 3 = 77.78 ± 14.39% vs 65.69 ± 18.90%; mesial position layer 2 = 74.83 ± 15.07% vs 64.37 ± 19.98%, adjusted p-value = 0.027 for both comparisons).

In Supplementary files S1 and S2, the results on bacterial viability and thickness of the SF-biofilm and bacterial viability of the TF-biofilm in test 1 as well as those on bacterial viability and thickness of the SF-biofilm in test 2, respectively, are shown.

- Factors affecting the bacterial diversity of the SF- and TF-biofilms

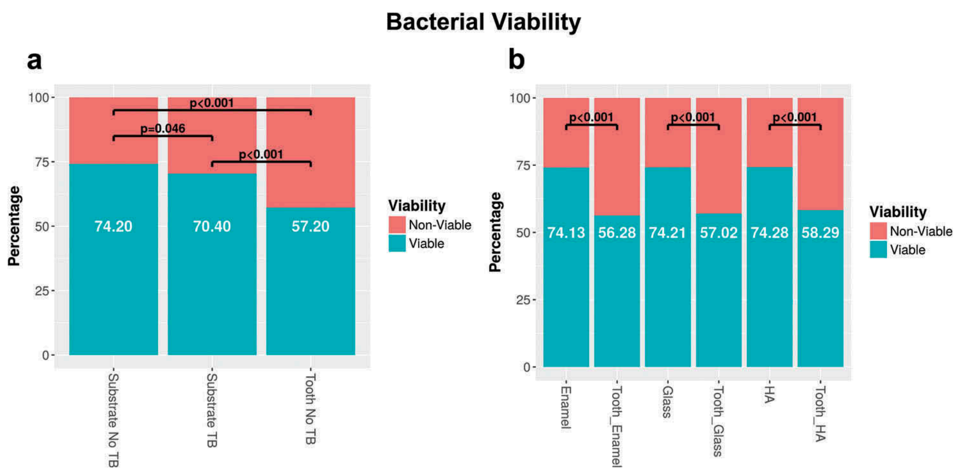

Figure 2. (**a**) Presentation of the mean total bacterial viability of the substrate-formed biofilm with and without toothbrushing in comparison to the tooth-formed biofilm without toothbrushing. (**b**) Presentation of the mean total bacterial viability of the biofilm formed on the artificial substrates (enamel, glass, and hydroxyapatite) in comparison to the tooth-formed biofilm, both without toothbrushing.

No TB = no toothbrushing; TB = toothbrushing; HA = hydroxyapatite. Each disc is compared to the corresponding contralateral tooth surface. (**a**) The standard deviations of the viability percentages obtained were from left to right: 14.34, 13.39, and 16.47, respectively. The median (interquartile range) obtained were, from left to right: 75.35 (19.15), 72.03 (16.44), and 58.86 (22.54), respectively. (**b**) The standard deviations of the viability percentages obtained were, from left to right: 15.62, 15.87, 12.54, 18.63, 15.15, and 15.15, respectively. The median (interquartile range) obtained were, from left to right: 56.84 (13.79), 75.84 (16.88), 60.84 (25.05), 73.19 (18.67), 62.71 (16.37), and 76.88 (19.76), respectively.

Table 1. Data on thickness, bacterial viability and bacterial viability by layers in the substrate-formed biofilm in both tests (test 1, without toothbrushing; test 2, with toothbrushing).

