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# Structure, viability and bacterial kinetics of an *in vitro* biofilm model using six bacteria from the subgingival microbiota

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*Background and Objective:* There are few *in vitro* models available in the scientific literature for study of the structure, formation and development of the subgingival biofilm. The purpose of this study was to develop and validate an *in vitro* biofilm model, using representative selected bacteria from the subgingival microbiota.

*Material and Methods:* Six standard reference strains were used to develop biofilms over sterile ceramic calcium hydroxyapatite discs coated with saliva within the wells of presterilized polystyrene tissue culture plates. The selected species represent initial (*Streptococcus oralis* and *Actinomyces naeslundii*), early (*Veillonella parvula*), secondary (*Fusobacterium nucleatum*) and late colonizers (*Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*). The structure of the biofilm obtained was studied using a vital fluorescence technique in conjunction with confocal laser scanning microscopy. The biofilm bacterial kinetics were studied by terminal restriction fragment length polymorphism analysis.

*Results:* After 12 h, initial and early colonizers were the first microorganisms detected adhering to the calcium hydroxyapatite discs. The intermediate colonizer *F. nucleatum* was not detected in the model until 24 h of incubation. Late colonizers *A. actinomycetemcomitans* and *P. gingivalis* could be measured inside the biofilm after 48 h. The biofilm reached its steady state between 72 and 96 h after inoculation, with bacterial vitality increasing from the hydroxyapatite surface to the central part of the biofilm.

*Conclusion:* An *in vitro* biofilm model was developed and validated, demonstrating a pattern of bacterial colonization and maturation similar to the *in vivo* development of the subgingival biofilm.

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The human oral cavity is a complex ecological environment where microorganisms, bathed in a liquid medium (saliva), have access to both hard and soft tissue surfaces to adhere, hence to develop biofilms. Human dental plaque is a dynamic and complex biofilm where bacteria from saliva are adhered to tooth surfaces, embedded in a matrix of polymers. The development and maturation of dental plaque as a biofilm has profound implications in

the etiology and progression of the most prevalent infections affecting humans, namely, dental caries and periodontal diseases (1).

Numerous studies have attempted to describe the bacterial composition of dental plaque in both healthy and diseased subjects, reporting the presence of hundreds of microbial species organized in communities (2–4). The number of bacterial species is currently undetermined and becomes increasingly higher as advances in molecular techniques allow for more specific identification profiles (5–9). Dental plaque usually develops at stagnant sites between teeth (approximal surfaces), in the pits and fissures on the occlusal surfaces of molars and premolars or at the tooth–gingival interface (gingival crevice). Dental plaque in approximal surfaces as well as in pits and fissures usually evolves into a cariogenic microbiota, with high proportions of streptococci and lactobacilli species. At the tooth–gingival interface, in periodontal health, gram-positive bacteria dominate the composition of the supragingival plaque, with high proportions of streptococci species. As gingival inflammation develops (gingivitis), this microbiota evolves to a more complex composition, with increasing proportions of gram-negative, anaerobic bacteria species. When the periodontal lesion (periodontal pocket) is established, there is a clear predominance of high proportions of gram-negative anaerobic species, such as *Porphyromonas gingivalis*, *Actinomyces* spp., *Tannerella forsythia* and *Treponema denticola* (2–4,10).

The composition of these oral biofilms has been studied using different bacterial identification methods, but there is, however, a lack of adequate knowledge of its structure, community dynamics and ecological determinants. This is probably due to the inherent heterogeneity and structural complexity of these biofilms and to the difficulties of studying *in vivo* dental plaque in health and disease. In order to overcome some of these difficulties, simplified *in vitro* models have been developed with the objective of studying the most salient features of these bacterial communities (11). With the purpose of mimicking the supragingival plaque biofilm, several investigators have developed specific-species biofilm models, using either microtitre plates or flow cell assays (12–14), by constructing oral devices (splints) carried by subjects that allow plaque accumulation (15–18), or by using a constant-depth film fermenter once dental plaque is inoculated (11,19,20).

There are, however, few models available in the scientific literature for study of the structure, formation and development of subgingival plaque. Some attempts have been made by placing inserts of different materials into periodontal pockets of periodontitis patients (21,22), or by using dispersed subgingival plaque or a selection of specific subgingival bacteria, in microtitre plate assays or in the constant-depth film fermenter systems (14,20,23–25). Very few of these studies, however, have provided accurate information on how subgingival plaque matures over time and the sequence of events that occurs in the development of a steady-state biofilm. The purpose of this study was, therefore, to develop and validate an *in vitro* biofilm model aiming to simulate the composition of the subgingival microbiota, comprising initial (*Streptococcus oralis* and *Actinomyces naeslundii*), early (*Veillonella parvula*), secondary (*Fusobacterium nucleatum*) and late colonizers (*P. gingivalis* and *Aggregatibacter actinomycetemcomitans*). Moreover, we attempted to study its structure and dynamics using both morphological (confocal laser scanning microscopy; CLSM) and molecular approaches (terminal restriction fragment length polymorphism; T-RFLP).

