Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 95–104

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01409.x

Early microbial succession in redeveloping dental biofilms in periodontal health and disease

F. R. Teles[1,2], R. P. Teles[1,2], N. G. Uzel[1*], X. Q. Song[1], G. Torresyap[1†], S. S. Socransky[1], A. D. Haffajee[1]

[1]Department of Periodontology, The Forsyth Institute, Cambridge, MA, USA and [2]Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, MA, USA

Teles FR, Teles RP, Uzel NG, Song XQ, Torresyap G, Socransky SS, Haffajee AD. Early microbial succession in redeveloping dental biofilms in periodontal health and disease. J Periodont Res 2012; 47: 95–104. © 2011 John Wiley & Sons A/S

Background and Objective: The development of dental biofilms after professional plaque removal is very rapid. However, it is not clear whether most bacterial species return at similar rates in periodontally healthy and periodontitis subjects or if there are differences in bacterial recolonization between supragingival and subgingival biofilms in periodontal health and disease.

Material and Methods: Supragingival and subgingival plaque samples were taken separately from 28 teeth in 38 healthy and 17 periodontitis subjects immediately after professional cleaning. Samples were taken again from seven teeth in randomly selected quadrants after 1, 2, 4 and 7 d of no oral hygiene and analyzed using checkerboard DNA–DNA hybridization. The percentage of DNA probe counts were averaged within subjects at each time-point. Ecological succession was determined using a modified moving-window analysis.

Results: Succession in supragingival biofilms from subjects with periodontitis and from healthy individuals was similar. At 1 d, *Streptococcus mitis* and *Neisseria mucosa* showed increased proportions, followed by *Capnocytophaga gingivalis, Eikenella corrodens*, *Veillonella parvula* and *Streptococcus oralis* at 1–4 d. At 4–7 d, *Campylobacter rectus, Campylobacter showae, Prevotella melaninogenica* and *Prevotella nigrescens* became elevated. Subgingival plaque redevelopment was slower and very different from supragingival plaque redevelopment. Increased proportions were first observed for *S. mitis,* followed by *V. parvula* and *C. gingivalis* and, at 7 d, by *Capnocytophaga sputigena* and *P. nigrescens*. No significant increase in the proportions of periodontal pathogens was observed in any of the clinical groups or locations.

Conclusion: There is a defined order in bacterial species succession in early supragingival and subgingival biofilm redevelopment after professional cleaning.

Dr Flavia R. F. Teles, DDS, MS, DMSc, Department of Periodontology, The Forsyth Institute, 245 First Street, Cambridge, MA 02142, USA
Tel: 617 892 8562
Fax: 617 262 4021
e-mail: fteles@forsyth.org

*Present address: Private Practice, Bala Cynwyd, PA, USA.
†Present address: Children's Hospital, Boston, MA, USA.

Key words: bacteria; biofilm; ecology; periodontal; succession; supragingival

Accepted for publication July 17, 2011

When dental plaque is removed by self-performed or professional procedures, there is an immediate and often visible reduction in the total number of organisms, followed, within hours, by a return of detectable plaque. Indeed, the re-establishment of dental biofilms in many people is so rapid that it is generally recommended that individuals brush their teeth at least twice daily. When the rate of dental biofilm return after professional plaque removal was measured by indices or quantitative assessment, it was estimated that biofilms returned to their precleaning levels in 1–2 d (1–5). What was not clear from these estimates was whether all bacterial species present in dental biofilms returned at similar rates in periodontally healthy and periodontitis subjects. Furthermore, it was not clear

whether there were differences in patterns of bacterial recolonization between supragingival and subgingival biofilms. A few studies have examined the shifts in microbial species that occur during *in vivo* supragingival or subgingival plaque development. Ritz (6) used selective media to demonstrate that streptococci and *Neisseria* were prominent at 1 d and that the proportion of *Actinomyces* species was initially low but had risen by 9 d. Nyvad & Kilian (7) used culture techniques to follow the early colonization of pieces of enamel and root surfaces mounted in acrylic appliances in human volunteers. They found that streptococci and gram-positive pleiomorphic rods dominated in the first 24 h. *Streptococcus mitis* and *Streptococcus oralis* were major contributors to the biofilm content, comprising 24–42% and 1–27% of the microbiota, respectively. Nyvad & Kilian (8) compared the streptococcal composition of 4-h biofilms that formed on pieces of enamel mounted on acrylic appliances in the mouths of adolescents. It was found that that the predominant streptococci were *S. oralis* and *S. mitis*, and that *Streptococcus sanguinis* was present at a higher proportion in the mouths of caries-inactive individuals than in the mouths of caries-active individuals. Socransky *et al*. (3) used predominant cultivable microbiota techniques to show that supragingival counts had increased at 1 d and plateaued from 2 to 16 d. *S. sanguinis* was detected at all time-points and at an increased proportion at 1 d. *Actinomyces* species were present in low proportions at 1 d but had increased by 16 d. Using the same techniques, Zee *et al*. (4) examined pooled plaque samples from 'rapid' and 'slow' plaque-forming subjects. *Streptococcus* species were dominant at day 1 but by day 14 their mean proportions had decreased and the proportions of *Actinomyces* species had increased.