SF-BIOFILM WITHOUT TOOTHBRUSHING PROTOCOL (TEST 1)					
Mean ± Standard Deviation, Median (Interquartile Range)					
			VIABILITY BY LAYERS (%)		
VARIABLE	THICKNESS (μm)	VIABILITY (%)	Layer 1	Layer 2	Layer 3
Substrate material					
Enamel	22.55 ± 4.47	74.13 ± 15.62	74.75 ± 16.87	74.21 ± 5.91	73.43 ± 17.63
	22.07 (5.12)	75.84 (16.88)	78.53 ± 19.09	76.45 ± 17.61	76.31 ± 20.86
Glass	22.57 ± 4.06	74.21 ± 12.54	75.34 ± 14.37	75.82 ± 13.19	71.45 ± 16.97
	22.38 (5.06)	73.19 (18.67)	77.16 (19.21)	76.44 (17.83)	71.22 (2.45)
Hydroxyapatite	22.29 ± 4.66	74.28 ± 15.15	77.91 ± 14.08	75.96 ± 15.97	68.98 ± 18.15
	23.13 (4.94)	76.88 (19.76)	82.61 (21.11)	81.55 (20.99)	70.53 (21.59)
Position of the intraoral device					
Upper	22.63 ± 3.82	76.11 ± 11.47	76.80 ± 13.37	76.55 ± 13.26	74.99 ± 12.04
	22.75 (2.90)	75.88 (13.88)	78.96 (19.04)	78.35 (18.57)	73.44 (13.37)
Lower	22.31 ± 4.88	72.30 ± 16.64	75.21 ± 16.68	74.10 ± 16.5	67.58 ± 21.1
	22.01 (7.04)	74.84 (22.21)	78.64 (20.04)	76.82 (18.33)	69.85 (31.91)
Position of the discs					
Distal	21.65 ± 3.84	70.51 ± 14.71	73.67 ± 14.77	73.11 ± 14.94	64.75 ± 19.31*
	22.00 (2.20)	68.38 (16.77)	75.11 (24.71)	72.70 (19.09)	64.84 (19.88)*
Medial	23.16 ± 4.68§	78.12 ± 13.23	78.53 ± 14.35	78.03 ± 14.87	77.78 ± 14.39*§
	23.88 (6.12) §	81.36 (12.05)	84.25 (23.10)	81.44 (19.55)	81.35 (19.12)* §
Mesial	22.61 ± 4.53	73.98 ± 14.48	75.80 ± 16.08	74.83 ± 15.07§	71.33 ± 16.44
	22.62 (6.59)	73.81 (12.80)	80.69 (14.99)	76.22 (11.50) §	71.42 (12.22)
SF-BIOFILM WITH TOOTHBRUSHING PROTOCOL (TEST 2)					
Mean ± Standard Deviation, Median (Interquartile Range)					
			VIABILITY BY LAYERS (%)		
	THICKNESS (μm)	VIABILITY (%)	Layer 1	Layer 2	Layer 3
Substrate material					
Enamel	18.23 ± 4.20	68.35 ± 15.88	69.75 ± 21.60	68.90 ± 17.32	66.41 ± 17.44
	17.79 (5.73)	72.03 (14.38)	76.75 (20.83)	71.32 (17.64)	68.82 (23.30)
Glass	20.25 ± 7.02	75.88 ± 13.55	79.06 ± 14.28	73.90 ± 18.07	70.00 ± 14.75
	19.60 (4.67)	73.15 (12.65)	79.25 (12.17)	73.07 (14.52)	67.12 (20.60)
HA	21.35 ± 6.88	68.12 ± 19.40	72.45 ± 19.65	65.71 ± 15.80	69.92 ± 23.71
	20.71 (4.88)	69.69 (11.21)	72.93 (12.67)	67.02 (14.18)	69.13 (14.05)
Position of the intraoral device					
Upper	19.12 ± 4.70	71.31 ± 14.97	75.80 ± 16.99	71.14 ± 16.83	66.98 ± 19.82
	19.25 (7.75)	74.97 (15.41)	77.45 (17.18)	72.62 (15.17)	69.34 (23.70)
Lower	19.91 ± 4.63	69.49 ± 11.72	72.15 ± 15.73	68.18 ± 13.94	68.13 ± 14.82
	20.82 (6.53)	69.11 (18.25)	74.87 (19.03)	66.24 (16.66)	70.59 (21.33)
Position of the discs					
Distal	19.67 ± 5.05	71.96 ± 10.06	75.21 ± 10.78	72.74 ± 11.18	67.93 ± 14.29
	20.98 (8.82)	72.65 (14.23)	76.74 (17.34)	71.32 (14.86)	64.75 (20.36)
Medial	18.92 ± 4.61§	70.35 ± 12.24	73.49 ± 16.09	71.89 ± 12.79	65.69 ± 18.90§
	18.35 (6.03) §	70.18 (15.68)	75.74 (12.53)	73.92 (16.90)	68.82 (28.56) §
Mesial	19.95 ± 4.37	68.88 ± 17.17	73.23 ± 21.13	64.37 ± 19.98§	69.04 ± 19.04
	20.20 (6.35)	71.51 (20.93)	79.22 (27.24)	65.34 (21.80) §	72.55 (17.49)

The maximum biofilm thickness of each field was divided into three zones or equivalent layers: the outer layer (layer 1), the middle layer (layer 2), and the inner layer (layer 3).
* This symbol indicates significant differences in the intra-test comparisons.
§ This symbol indicates significant differences in the inter-test comparisons.