## Material and methods

### Bacterial strains

Standard reference strains of *S. oralis* CECT 907T, *V. parvula* NCTC 11810, *A. naeslundii* ATCC 19039, *F. nucleatum* DMSZ 20482, *A. actinomycetemcomitans* DSMZ 8324 and *P. gingivalis* ATCC 33277 were used in this study. Bacteria were grown on blood agar plates (blood agar oxoid no. 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg/mL hemin (Sigma, St Louis, MO, USA) and 1.0 mg/mL menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% $H_2$, 10% $CO_2$ and balance $N_2$) at 37°C for 24–72 h.

### Growth kinetics of standard reference strains

Each of the six standard reference strains was grown in modified brain–heart infusion medium by anaerobic incubation at 37°C for 24–72 h. This protein-rich medium contains brain–heart infusion (Becton, Dickinson and Company; Becton, Dickinson Co., Franklin Lakes, NJ, USA) supplemented with 2.5 g/L mucin (Oxoid), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Sigma), 2.0 g/L sodium bicarbonate (Merck), 5.0 mg/mL hemin (Sigma), 1.0 mg/mL menadione (Merck) and 0.25% (v/v) glutamic acid (Sigma). The growth kinetics were evaluated by generating growth curves of each experiment in triplicate. After incubation, the bacteria were harvested in the late exponential growth phase and added to fresh modified brain–heart infusion medium in order to begin the growth curve at the lag phase [0.05 of optical density (OD) at 550 nm]. At specified time intervals, measurements of the OD at 550 nm were made and 100 μL aliquots taken. These aliquots were diluted serially in phosphate-buffered saline (PBS; pH 7.2; 0.1 M), plated in duplicate on supplemented blood agar plates, and incubated in anaerobic conditions at 37°C for 4–7 d. After incubation, the counts of the total number of colony-forming units (CFU) per millilitre of sample were calculated.

### Preparation of saliva

Whole and unstimulated saliva samples collected in sterile plastic tubes were obtained from healthy volunteers at least 1.5 h after eating, drinking or tooth brushing. Aliquots of 10.0 mL were treated with 2.5 mM DL-dithiothreitol (Sigma) for 10 min with stirring to reduce salivary protein aggregation. Treated saliva was then centrifuged (10 min, 4°C, 30,000 *g*)

and the supernatant was diluted 50% with PBS, filtered, sterilized through a 0.22 μm pore size Millex GV low-protein-binding filter ×50 (Millipore; Millipore Corp., Bedford, MA, USA) and stored at −20°C. The efficacy of this protocol was assessed by plating processed saliva samples onto supplemented blood agar plates, incubating for 72 h at 37°C and observing no growth on either aerobically or anaerobically incubated plates.

### Biofilm development assays

The six standard reference strains selected were used to develop biofilms over sterile ceramic calcium hydroxyapatite discs (HA), 7 mm in diameter and 1.8 mm thick (SD 0.2 mm; Clarkson Chromatography Products, Williamsport, PA, USA), within the wells of presterilized polystyrene, 24-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany). The bacterial concentration was adjusted by spectrophotometry in order to obtain a solution in modified brain–heart infusion medium containing $10^3$ CFU/mL for *S. oralis*, $10^5$ CFU/mL for *V. parvula* and *A. naeslundii*, and $10^6$ CFU/mL for *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*. The HA discs were coated with 50% sterile saliva for 4 h at 37°C in sterile plastic tubes and then placed in the wells of a 24-well tissue culture plate. Each well was then filled with 1.5 mL pooled bacterial culture prepared and incubated in anaerobic conditions (10% $H_2$, 10% $CO_2$ and balance $N_2$) at 37°C for up to 7 d (1, 12, 24, 48, 72, 96, 120, 144 or 168 h), with a change to fresh medium at 48 h intervals after 72 h of incubation. The plates employed for assessing the sterility of the culture medium were used as controls.

### Confocal laser scanning microscopy analysis of biofilms and statistical analysis

Before the CLSM analysis, the discs were sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 s) three times, in order to remove nonadherent bacteria.