Studies of very early biofilm development using molecular techniques demonstrated that *S. mitis* and *S. oralis* were present in high numbers in supragingival biofilm 6 h after tooth polishing (1). Ramberg *et al*. (5) used similar techniques to study early biofilm development in the mouths of subjects with minimal gingivitis. After an intense 2-wk preparatory phase involving repeated professional cleaning, the dominant taxa in 0 time supragingival plaque samples were *Actinomyces* species. During 4 d of no oral hygiene, there was an increase in the levels of *Streptococcus, Capnocytophaga, Campylobacter* and *Fusobacterium* species as well as an increase in the level of *Aggregatibacter actinomycetemcomitans*.

Quirynen *et al*. (9) used molecular and culture techniques to examine the subgingival colonization of 'pristine' periodontal pockets by following the development of subgingival biofilms on recently inserted implants. Species were detected at similar frequencies from the 1-wk microbiota around implants and from the undisturbed subgingival plaque of shallow tooth sites, but counts of red-complex and orange-complex species were higher for tooth sites. Longer-term studies by the same group demonstrated little change in the already established complex microbiota between 2 and 26 wk, except for an increase in red-complex and orange-complex species (10).

The above investigations provided a starting point for understanding changes in species composition during *in vivo* biofilm development. However, there was a need for larger-scale supragingival biofilm studies and for studies that would involve subgingival plaque biofilm samples from periodontally healthy and periodontitis sites. In an earlier publication (11), we described microbial shifts in redeveloping supragingival and subgingival dental biofilms over a 7-d period in the absence of oral hygiene in periodontally healthy and periodontitis subjects. It was shown that supragingival biofilm redevelopment was similar in both groups. Mean total DNA probe counts (i.e. mean number of bacterial cells) reached precleaning levels by 2 d, with *Veillonella parvula* and *Neisseria mucosa* showing the greatest increase. Redevelopment of subgingival biofilm was somewhat different. Significant differences between clinical groups were present in subgingival biofilm samples by 7 d for 17 species, including *Actinomyces* and *Fusobacterium* as well as *A. actinomycetemcomitans, Prevotella intermedia* and *Porphyromonas gingivalis,* all of which were present at higher levels in the periodontitis group.

Species counts were useful for determining increases in total biomass and indicating the species whose numbers became markedly elevated. However, changes in the proportions of species are more sensitive for the detection of abrupt shifts in taxa than counts; particularly for species that are present in low numbers in biofilm samples. The finding of such abrupt changes during a specific time-period allows the identification of an orderly and predictable pattern of change in a community over time, known as ecological succession (12). In the present study, changes in the proportions of species were used to seek evidence of bacterial succession and to identify increases followed by decreases in proportions, highlighting so-called 'microbial blooms'. Therefore, the purpose of the present investigation was to examine the order of species succession during supragingival and subgingival biofilm redevelopment after professional dental cleaning in periodontally healthy and diseased subjects.

Material and methods

Details regarding the subject population, clinical monitoring, dental cleaning, microbial sample-taking and enumeration have been described by Uzel *et al*. (11) and are briefly presented here.

Subject population

Thirty-eight periodontally healthy subjects and 17 patients with chronic periodontitis were enrolled, according to the following criteria.

Inclusion criteria for healthy subjects: > 20 years of age; > 24 teeth; no pocket depth or attachment level measurements > 3 mm; < 20% of sites with overt gingival redness and/or bleeding on probing; and willingness and ability to sign informed consent.

Inclusion criteria for periodontitis subjects: > 20 years of age; > 20 teeth; > 8 teeth with pocket depth

and/or attachment level > 4 mm; and willingness and ability to sign informed consent.

Exclusion criteria: pregnancy or breastfeeding; periodontal or antibiotic therapy in the previous 3 mo; systemic conditions that might influence the course of periodontal disease or treatment (e.g. diabetes, AIDS); systemic conditions that require antibiotic therapy for routine periodontal procedures (e.g. heart conditions, joint replacements); soft tissue lesions (e.g. leukoplakia, lichen planus); and smoking.

Attempts were made to recruit approximately equal numbers of male and female subjects. In addition, subjects of any racial/ethnic group were accepted for the study. All subjects were recruited at The Forsyth Institute. The study was approved by The Forsyth Institute Institutional Review Board and all subjects signed informed consent before entering the study. The baseline clinical characteristics of the subjects in the two groups are shown in Table 1.