Regarding 16S rRNA pyrosequencing, the averages of the raw sequences per biofilm sample were 4,980 and 3,381 after applying the quality protocol. When we compared each type of substrate with its corresponding TF-biofilm samples, the SF-biofilm had similar observed OTU and ChaO1 index values to those detected in the tooth surfaces (84–91 vs 85–91 and 85.20–95.50 vs 85.00–93.00, respectively; adjusted p-value > 0.05), as was also the case with the diversity indexes (Shannon index = 1.28–2.44 vs 1.74–2.89, respectively; Simpson index = 0.52–0.82 and 0.64–0.89, respectively; adjusted p-value > 0.05). Among the artificial substrates, no significant differences were detected in any of the diversity parameters (Figure 3).

The PCoA revealed no clustering of the biofilm samples when taking into account factors such as the type of substrate or the position of the IDODS. On the contrary, the biofilm samples belonging to the same volunteer tended to cluster together, regardless of substrate type (Figure 4). The Permanova test confirmed that only the variable 'patients' had a significant effect on the global structure of the biofilm microbiota (p-value < 0.0001).

The percentage of unclassified OTUs at the genus level was 4.3%. Twenty-three bacterial genera were identified in all the samples. The most abundant genera in the biofilm samples were *Streptococcus, Fusobacterium, Veillonella, Neisseria, Gemella, Prevotella, Alloprevotella, Porphyromonas, Aggregatibacter*, and *Leptotrichia.*