Noninvasive confocal imaging of fully hydrated biofilms was carried out by means of a fixed-stage TCS SP2 confocal microscope (Leica Microsystems, Milton Keynes, UK) incorporating a 488 nm Ar/Ar–Kr laser scan head mounted on a vibration-free platform. The objective lenses used were ×10 dry and ×63 water-immersion lenses (Leica Microsystems). Specimens were stained with LIVE/DEAD® BacLightTM Bacterial Viability kit solution (Molecular Probes BV, Leiden, The Netherlands) at room temperature. The 1:1 ratio of fluorocromes and 9 ± 1 min of staining time were used to obtain the optimal fluorescence signal at the corresponding wavelengths (SYTO9®, 515–530 nm; Propidium Iodide, > 600 nm). At least three separate and representative locations on the HA discs covered with biofilm, which contained stacks (or 'towers') that could be isolated within the confocal view field, were selected for these measurements. Within each area, the thickest point was measured by determination of the upper and lower boundaries of the biofilm. The CLSM control software was set to take a *z*-series of scans (*xyz*) of 0.5 μm thickness (8 bits, 1024 × 1024 pixels). Image stacks were analysed by using the Leica Confocal Lite® software (Leica Microsystems). In order to quantify the biomass and cell viability within the biofilm, total fluorescence staining of the confocal micrographs was analysed using the image analysis program MetaMorph® 7.6 software (Molecular Devices Corp., Sunnyvale, CA, USA). The program was used to calculate the percentage of the biomass and cell viability from stacks of two-channel images by measuring voxel intensities. Fluorescence intensity thresholds were manually set for each of the fluorescence colours. The resulting biofilm contents were analysed for statistical significance.

spss version 13.0 software (SPSS Inc., Chicago, IL, USA) was used for all data analyses. The Kolmogorov–Smirnov test was used to evaluate the normality of the distribution, by measuring the skewness and kurtosis for each variable. The data for all variables were expressed as means and SD. Statistical significance was established at the 95% confidence level.

### Extraction of DNA

Before DNA extraction, the discs were sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 s) three times, in order to remove nonadherent bacteria.

The biofilm samples were then collected in sterile plastic tubes that contained 1.0 mL of PBS, and were separated from the HA disc by vortexing for 3 min. The DNA was extracted from the bacterial cell pellet obtained using the DNA extraction kit GNome DNA Kit® (MP Biomedicals, LLC, Cleveland, OH, USA) following the manufacturer's instructions. Bulk nucleic acids were precipitated from solution with ethanol followed by centrifugation. The DNA precipitate was washed with 70% ethanol, dried, and resuspended in 50 μL of sterile water. The bacterial genomic DNAs used as controls were extracted from pure cultures following the same protocol. Genomic DNA was stored at −20°C until analysis.

### PCR amplification of the 16S rDNA

The primers used for the PCR amplification of 16S rRNA gene sequence were 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1544R (5′-AGAAAGGAGGTGATCCAGCC-3′).
Forward primer 27F was labeled at the 5′ end with the 6′-carboxyfluorescein (6-FAM), which was synthesized by Invitrogen (Carlsbad, CA, USA). Amplification reactions were carried out in triplicate, performed in a total volume of 50 μL containing template DNA (100 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer and 2.5 units of Taq DNA polymerase (Invitrogen). The 16S rRNA genes were amplified in an Applied Biosystems Thermocycler model 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) using the following program: 94°C for 3 min, followed by 35 cycles consisting of 94°C for 30 s, 55°C for 1 min and 72°C for 3 min, with a final extension cycle of 72°C for 10 min. Amplified 16S rDNA was verified by electrophoresis on a 1.0% agarose gel, in 1× TAE

buffer. To remove primers and to concentrate amplicons, the Illustra Kit® (GE Healthcare, Chalfont St.Giles, Bucks UK) was used according to the manufacturer's protocol. Purified 16S rDNAs were stored at −20°C until analysis.

### Terminal restriction fragment length polymorphism analysis

The restriction enzymes were selected according to Moyer *et al*. (26). To assess the reproducibility of T-RFLP analysis, three different PCRs from a single DNA sample were performed and combined. Purified PCR product (15 μL) was digested with 30 units (1.5 U/μL) of either *Rsa*I or *Msp*I (Roche Diagnostics, Meylan, France) in a total volume of 20 μL at 37°C for 4 h. The restriction digest product (1 μL) was mixed with 13.75 μL of de-ionized formamide and 1 μL DNA fragment-length standard. The standard size marker was GS 500 LIZ (Applied Biosystems, Carlsbad, CA, USA), including 35, 50, 75, 100, 139, 150, 160, 200, 300, 350, 400, 450, 490 and 500 bp. Each sample was denatured at 95°C for 5 min and then immediately placed on ice for 5 min. The length of the terminal restriction fragment (T-RF) was determined on a 3730 DNA analyser (Applied Biosystems) at 15 kV, 8 mA and 60°C for 40 min for each sample. Fragment sizes were estimated by using the Peak Scanner software version 1.0 (Applied Biosystems). As the excised mixture of amplicons was analysed in a DNA sequencer, only the terminal fragments were read. The resulting T-RFLP profile was expressed in an electropherogram representing the total fluorescence as a peak instead of the band in the electrophoresis gel. The abundance of each T-RF was determined based on fluorescence intensity and expressed as peak height. Terminal restriction fragments with a peak height of $< 400$ fluorescence units were excluded from the analysis.

Predicted T-RFLP patterns for the 16S rDNAs of each of the bacterial species used in the study were obtained from pure cultures by enzyme digestion with *Rsa*I or *Msp*I.