Clinical monitoring and treatment protocols

All subjects were clinically monitored at entry. Clinical measurements were taken at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) on all teeth excluding third molars (a maximum of 168 sites per subject), as previously described (13). The clinical parameters were measured in the following order: (i) gingival index (14); (ii) plaque index (15); (iii) pocket depth (mm); (iv) attachment level (mm); (v) bleeding on probing (0 or 1); and (vi) suppuration (0 or 1).

Pocket depth and attachment level measurements were made to the nearest mm using a North Carolina periodontal probe. The measurements were performed twice, and the average of the pair of measurements was used for analysis. The first set of supragingival and subgingival plaque samples were taken before making the clinical measurements. Samples were taken by the same calibrated examiner at all sampling visits for a given subject.

Scaling and root planing or dental prophylaxis— At the entry visit, after the initial monitoring and sampling, all periodontitis subjects received full-mouth scaling and root planing (SRP) at a single visit, using manual curettes and ultrasonic devices, followed by polishing and flossing. Periodontally healthy subjects received a dental prophylaxis using a rubber cup and paste, followed by dental flossing. After the initial prophylaxis or SRP, subjects refrained from oral hygiene procedures for 7 d.

Table 1. Mean clinical parameters (± standard deviation) of subject groups at baseline

Parameter	Healthy Subjects	Periodontitis patients	*p* (Mann Whitney)
No. of subjects	38	17	
Age (years)	32.3 ± 9.3	44.9 ± 11.9	< 0.001
Number of missing teeth	0.9 ± 1.6	2.2 ± 2.4	< 0.05
Percentage men	39	35	NS
Percentage past smokers	29	29	NS
Pocket depth (mm)	1.9 ± 0.3	2.7 ± 0.3	< 0.001
Attachment level (mm)	1.5 ± 0.6	3.0 ± 1.2	< 0.001
Plaque index	1.2 ± 0.7	1.6 ± 0.3	NS
Percentage of sites with:			
Redness	47.7 ± 30.7	62.3 ± 34.8	< 0.001
BOP	7.3 ± 6.9	27.2 ± 12.8	< 0.001
Suppuration	0.0 ± 0.0	0.2 ± 0.4	< 0.01
PD			
> 6 mm	0.0 ± 0.0	0.8 ± 1.0	< 0.001
4–6 mm	0.9 ± 2.2	15.3 ± 7.2	< 0.001
< 4 mm	99.1 ± 2.2	83.8 ± 7.3	< 0.001
AL			
> 4 mm	0.0 ± 0.0	5.3 ± 10.7	< 0.001
4–6 mm	1.8 ± 6.6	21.6 ± 17.2	< 0.001
< 4 mm	98.2 ± 6.6	73.1 ± 26.1	< 0.001

Data are given as percentage or mean ± standard deviation.
AL, attachment level; BOP, bleeding on probing; NS, not significant; PD pocket depth.

Microbiological sample taking and enumeration of organisms

Individual supragingival and subgingival plaque samples were taken separately from the mesiobuccal aspect of up to 28 teeth in each subject at entry and immediately after tooth cleaning. Thus, up to 28 samples per subject were taken at two visits (at baseline and immediately after professional cleaning) from two locations (supragingival and subgingival) for a total of up to 6160 samples (55 subjects × 28 teeth × 2 visits × 2 locations). Each quadrant in each subject was randomly assigned to be sampled at the 1-, 2-, 4- and 7-d time-points (i.e. up to seven teeth from the same quadrant were sampled at a given time-point). Seven supragingival and separately seven subgingival samples were taken at those time-points, providing up to 3080 additional samples (55 subjects × 7 teeth × 4 visits × 2 locations).

Supragingival plaque samples were taken separately from each tooth using individual sterile Gracey curettes. After removal of any remaining supragingival plaque, subgingival plaque samples were taken separately from each tooth and evaluated as described earlier in the Material and methods. Each sample was placed in individual tubes containing 0.15 mL of 10 mM Tris–HCl/0.1 mM EDTA (TE) pH 7.6, and then 0.15 mL of freshly prepared 0.5 M NaOH was added. The samples were boiled for 5 min, neutralized using 0.8 mL of 5 M ammonium acetate, placed into the extended slots of a Minislot (Immunetics, Cambridge, MA, USA) and then concentrated onto a positively charged nylon membrane (Roche, Indianapolis, IN, USA) by vacuum and fixed to the membrane by exposure to ultraviolet light followed by baking at 120°C for 20 min. The counts of the 41 species in each sample were determined using checkerboard DNA–DNA hybridization (16,17).

Data evaluation

The percentage of DNA probe counts of each of the 41 test species in the individual supragingival and subgingival biofilm samples was computed. The percentage value for each species was averaged within each subject at each time-point and then averaged across subjects at that time-point in the two clinical groups separately. Up to 28 supragingival and 28 subgingival samples were averaged per subject immediately after tooth-cleaning (day 0), and seven samples were averaged per subject at days 1, 2, 4 and 7.