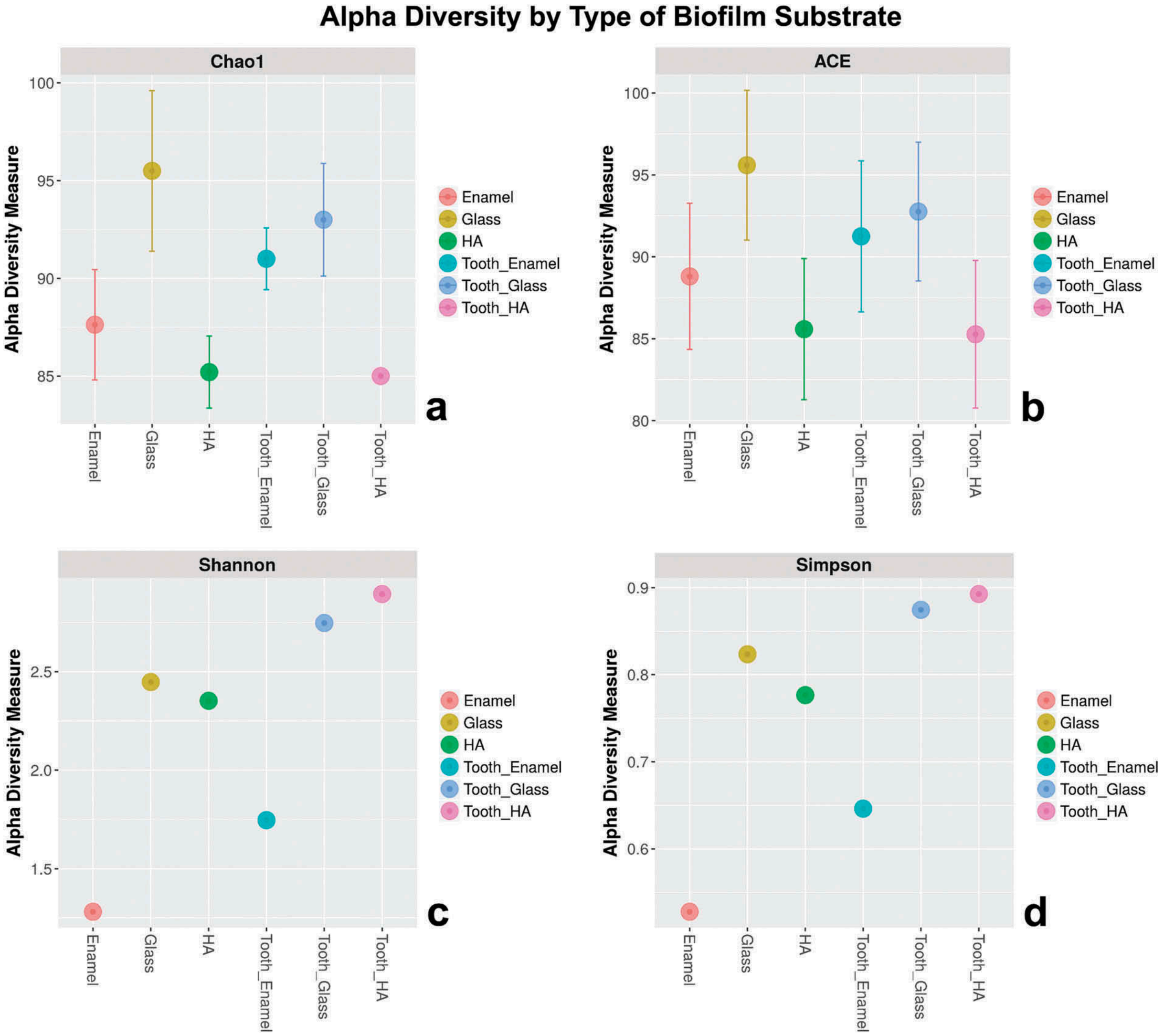

Figure 3. Alpha diversity estimators including Chao1, ACE, Shannon and Simpson indexes as per the type of biofilm formed on artificial substrates and tooth surfaces.

HA = hydroxyapatite. The vestibular position of the disc marked the vestibular position of the contralateral tooth surface to be scraped.

Streptococcus and *Fusobacterium* were the most abundant genera on the artificial substrates (56.95%–23.62% and 65.92%–13.06%, respectively), while on the tooth surfaces *Streptococcus* (45.69%–19.72%), *Fusobacterium* (56.91%–6.81%), *Veillonella* (27.72%–2.38%), and *Neisseria* (12.12%–3.37%) were the most abundant (Figure 5).

In Figure 6, the volcano plots representing the differential abundance at the genus and OTU levels between the different artificial substrates and the corresponding tooth surfaces are shown. In the SF-biofim on the enamel discs, five genera and two species showed significant differences to the TF-biofilm in the differential abundance analysis. All these genera and species were detected in low abundances (<1.5%) and included: *Abiotrophia defectiva, Alloprevotella* spp., *Bergeyella* spp., *Capnocytophaga* spp., *Leptotrichia* spp., and *P. naceiensis*. The abundance of these taxa represented 2.98% of the genera and 0.50% of the OTUs in the TF-biofilm. Except for *Alloprevotella* spp. and *P. naceiensis* (Log2foldchange = 3.45 and 4.16, respectively), all the genera and *A. defectiva* were significantly more abundant in the TF-biofilm samples (Log2foldchange ranging from −3.66 to −2.60).

In the SF-biofilm on the hydroxyapatite discs, three genera and one species (*Capnocytophaga* spp., *Prevotella* spp., *P.. naceiensis*, and *Rothia* spp.; all detected in abundance <4%) presented with a differential abundance in comparison to the TF-biofilm samples. The abundance of these taxa represented 1.18% of the genera and 0.000001% of the OTUs in the TF-biofilm. Unlike *Capnocytophaga* spp. and *Rothia* spp. (Log2foldchange = −3.14 and −4.35, respectively), *Prevotella* spp. and *P. naecensis* were significantly more abundant in the SF-biofilm formed on the hydroxyapatite discs (Log2foldchange = 4.22 and 4.48, respectively).

In the SF-biofilm on the glass discs, no genus was detected that showed a differential abundance in contrast to the TF-biofilm, and only *A. defectiva* was

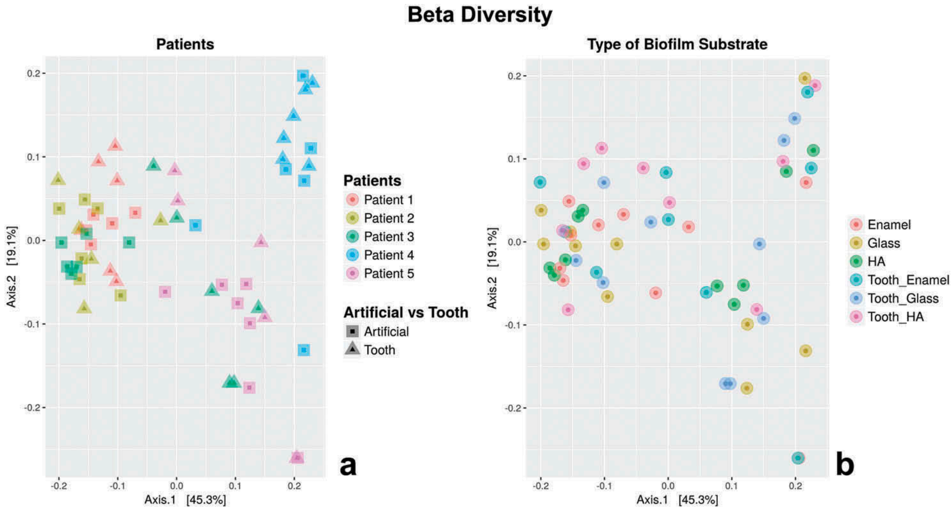

Figure 4. Principal coordinates analysis (PCoA) evaluating the global bacterial structure of the biofilm samples per patient and type of biofilm formed on artificial substrates and tooth surfaces.