## Results

### Bacterial growth kinetics

Comparisons of the OD obtained at 550 nm with direct counts showed that an OD of 1.0 corresponded to a direct count of $1.0 \times 10^9$ cells/mL for *S. oralis*, reaching the stationary phase in 4 h; $1.5 \times 10^9$ cells/mL for *A. naeslundii*, reaching the stationary phase in 22 h; $3.6 \times 10^{10}$ cells/mL for *V. parvula*, reaching the stationary phase in 42 h; $6.0 \times 10^8$ cells/mL for *F. nucleatum*, reaching the stationary phase in 24 h; $1.8 \times 10^9$ cells/mL for *A. actinomycetemcomitans*, reaching the stationary phase in 30 h; and $4.0 \times 10^9$ cells/mL for *P. gingivalis*, reaching the stationary phase in 49 h. These results ensured similar initial conditions for the *in vitro* biofilm assay.

### Structural analysis of the biofilm and viability assessment

Biofilms were collected at different times (1, 12, 24, 48, 72, 96, 120, 144 or 168 h) and analysed by CLSM. To confirm the repeatability of the biofilm-generating procedure, three independent trials (on three different occasions) with trios of biofilms were carried out. Figure 1 shows representative confocal micrographs depicting two-dimensional maximum projection images of the biofilms per time point. Viability of the biofilms was analysed using CLSM images stained with LIVE/DEAD® BacLight™ Bacterial Viability kit solution (Molecular Probes), clearly delineating the viable and nonviable bacteria as green- and red-stained cells, respectively. Table 1 shows the evolution of the biofilm thickness and vitality.

One hour after inoculation, some multicellular aggregates, probably originating from the inoculum, could

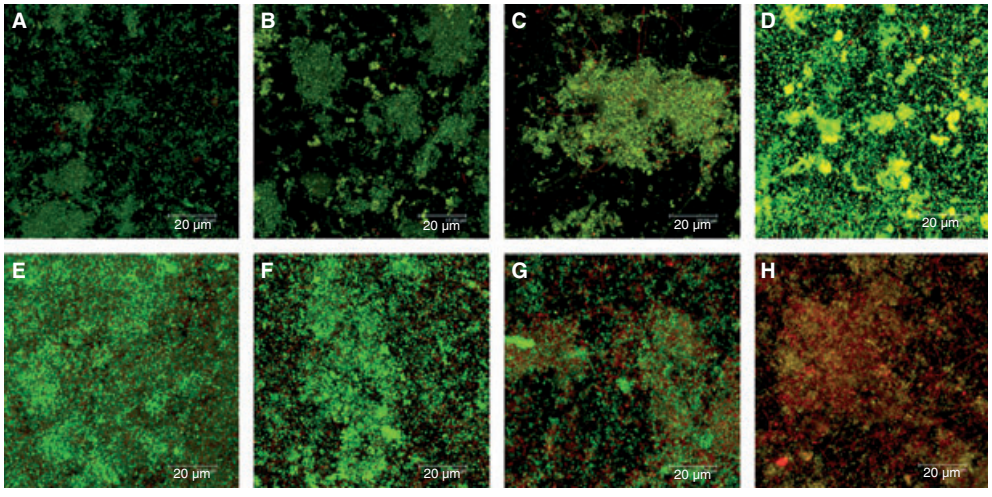


*Fig. 1.* Confocal micrographs representing a two-dimensional maximum projection of the series along a fixed axis of the biofilms after 12 h (A), 24 h (B), 48 h (C), 72 h (D), 96 h (E), 120 h (F), 144 h (G) and 168 h (H) of growth. BacLight Live/Dead stain was used to assess the vitality of cells.

*Table 1.* Observed changes in biofilm vitality and thickness

| Biofilm sample | 12 h ($n$ = 9) | 24 h ($n$ = 9) | 48 h ($n$ = 9) | 72 h ($n$ = 9) | 96 h ($n$ = 9) | 120 h ($n$ = 9) | 144 h ($n$ = 9) | 168 h ($n$ = 9) |
|---|---|---|---|---|---|---|---|---|
| Vitality (%) | | | | | | | | |
| Mean | 78.4 | 80.7 | 78.2 | 60.7 | 57.7 | 54.5 | 46.2 | 23.7 |
| Standard deviation | 9.1 | 9.9 | 13.5 | 9.5 | 17.1 | 10.6 | 11.3 | 8.4 |
| Median | 78.2 | 79.4 | 80.9 | 58.1 | 56.6 | 55.5 | 40.9 | 24.1 |
| Interquartile range | 9.5 | 7.3 | 17.3 | 13.4 | 12.0 | 21.4 | 14.5 | 9.3 |
| Thickness (μm) | | | | | | | | |
| Mean | 29.6 | 30.7 | 25.8 | 35.1 | 35.5 | 25.6 | 21.1 | 21.2 |
| Standard deviation | 5.7 | 12.4 | 11.0 | 16.9 | 11.9 | 12.6 | 4.3 | 5.2 |
| Median | 27.4 | 25.6 | 24.2 | 36.0 | 37.9 | 22.4 | 20.0 | 20.0 |
| Interquartile range | 9.0 | 10.5 | 16.8 | 10.0 | 21.0 | 3.9 | 2.1 | 2.0 |