Major significant increases or decreases over time in the proportions of species in each location (supragingival or subgingival) in each clinical group (periodontally healthy or periodontitis) were sought using a modification of a 'moving-window' approach (18). The moving-window approach is commonly used in macro-ecology to identify changes in communities, patterns of community assembly and factors associated with the development of community structure in forests, lakes and soil (19–21). It has also been used for the study of environmental microbial ecology (22,23). It allows the analysis of multivariate data across a gradient, which, in the present study, was time (7 d), and is particularly useful for the detection of sharp transitions in species composition in one ecosystem (24).

The mean proportions of each species at each time-point in a location/clinical group were compared from the first time-point with mean proportions of the same species in samples from the same subjects at each of the later time-points, and a t statistic of > 1.96 between the tested time-points was considered to be different. Thus, the t statistic was used as a measuring stick to discriminate meaningful differences in the mean proportions of a species at two time-points. These differences were considered to be significant.

The technique of visual inspection of the data (18) revealed that in some

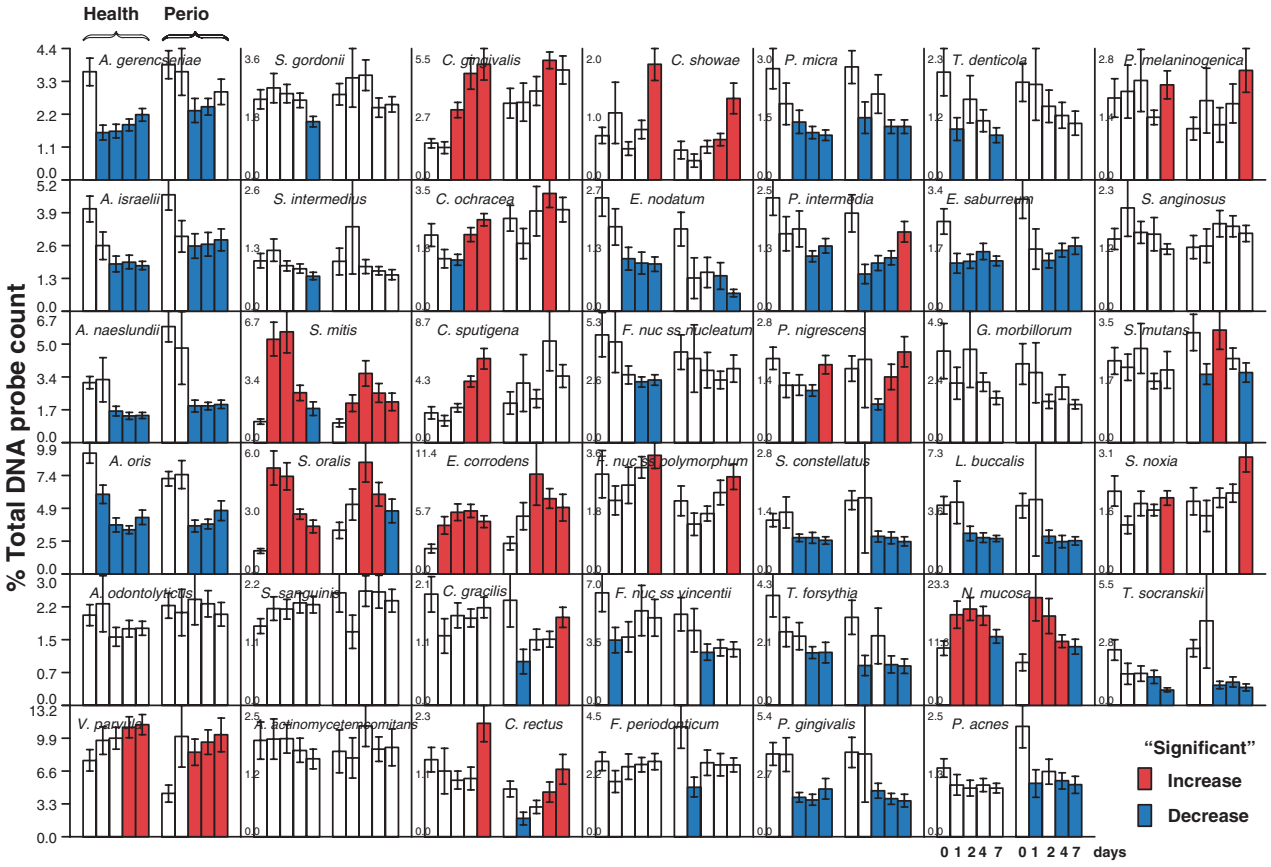

Fig. 1. Bar charts of the mean percentage of the total DNA probe count of 41 bacterial species in samples of supragingival biofilm taken immediately after dental cleaning (day 0) and after 1, 2, 4 and 7 d of biofilm accumulation in the absence of oral hygiene procedures from periodontally healthy subjects (left set of bars in each panel) and from