HA = hydroxyapatite. The vestibular position of the disc marked the vestibular position of the contralateral tooth surface to be scraped.

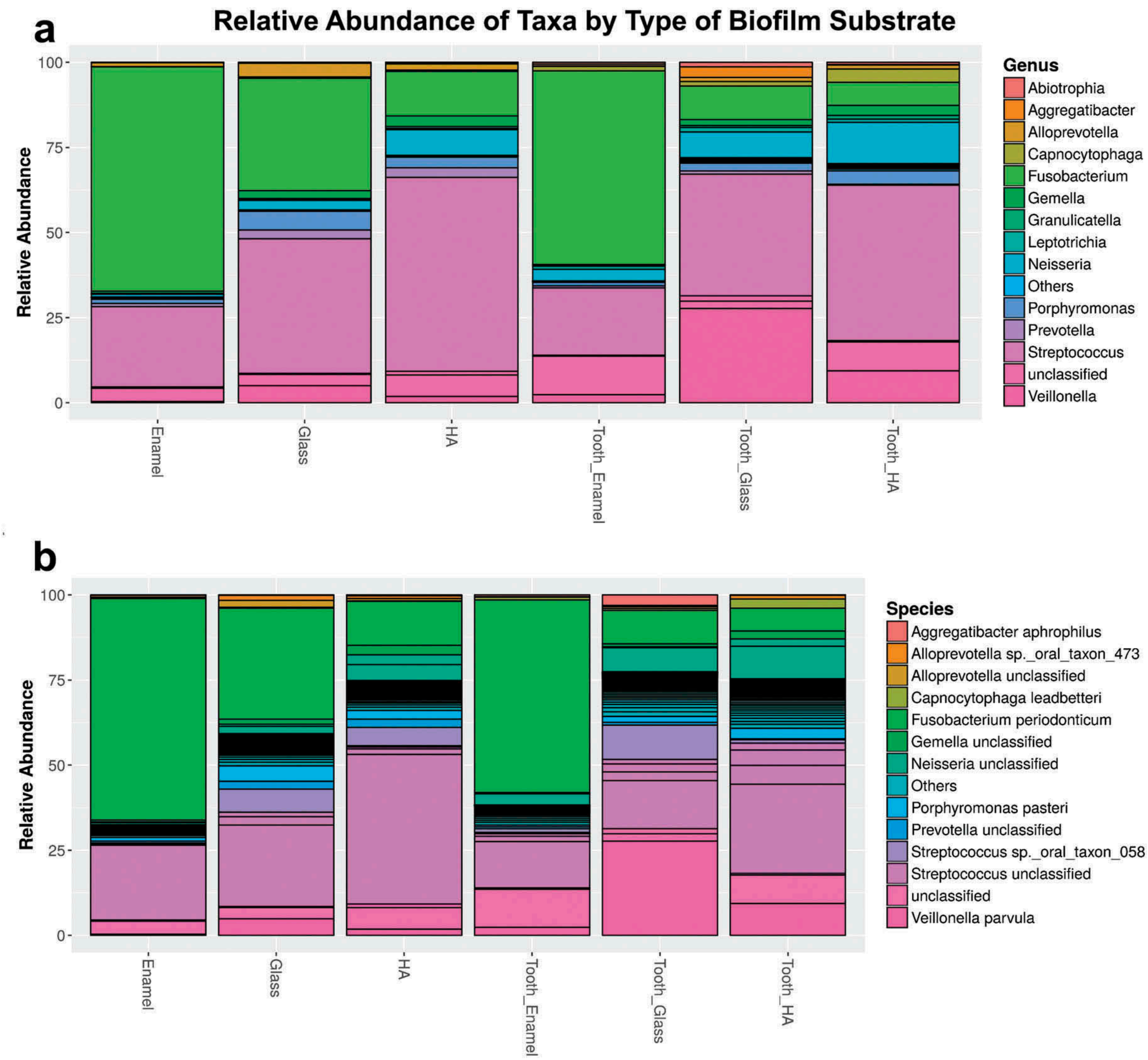

Figure 5. Graphic presentation of the relative abundance of the bacterial taxa present in the artificial substrates and tooth surfaces (genera and species with a mean abundance of >0.5% were included).