be seen sparsely attached to the HA surface. After 12 h of *in situ* formation, biofilms consisted mostly of a large cell population arranged either as single cells or as short streptococcal chains, attached to the saliva-coated HA surface. Some stacks could be identified, mostly comprised of viable bacteria, with 78.4% (SD 9.1%) of viable cells and a measurable mean thickness of 29.6 μm (SD 5.7 μm). After 24 h of incubation, bacterial cells were distributed in clusters of microcolonies and were arranged in larger stacks (mean height of 30.7 μm; SD 12.4 μm) against a background of single cells attached to the saliva-coated HA discs; 80.7% (SD 9.9%) of the bacterial population was alive. At this moment, spindle-shaped rods, indicating *F. nucleatum*, were observed inside the biofilm. After 48 h, the biofilm appeared firmly attached to the saliva-coated surface of the HA disc. The biomass was doubled, with a cell viability of 78.2% (SD 13.5%), and the biofilm developed into a multilayered consortium of bacteria, with the formation of stacks reaching up to 36 μm. The biofilm continued to develop for 72 h with an increased biomass but with decreased cell vitality, with values close to 60.7% (SD 9.5%). The entire disc surface was covered with cells, combining a continuous layer of cells with clusters forming prominences. Between 96 and 144 h of incubation, no relevant changes occurred in the biofilm structure, suggesting that the biofilm had reached a steady state. The height of the biofilm reached up to 38 μm, but vitality continued to decline, with values between 46.2% (SD 11.3%) and 57.7% (SD 17.1%). After 168 h of incubation, biofilm biomass and cell vitality decreased dramatically, with only 23.7% (SD 8.4%) of cells alive.

The cell viability profiles were also assessed throughout the depth of the biofilm, along the $z$-axis. The 72-h-old biofilms (steady state) showed an increase of the cell viability percentage with depth. In the bottom half of the biofilm, however, the profiles of viability tended to fall with increasing depth. In this biofilm model, therefore, a lower percentage of vital organisms was found near the HA disc surface, while the percentage increased in the $z$-axis towards the central parts. Along the $x/y$-axis, no general pattern for vitality distribution could be identified.

Using 72-h-old biofilms (steady state), the reproducibility of this biofilm model was assessed, based on the evaluation of the biomass and cell vitality within the biofilm. The statistical analysis showed that there was a homogeneous distribution of vitality among the samples ($p$ = 0.959). Steady-state biofilms presented a mean vitality of 60.7% (SD 9.5%) and median of 58.1% with an interquartile range of 13.4%.

### Single organism terminal restriction fragments

In order to identify individual organisms by their T-RFLP pattern within the *in vitro* biofilm, T-RF lengths were determined for all organisms used (Fig. 2). For each bacterium, the observed T-RF lengths were different by a maximum of 4 bp within three trials after digestion with *Rsa*I and *Msp*I (Table 2), and an extra T-RF was observed for some organisms (Fig. 2), possibly indicating 16S rDNA sequence heterogeneity in these organisms. Digestion with *Msp*I resulted in similar length T-RFs for two different organisms (Table 2).

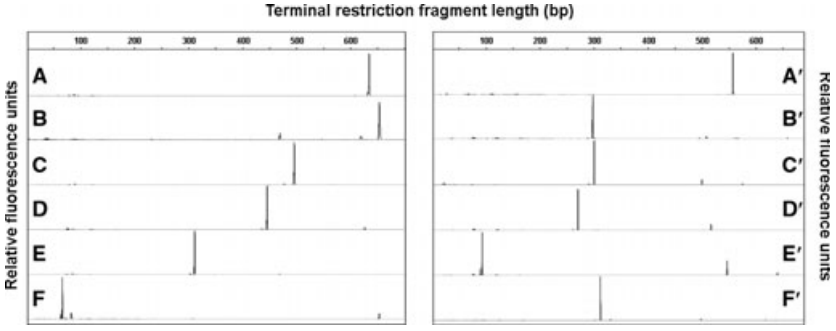


*Fig. 2.* Terminal restriction fragment length polymorphism patterns derived from *Rsa*I and *Msp*I digestion, respectively, of 16S rDNAs from the following individual bacteria used: *S. oralis* (A–A′), *A. naeslundii* (B–B′), *V. parvula* (C–C′), *F. nucleatum* (D–D′), *P. gingivalis* (E–E′) and *A. actinomycetemcomitans* (F–F′).