subjects with periodontitis (right set of bars in each panel). The percentage of the total DNA probe count was computed for each species in each sample, averaged within the subject at that time-point, and then averaged across subjects for the individual time-points, separately for subjects who did or did not have periodontitis. The bars indicate the mean values and the whiskers indicate the standard error of the mean. Note that the y-axis values differ for each species and are indicated in each panel. The red bars indicate a significant increase in the mean proportion from a mean value for an earlier time-point for that species in that clinical group (see data analysis). Similarly, the blue bars indicate a significant reduction in mean proportions of species from an earlier time-point. Species are ordered according to subgingival microbial complexes (29).

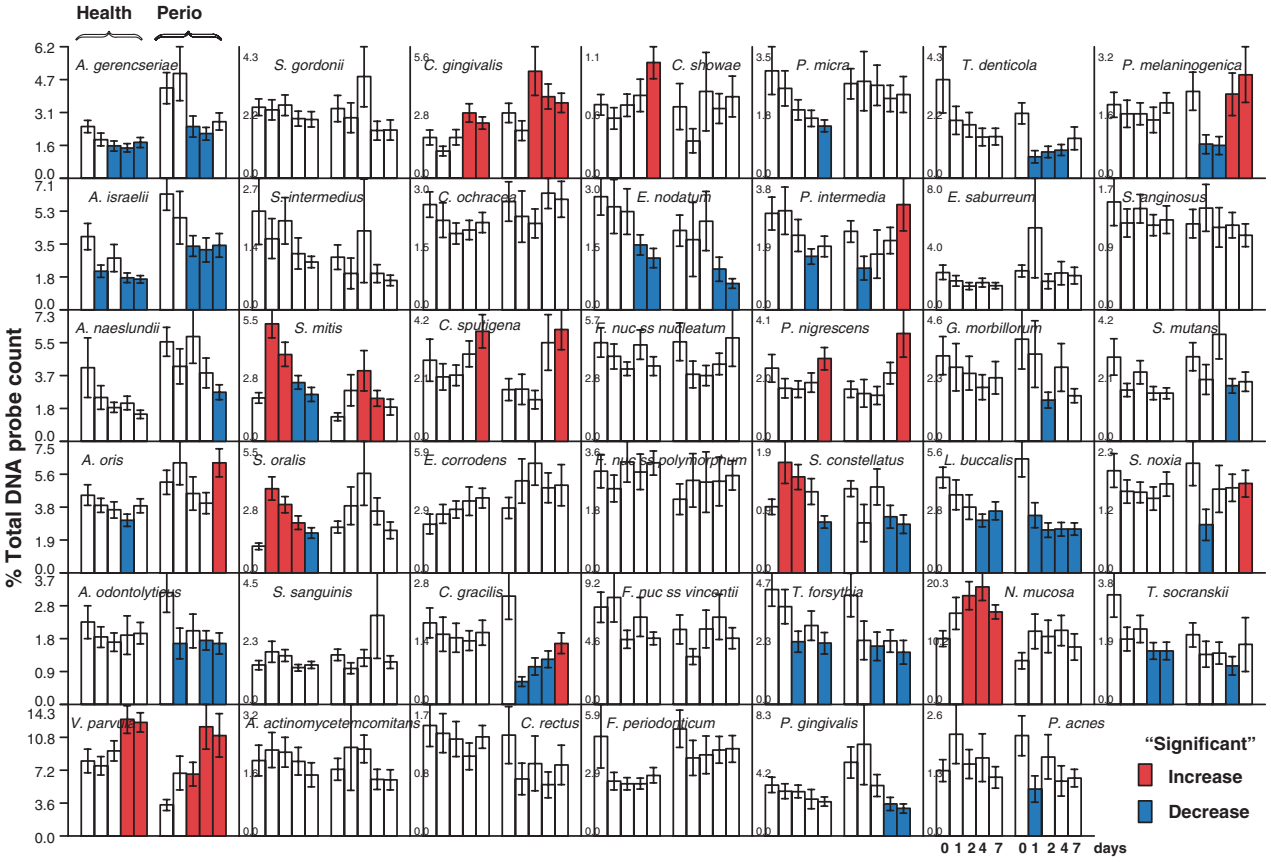

Fig. 2. Bar charts of the mean percentage of the total DNA probe count of 41 bacterial species in samples of subgingival biofilm taken immediately after dental cleaning and after 1, 2, 4 and 7 d of biofilm accumulation in the absence of oral hygiene procedures. The subject population, computation of mean species proportions and determination of significant increases or decreases in proportions were performed as described for Fig. 1.

instances (see for example, *Capnocytophaga gingivalis* in periodontally healthy subjects in Fig. 1), increases did not occur in the samples immediately after cleaning (day 0), but at later visits, such as from 2 to 4 d and from 2 to 7 d in the cited example. Thus, the moving window sought differences, not just from day 0 to days 1, 2, 4 and 7, but also from day 1 to days 2, 4 and 7, from day 2 to days 4 and 7, and from day 4 to day 7.