HA = hydroxyapatite. The vestibular position of the disc marked the vestibular position of the contralateral tooth surface to be scraped. Very low abundance genera and species, whose names do not appear in the legend, are represented by a black line. The accumulation of black lines causes the appearance of a black area.

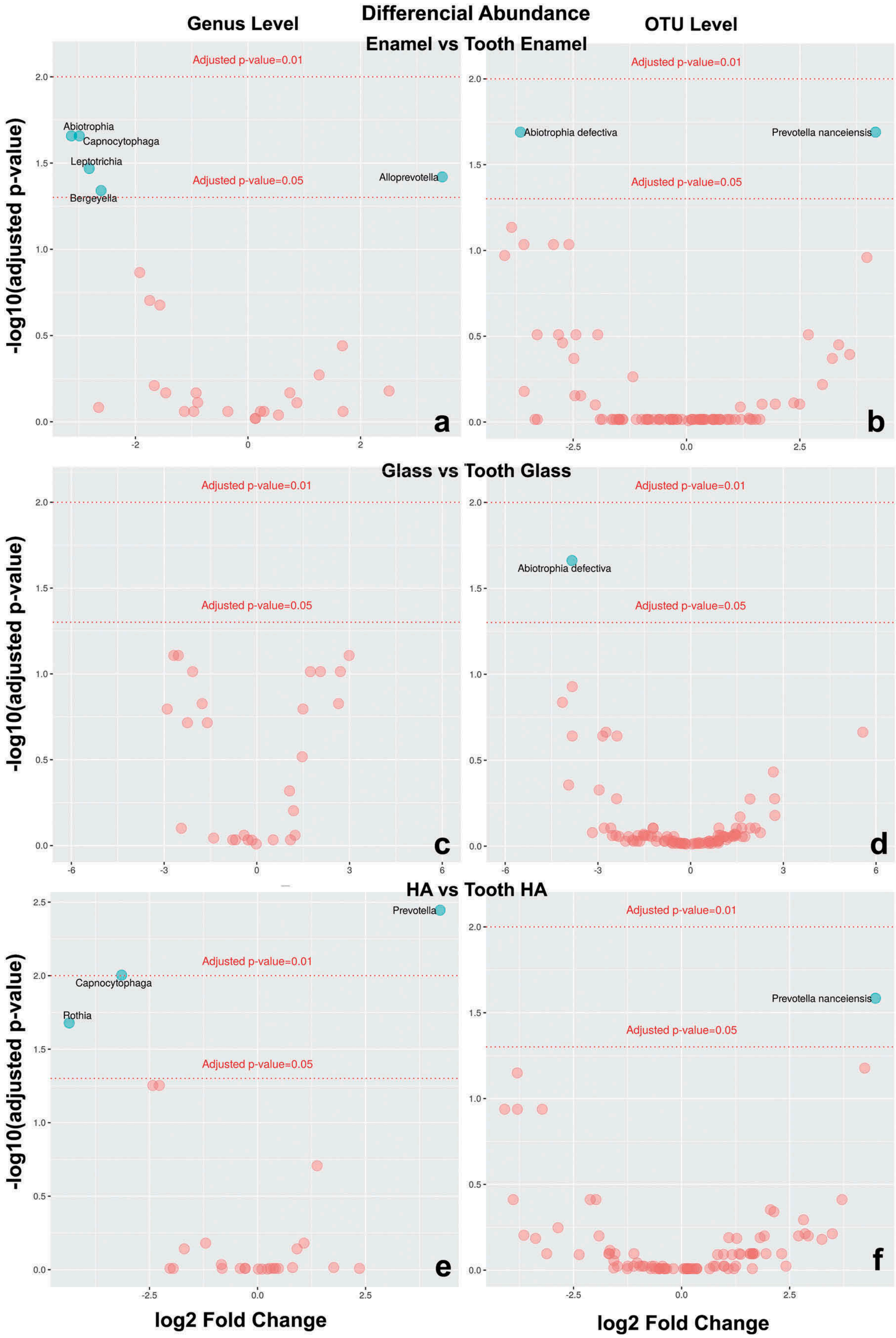

Figure 6. Volcano plots representing the differential abundance at the genus and OTU levels between the different artificial substrates and corresponding tooth surfaces.