*Table 2.* Observed length of terminal restriction fragments (T-RFs) of 16S rDNA amplified from the six individual bacteria used

| Bacterial strain | Length (bp) of the 5′ T-RFs after digestion with | |
|---|---|---|
| | *Rsa*I | *Msp*I |
| *Streptococcus oralis* | 631 ± 1 | 556 ± 1 |
| *Veillonella parvula* | 495 ± 1 | 299 ± 1 |
| *Actinomyces naeslundii* | 650 ± 4 | 297 ± 3 |
| *Fusobacterium nucleatum* | 440 ± 1 | 267 ± 1 |
| *Porphyromonas gingivalis* | 314 ± 1 | 93 ± 1 |
| *Aggregatibacter actinomycetemcomitans* | 70 ± 1 | 307 ± 1 |

### Bacterial community T-RFLPs from *in vitro* biofilm samples

Universal primers 6-FAM-27F and 1544R were used to amplify 16S rRNA genes of oral bacteria within the generated biofilms. The T-RFLP patterns were derived from the *Rsa*I and *Msp*I digestion of amplified 16S rDNAs. Each T-RFLP profile consisted of the ratio between the number of fragments with unique lengths and the relative abundance of each fragment, as reflected by the size of each peak in the electropherogram, where the *x*-axis depicts the fragment size and the *y*-axis the fragment fluorescence intensity. Each peak corresponds to one genetic variant in the biofilm, while its height corresponds to its relative abundance in the bioflm.

Figure 3 shows one of the three sets of T-RFLP profiles per biofilm, selected arbitrarily, from 12 to 168 h of incubation from the *Rsa*I digestion. Visual inspection of the T-RFLPs patterns in Fig. 3 provides confirmation of the differences between the bacterial communities sampled at different times of incubation. The proportion of the bacterial species integrating into the biofilm increased gradually between 12 and 48 h of incubation. Within the first 1–12 h of incubation, biofilms demonstrated three different T-RFs that corresponded to the T-RFLP pattern of the initial and early colonizers *S. oralis* (632 ± 1 bp), *A. naeslundii* (650 ± 4 bp) and *V. parvula* (495 ± 1 bp; Table 2). These results are consistent with the observed CLSM patterns at this incubation time, where the biomass was represented either by single cells or short streptococcal chains. After 24 h, a new T-RF emerged, with a size of 440 ± 1 bp, corresponding to *F. nucleatum*. At the same time, the peak heights (relative fluorescence units) of *S. oralis*, *A. naeslundii* and *V. parvula* T-RFs increased, indicating higher numbers of each bacterial population, although *A. naeslundii* showed a slower growth rate compared with *S. oralis* and *V. parvula*. These results are consistent with the significant increase of biomass measured by CLSM at this incubation time. After 48 h of *in situ* formation, two new T-RFs could be identified at 70 ± 1 and 315 ± 1 bp, corresponding to *A. actinomycetemcomitans* and *P. gingivalis*, respectively. Therefore, after 48 h of incubation, the six bacterial species inoculated were part of the biofilm. Between 48 and 96 h, biofilms demonstrated similar T-RLFP patterns, with an increase in peak heights, indicating an increase in the bacterial population, thus demonstrating the maturation of the biofilm, also in conformity with the CLSM results. After 120 h of incubation, the peak heights of T-RFs tended to diminish progressively and were clearly reduced after 168 h. The results obtained by digestion with *Msp*I were in agreement with the generated T-RFLP patterns after digestion with *Rsa*I (data not shown).

Dynamic profiles for each biofilm species resulting from averaging three independent experiments are shown in Fig. 4A. These profiles are based on the T-RFLP analysis after digestion with *Rsa*I. Figure 4B shows the relative proportion of each species from 1 to 168 h biofilm samples, obtained from the T-RFLP patterns after *Rsa*I digestion of 16S rDNAs. These proportions were generated by dividing the relative fluorescence per species by the total relative fluorescence of the

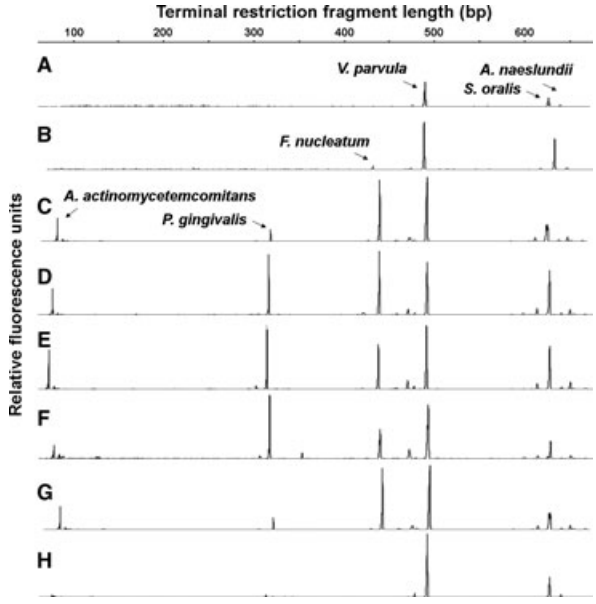


*Fig. 3.* Terminal restriction fragment length polymorphism patterns derived from *Rsa*I digestion of 16S rDNAs from biofilm samples after the following incubation times: 12 h (A), 24 h (B), 48 h (C), 72 h (D), 96 h (E), 120 h (F), 144 h (G) and 168 h (H). Arrows indicate fluorescence peaks corresponding to each terminal restriction fragment present in the biofilm bacteria, *S. oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum*, *P. gingivalis* and *A. actinomytemcomitans*.