Results

Bacterial succession in supragingival dental biofilms in periodontally healthy individuals

Figure 1 presents the mean proportions of 41 bacterial taxa in supragingival biofilms obtained from periodontally healthy subjects (as well as periodontitis patients) immediately after prophylaxis (day 0) and after 1, 2, 4 and 7 d of biofilm accumulation in the absence of self-performed oral hygiene. The first species showing a significant increase in mean proportions, at 1 d, were *S. mitis, S. oralis, Eikenella corrodens* and *N. mucosa*. *S. mitis* and *S. oralis* maintained their high proportions at day 2 but began to decrease thereafter. *E. corrodens* increased from immediate postcleaning (day 0) mean values to significantly higher values at 1, 2, 4 and 7 d, while *N. mucosa* increased at 1, 2 and 4 d and declined slowly thereafter. *C. gingivalis* began to increase significantly at 2 d and the mean proportions continued to show an increase at 4 and 7 d. The mean proportions of *V. parvula, Capnocytophaga ochracea* and *Capnocytophaga sputigena* showed a significant increase at 4 d. At 7 d, significant increases were observed for *Campylobacter rectus* and *Campylobacter showae*. Major significant decreases were observed in the mean proportions of *Actinomyces* species, *Fusobacterium nucleatum* subspecies, *P. intermedia, P. gingivalis, Treponema denticola, Eubacterium nodatum, Parvimonas micra* and *Streptococcus constellatus* after cleaning.

Bacterial succession in supragingival dental biofilms in subjects with periodontitis

At day 1, only the mean proportions of *S. mitis* and *N. mucosa* increased significantly in the supragingival samples from subjects with periodontitis (Fig. 1). By day 2, significant increases were observed in the mean proportions of *S. oralis, E. corrodens* and *V. parvula*. The mean proportions of *C. gingivalis, C. ochracea, C. rectus, Prevotella nigrescens* and *C. showae* increased by 4 d and the mean proportions of *F. nucleatum* ss

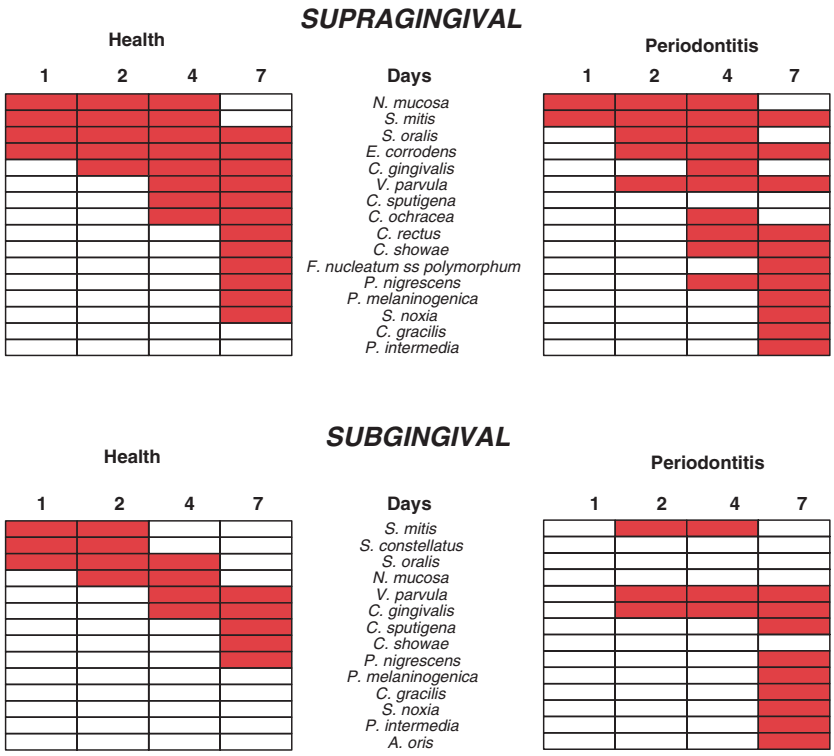

Fig. 3. Taxa that showed 'significant' increases in mean proportions during the 7 d of biofilm redevelopment in the absence of oral hygiene procedures. The top pair of panels indicate supragingival samples, and the bottom pair of panels indicate subgingival samples. Panels to the left represent samples from periodontally healthy subjects and panels to the right represent data from subjects with periodontitis. The red rectangles indicate time-points at which the mean proportions were significantly higher than the mean proportions in samples from an earlier time-point (see Material and methods). The taxa were ordered according to the earliest 'significant' increases in periodontally healthy subjects.