HA = hydroxyapatite. The vestibular position of the disc marked the vestibular position of the contralateral tooth surface to be scraped. A -log10 adjusted p-value = 1.3, which equals adjusted p-value = 0.05; a -log10 adjusted p-value = 2.0, which equals adjusted p-value = 0.01. A positive value of Log2foldchange indicates that taxa were more abundant on artificial substrates than on tooth surfaces; a negative value of Log2foldchange that taxa were less abundant on artificial substrates than on tooth surfaces.

significantly more abundant in the TF-biofilm (Log2foldchange = −3.84). The abundance of this taxon represented 1.31% of the OTUs in the TF-biofilm.

There were no significant differences in abundance between the different artificial substrates. In Supplementary files S3 and S4, the results on the relative abundance of taxa at genus and OTU levels of the SF-biofilm and TF-biofilm are shown, as well as several differential abundance analyses.

Discussion

The present investigation demonstrates for the first time that an intraoral device (IDODS) with different substrates such as enamel, glass, and hydroxyapatite is capable of successfully representing the bacterial composition of a 2-day supragingival tooth biofilm.

- Thickness, bacterial viability, and diversity of the SF-biofilm compared to the TF-biofilm

In the present study, the bacterial viability of the TF-biofilm harvested in test 1 was lower than that from the discs (a viability difference of approximately 15–20%). A possible reason for these notable differences could be the fact that the TF-biofilm was obtained by physical removal with a sterile curette. This procedure can disrupt the integrity of the bacterial cell membrane, with the effect being that the TF-biofilm is less viable than that formed on an artificial substrate that is not further manipulated. Moreover, the TF-biofilm collected with a curette has a stacked and unstructured shape, making it impossible to measure the thickness or the viability in different layers. Consequently, the SF-biofilm in the IDODS has the advantage that it can be withdrawn from the oral cavity without difficulty and subsequently observed and analysed without collapsing its original structure.

In the present series, the SF-biofilm thickness or bacterial viability was not conditioned by the type of the artificial substrate used, ranging from 19 μm to 24 μm and 69% to 79%, respectively. Coinciding with our findings, Bevilacqua et al. [43] recently examined the amount of biofilm formed on differently treated titanium surfaces both *in situ* and *in vitro* by CLSM. Unlike the results observed in the *in-vitro* biofilm, *in-situ* experiments showed no difference in the amount of biofilm formed on the different surfaces after a 24-h incubation in the mouth of healthy volunteers. Consequently, these authors concluded that quantitative differences observed *in-vitro* models among surfaces with distinct roughness may not be predictive of different colonisation rates *in situ* [43].

Regarding bacterial diversity, it was only in the *in-situ* study by de Melo et al. [9] that dental plaque samples were compared to SF-biofilm samples using 16S rRNA gene amplicon sequencing. However, these authors analysed 24-h biofilms and evaluated different substrates to those used in the present series (titanium discs vs supra/subgingival tooth surfaces from the same four subjects). Likewise, we have only identified one *in-vitro* investigation, the aim of which was to evaluate the effect of two artificial substrates (glass vs hydroxyapatite) on saliva-derived biofilms using 16S rRNA gene sequencing [44]. These methodological differences mean that any comparison of these papers to the present study is difficult and must be done with care.

De Melo et al. [9] detected that the 24-h biofilm obtained from titanium surfaces had 60% fewer OTUs than a biofilm obtained from tooth surfaces. In contrast, in our series, the SF-biofilm of the different materials (enamel, glass, and hydroxyapatite) had a bacterial richness and an evenness similar to that found in the TF-biofilm. Equally, these diversity parameters were similar between the artificial substrates, which is in line with previous findings comparing glass and hydroxyapatite for *in-vitro* saliva-derived biofilm formation [44].

Regarding the biofilm's global structure, the variable 'patients' had a significant effect on the global microbiota composition, regardless of the type of substrate, confirming that the supragingival biofilm has a subject-dependent character [12,45]. The most abundant genera found in the biofilm samples were *Fusobacterium, Streptococcus, Veillonella, Neisseria, Gemella, Prevotella, Alloprevotella, Porphyromonas, Aggregatibacter*, and *Leptotrichia. Streptococcus* and *Fusobacterium* were the most abundant genera on the artificial substrates, whereas on the tooth surfaces the most abundant were *Streptococcus, Fusobacterium, Veillonella*, and *Neisseria*. Coinciding with the results of Wake et al. on hydroxyapatite discs [14], we have shown that, 48 h after the initiation of biofilm formation, Gram-negative anaerobic bacteria such as *Fusobacterium* are predominant, together with *Streptococcus*, which are more dominant in early phases. In the differential abundance analysis, there were differences in very few genera and species, all of them of low abundance, between the SF-biofilm, especially on the enamel discs, and the TF-biofilm. In none of the case, these specific differences in some taxa did not condition the overall compositional structure of supragingival biofilm. This finding implies that, with the *in-situ* use of these substrates on IDODS, we can reliably reproduce the supragingival biofilm and extrapolate our observations on the efficacy of antimicrobial agents obtained from the SF-biofilm to the TF-biofilm. These results concur with those previously published by other authors, but on titanium surfaces, and confirm that the influence of surface characteristics on bacterial adhesion is compensated by the development of the oral biofilm [9,46].