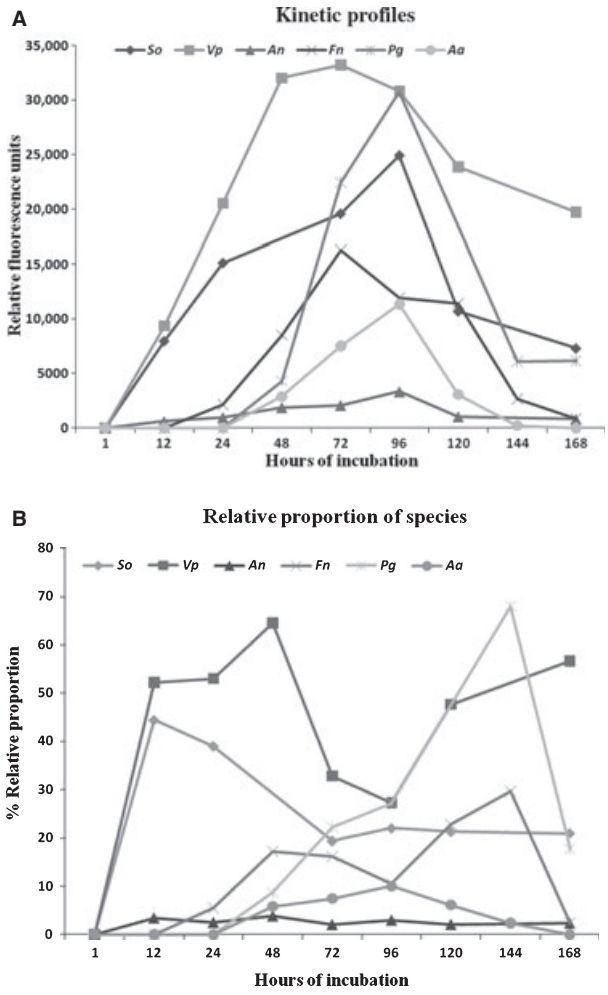


*Fig. 4.* (A) Kinetic profiles of the six standard strains in the biofilm, obtained from terminal restriction fragment length polymorphism patterns derived from *Rsa*I digestion of 16S rDNAs from biofilm samples from 1 to 168 h. (B) Kinetic profiles of the relative bacterial proportions within the biofilm obtained from the terminal restriction fragment length polymorphism patterns after *Rsa*I digestion of 16S rDNAs. *S. oralis* (So), *V. parvula* (Vp), *A. naeslundii* (An), *F. nucleatum* (Fn), *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg).

sample. This total fluorescence is an indication of the total DNA quantity represented by each profile and was calculated in each sample from the sum of all peak heights ≥400 fluorescence units (400 fluorescence units being the threshold for baseline noise).

## Discussion

In this investigation, we have attempted to develop an *in vitro* biofilm model using well-defined species simulating the composition of the *in vivo* subgingival plaque. The selected species are frequently found in the subgingival plaque (2,4,5,8) and include initial, early, intermediate and late colonizers, belonging to different clusters or complexes, as described by Socransky *et al.* (27). Among them, relevant periodontal pathogens are included, such as *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*. This six selected bacterial species also exemplify a wide range of metabolic and physiological characteristics, which represent a diverse array of oral bacteria (13).

Very few subgingival biofilm models have been reported in the literature, probably due to the inherent difficulties in obtaining reproducible growth of these microorganisms. The growth medium is one of the key factors in *in vitro* biofilm development, because it strongly influences intra- and interspecies aggregation and co-aggregation (12). In this biofilm model, we developed a complex medium with a high proportion of proteins, reproducing the carbon and energy sources available to bacteria within the oral cavity. With the use of this medium, together with the inoculated six selected bacterial species on natural saliva-coated HA discs in anaerobic conditions, we were able to develop a biofilm. The experimental conditions in this model also included a discontinuous flow system in the cell culture plates, with the aim of minimizing detachment forces and avoiding liquid–air interfaces during the renewal of fresh culture medium (12). This flow system only dislodged a loose halo of bacteria on the surface of the biofilm, but not the cells attached to the disc surface. These experimental conditions have the advantage over other systems, such as the constant-depth film fermenter or flow cell assays, which utilize fluid flows or scraper blades that generate continuous detachment forces, with the risk of modifying the biofilm structure (11,14,20).