polymorphum, P. intermedia, Campylobacter gracilis, Prevotella melaninogenica and *Selenomonas noxia* increased by 7 d. A decrease in the mean proportions in supragingival biofilm samples obtained from periodontitis subjects was observed for *Actinomyces* species, *P. gingivalis, Tannerella forsythia, E. nodatum, F. nucleatum* ss *vincentii, S. constellatus* and *Treponema socranskii.*

Bacterial succession in subgingival dental biofilms in periodontally healthy individuals

At 1 d, only the mean proportions of *S. mitis, S. oralis* and *S. constellatus* were increased significantly in the subgingival biofilms of periodontally healthy subjects (Fig. 2). At 2 d, *N. mucosa*, at 4 d, *V. parvula* and *C. gingivalis,* and at 7 d *C. sputigena, C. showae* and *P. nigrescens* also showed significant increases in the periodontally healthy subject group. The mean proportions of *Actinomyces israelii, Actinomyces gerencseriae, T. forsythia, E. nodatum, P. intermedia, Leptotrichia buccalis, P. micra,* and *T. socranskii* decreased during biofilm redevelopment in this group.

Bacterial succession in subgingival dental biofilms in subjects with periodontitis

The earliest significant increase in mean proportions occurred at day 2 for *V. parvula, S. mitis* and *C. gingivalis* in subgingival biofilm samples from subjects with periodontitis (Fig. 2). From days 4 to 7, the mean proportions of *Actinomyces oris, C. sputigena, C. gracilis, S. noxia, P. intermedia, P. nigrescens* and *P. melaninogenica* also increased significantly. Notable decreases in mean proportions occurred for *Actinomyces* species, *S. constellatus, E. nodatum, T. forsythia, P. gingivalis* and *T. denticola.*

Comparison of bacterial succession in periodontal health and disease

Figure 3 summarizes the significant increases in mean proportions that took place in supragingival and subgingival biofilm samples from subjects who were periodontally healthy or had periodontitis. In supragingival samples from both periodontally healthy and periodontitis subjects, early increases (at 1 and 2 d) in the mean proportions were seen for *S. mitis, S. oralis, E. corrodens* and *N. mucosa*. The mean proportion of *V. parvula* increased significantly at 2 d in periodontitis subjects and at 4 d in healthy individuals. The mean proportion of *C. gingivalis* increased significantly at 2 and 4 d in both clinical groups. At 4–7 d, increases were observed for both clinical groups in the mean proportions of *C. rectus, C. showae, C. ochracea* and *P. nigrescens*. The species that showed a significant increase in periodontitis subjects, but not in healthy subjects,

included *C. gracilis* and *P. intermedia*. The mean proportion of *C. sputigena* increased in healthy subjects but not in those with periodontitis.

In subgingival samples, increases in the mean proportions of specific species started at 1 d in samples from healthy subjects and at 2 d in the samples from patients with periodontitis. At 1–2 d, only the proportions of *S. mitis* increased significantly in subgingival biofilm samples from both healthy subjects and patients with periodontal disease. The mean proportions of *V. parvula* and *C. gingivalis* increased from days 2 to 4, whereas the mean proportions of *C. sputigena* and *P. nigrescens* increased in both clinical groups at 7 d. In subgingival samples from periodontal healthy subjects, *S. oralis* and *S. constellatus* increased significantly at day 1, *N. mucosa* at 2 d and *C. showae* at 7 d. In subgingival samples from periodontitis subjects, *P. melaninogenica* increased significantly at 4 d, and *C. gracilis, A. oris, P. intermedia* and *S. noxia* increased significantly at 7 d.

Discussion

The purpose of the present investigation was to define the early ecological succession of bacterial species during 7 d of no oral hygiene after professional removal of supragingival and subgingival plaque from periodontally healthy individuals and from subjects with periodontitis.

Information about ecological succession in periodontal biofilms is limited to a few studies of the development of supragingival plaque in healthy individuals (1–5,7,8,25) and subgingival biofilm formation around implants (9,10). Hence, there is a gap in the knowledge of microbial succession in supragingival and subgingival plaque, in both periodontal health and disease. This information is important because dental biofilms have a direct impact on periodontal stability, disease initiation and progression (26–30). The identification of critical periods for pathogen colonization and proliferation would be helpful in the prevention and management of periodontal diseases.

Studies to date using *in vitro* and *in situ* models have provided guidance in understanding possible growth requirements, spatial organization, as well as synergistic and antagonistic relationships among different species and perhaps can provide clues to possible ecological succession (31–37). However, only *in vivo* studies can demonstrate actual ecological succession and provide the opportunity to test conceptual models or assumptions.