In fact, it has been demonstrated that biofilm developed *in-vitro* is more easily influenced by surface features than biofilm formed *in-situ* [43]. In the *in-situ* biofilm, the interactions between members of the complex bacterial community, the presence of a wide range of nutrients, and other factors such as the intraoral shear forces originating from the muscles or tongue and the salivary flow can condition the SF-biofilm characteristics [47].

Interestingly, the SF-biofilm on the glass discs was the one with the greatest compositional similarity to the TF-biofilm; whereas no abundance differences were found between the artificial substrates. Consequently, the authors recommend the use of glass discs for developing the SF-biofilm, as other substrates have various disadvantages such as background autofluorescence [48]. In our experience, other significant drawbacks of the hydroxyapatite and enamel discs include their fragility and their higher cost.

It is important to note that the results of the differential abundance analyses in the present study may be influenced by the statistical protocol applied [31]. This protocol minimises the rate of false positives, unlike other commonly used approaches to microbiome data, such as comparisons of proportions. On the other hand, considering the limited sample size used in the 16S rRNA gene pyrosequencing, it would be appropriate to verify our findings on the bacterial diversity of the SF-biofilm vs the TF-biofilm in future research using a larger number of participants and biofilm samples.

- Factors influencing the thickness, bacterial viability, and diversity of the SF-biofilms

In the present series, thickness and viability, as well as the biofilm's global structure and the bacterial composition of the SF-biofilm, were not affected by the IDODS or substrate position. Therefore, IDODS can be used in both the upper or lower arches and represent dental plaque well. The best position for IDODS will depend on a study's objective. Artificial substrates in the oral cavity are preferentially positioned buccally as bacterial accumulation is very limited on the lingual side [49]. Based on this premise, other authors [11] have concluded that buccally positioned substrates did not differ much regarding SF-biofilm thickness when taking into account the fact that they were located in the upper or lower jaw. This observation is corroborated by the results of the present research, both in the inter-arch and intra-arch positions.

On the other hand, this is the first study to test the influence of toothbrushing during the SF-biofilm formation period. So far, this variable has not been evaluated, with studies found in which the volunteers did not brush their teeth at all [50,51], while there are others in which they did [7–9,52,53]. Our results revealed that the thickness of the SF-biofilm formed after 48 h and its bacterial viability were significantly affected by the toothbrushing protocol, as these microscopic parameters were higher in test 1 (without toothbrushing), especially in the medial-mesial positions. Consequently, toothbrushing is an essential factor in the development of SF-biofilms, partly because volunteers can eliminate any leftovers that may be a potential source of nutrients for the oral biofilm or because early colonisers are favoured by the constant removal of plaque.

Conclusion

An IDODS carrying various artificial substrates (enamel, glass, and hydroxyapatite) allows the development of an *in situ,* 2-day supragingival biofilm. Based on our findings, the biofilm's global structure has a subject-dependent character but is not conditioned by the substrate material or the device position. The bacterial composition of the biofilm on the artificial substrates was similar to that on the tooth surfaces, with significant differential abundance only detected in very few genera and species of low abundance. Unlike the substrate material and the device/substrate position, the toothbrushing practice does influence the bacterial characteristics (thickness and bacterial viability) of substrate-formed biofilms and is, therefore, a clinical aspect to consider in supragingival biofilm studies. Accordingly, *in-situ* SF-biofilms after two days of maturation appear to be highly representative of naturally occurring supragingival biofilms providing promising opportunities for oral microbiology research.

Data availability statement

Sequence data that support the findings of this investigation were deposited in the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP131455, https://www.ncbi.nlm.nih.gov/sra/SRP131455).

Acknowledgments

The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary information files.

Disclosure statement

No potential conflict of interest was reported by the authors.

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