The structure and spatial distribution of viable and nonviable bacteria in the generated biofilm was evaluated using CLSM in conjunction with fluorescence staining. This is a noninvasive and nondestructive microscopic technique, frequently used in studies assessing the biofilm structure, from dental plaque biofilm models generated in mouth splints worn by healthy volunteers, from *in vitro* models using dispersed plaque bacteria, or from constant-depth film fermenter models using a defined multispecies inoculum (15,16,18–20). All these studies have reported different structural descriptions, but in general there is agreement with the results reported in this investigation that generated biofilms show an uneven spatial distribution of vital and dead microorganisms. In the present biofilm model, the viability profiles showed that in steady-state biofilms (72-h-old biofilms), the percentage of vital microorganisms was lower adjacent to the HA disc surface, but increased in the *z*-axis towards the central parts, which contained a greater percentage of viable bacteria.

There were higher proportions of viable bacteria in the upper layers of the biofilm compared with the deeper layers. This may be due to the scarcity of essential nutrients and/or physicochemical conditions (e.g. pH, redox potential) in the deeper biofilm layers, which results in dead or physiologically inactive cells showing lower fluorescence intensity (11). The bacteria in the deeper layers of the biofilm are likely to be dependent on one or more metabolic end-products derived from the organisms located at the center of the stacks, hence dying from deprivation of these essential nutrients. This dead biological material is probably an important factor in the initial phase of dental biofilm development, because bacteria in the intermediate layer may benefit from its close proximity to dead cells in the basal layer and use this cellular material in the basal layer as a source of nutrients (15). This finding of dead bacterial layers adjacent to the HA disc surface has been previously described in histochemical and electron-microscopic studies (16).

The initial attachment of bacteria to a hard surface in a liquid-phase medium is essential to the biofilm formation. Even though the six selected bacterial species were simultaneously exposed to a substratum, the sequence of their incorporation into the biofilm model shown in this investigation confirmed the pattern that occurs in subgingival plaque formation *in vivo* (28). Although the research on the composition of bacterial communities within biofilms has used different identification methods, the advent of molecular biological techniques has provided a more comprehensive, rapid and precise bacterial characterization than the first attempts using conventional culture-dependent methods (4,7–9,14,23,29,30). In the present report, the bacterial dynamics inside the biofilm were described by determining T-RFLP of genes encoding 16S rRNA. The analysis of T-RFLP has been demonstrated to be a well-suited molecular approach for studying complex bacterial communities, because the differences in the sizes of T-RFs reflect differences in the sequences of 16S rRNA genes (i.e. sequence polymorphisms), thus detecting phylogenetically distinct populations of organisms and providing a rapid and reproducible description of the bacterial population dynamics (31–33). Determination of the length of fluorescently labeled T-RFs by automated DNA sequencers allowed a highly precise fragment length determination, using internal size standards in every profile, and provided numerical data of high resolution. The ability of this method to differentiate microbial communities has been previously validated (29,30,34).

In this investigation, the T-RFLP analysis resulted in the initial colonization by *S. oralis* and *A. naeslundii* that appeared attached to the HA disc after 12 h, probably by adhering directly to the acquired pellicle. Also as an early colonizer, *V. parvula* was identified at 12 h of incubation. The intermediate colonizer found was *F. nucleatum*, which was not detected in this model until 24 h of incubation. Late colonizers included *A. actinomycetemcomitans* and *P. gingivalis*, which were identified within the biofilm after 48 h of *in situ* formation. Biofilms showed an increase in the bacterial population between 72 and 96 h after inoculation, reaching the steady state at this time. After 96 h, the peak heights decreased as the biofilm aged. In these old biofilms, the presence of dead bacteria, as confirmed by CLSM, would result in DNA degradation and therefore in altered T-RF peak heights.

This biofilm behavior is in agreement with other studies analysing biofilm communities of subgingival plaque either *in vivo* (21,22) or *in vitro*, using dispersed subgingival plaque (20,24), as well as those models using selected bacterial consortia (14,23,25). Periasamy & Kolenbrander (14) demonstrated that *P. gingivalis* could not grow as a single species or together with the initial colonizer *S. oralis*, but showed a positive growth when inoculated with other bacterial species, such as *Veillonella* spp. (early colonizer), *F. nucleatum* (secondary colonizer) and *A. actinomycetemcomitans* (late colonizer). We were able to demonstrate in the present model that six bacterial species grew in consortia and developed into a well-defined biofilm structure.

In conclusion, an *in vitro* biofilm model has been developed and validated, using defined multibacterial species from the subgingival plaque, which included initial (*S. oralis* and *A. naeslundii*), early (*V. parvula*), secondary (*F. nucleatum*) and late colonizers (*P. gingivalis* and *A. actinomycetemcomitans*). The combined use of confocal laser scanning microscopy and terminal restriction fragment length polymorphisms of genes encoding 16S rRNA demonstrated the usefulness of these techniques for studying the spatial distribution and dynamics of specific members of the bacterial communities in a biofilm model. This model was reproducible, and as it is technically simple to prepare, maintain and analyse, it is very suitable for studying the development, structure and dynamics of the subgingival biofilm, as well as for undertaking comparative studies to test different antimicrobial approaches.

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