In the present study, we demonstrated that supragingival plaque redevelopment was similar in periodontal health and disease, but the redevelopment of subgingival plaque was quite different in the two clinical groups. In supragingival plaque, specific taxa increased or decreased at similar time-points in both groups. The proportions of *S. mitis* and *N. mucosa* were significantly elevated at day 1, corroborating their proposed role as early colonizers (1–4,6). This role might be a result of their ability to attach to hydroxyapatite or to the salivary glycoproteins that cover hard surfaces, an ability to proliferate in the presence of oxygen and to metabolize dietary or salivary sources of carbohydrate. The proportions of *C. gingivalis* and *E. corrodens* increased significantly in both groups on days 2–4. This time frame coincides with the period when plaque biomass typically surpasses precleaning levels in studies of plaque development in the absence of oral hygiene (4,5,11). Interestingly, those species have been shown to contribute significantly to the increase of biomass in supragingival biofilms (38).

At 4 d, the mean proportions of *V. parvula* showed a 5% increase relative to that of the postcleaning plaque and an increase of almost 10% by day 7. This increase was observed days after the increase in *S. mitis* and *S. oralis,* supporting a likely metabolic food chain in dental biofilms (39). We also observed that a more complex bacterial community ensued in both clinical groups following the increase in proportion of *V. parvula*. This finding is in accordance with a recent publication that regarded *Veillonella* as a critical genus which guides the development of multispecies communities when saliva is the main nutritional source (40). Conceivably, *Streptococcus* species set the stage for the growth of *Veillonella*, which, in turn, set the stage for other taxa.

On days 4 and 7, significant increases in both groups occurred mostly among orange-complex species, including *C. showae, C. rectus, P. nigrescens* and *F. nucleatum* ss *polymorphum*. Additional orange-complex taxa increased in proportions in the periodontitis group, including *P. intermedia* and *C. gracilis*. Orange-complex species have been associated with gingivitis and periodontitis (41,42). In addition, local inflammation has been shown to influence the composition of the supragingival microbiota (43,44). Hence, it is possible that inflammatory responses elicited by dental-cleaning procedures, incomplete healing after mechanical therapy or early plaque redevelopment might have favored their growth and thus might have influenced microbial succession. Pathogenic species, such as *E. nodatum, T. forsythia, P. gingivalis* and *T. denticola*, decreased in both clinical groups. This finding is in accordance with other studies (1,5) and suggests that the habitats which were once conducive to the growth of these fastidious strict anaerobes were disrupted by cleaning procedures and these habitats might take much longer than 7 d to re-establish.

Overall, the subgingival environment exhibited fewer significant changes in the proportions of taxa (Fig. 2), suggesting that this ecosystem may take longer than supragingival biofilms to redevelop. This may be partly because of the physical confinement of this location, which is surrounded by hard and soft surfaces and thus has limited access to certain dietary nutrients. This seclusion might also have shielded the site from potential colonizing bacterial cells that can be disseminated after supragingival and subgingival debridement (45). This suggests that the likely source of recolonizing species in subgingival biofilm is the bacterial cells left behind after cleaning. Professional cleaning might have altered the surfaces for attachment, the reservoir sources for

recolonization and the tissue source of nutrients, all factors that can affect biofilm development (44,46–49).

Significant changes in subgingival biofilm development began somewhat later in periodontitis patients in comparison with periodontally healthy subjects, although, by day 7, more significant increases were observed in the former. At that time-point, three orange-complex species – *C. gracilis, P. intermedia* and *P. nigrescens* – were significantly elevated in the periodontitis group. This finding might suggest a possible shift towards a pathogenic microbiota, even though the proportions of 'classic' periodontal pathogens showed no significant increase in either group. In fact, the proportions of *E. nodatum, P. gingivalis, T. forsythia* and *T. denticola* decreased in periodontitis subjects. These findings are in accordance with those of Quirynen *et al.* (9). Although biofilm development may be somewhat different around implants, it is worth reporting that the authors found that subgingival colonization of shallow and moderate pockets around implants were more similar to the undisturbed microbiota present in shallow pockets around teeth than moderate pockets associated with teeth. These observations describe the level of taxa 1 wk after abutment connection and remained virtually unchanged until 4 wk. Among all shallow and moderate sites, the implant-associated pocket sites had the lowest levels of orange-complex species among all shallow and moderate sites and also extremely low levels of 'classic' periodontal pathogens. The authors suggested that these complexes might take longer to establish, in part because they might require the presence of appropriate conditions, provided by earlier colonizers. In a follow-up paper, the observation period was extended to 26 wk (10). After week 2, a clear increase in the levels of all taxa was observed. Increases in the levels of orange-complex and red-complex species began by week 4, were clear at week 13 and continued to week 26.

While biomass was restored within days after careful dental cleaning in the subjects in this study (11), the climax community typical of the supragingival and subgingival tooth surfaces was not fully reestablished (50). *Actinomyces* species were present at lowered proportions in supragingival biofilms at 7 d, but data in the literature suggest that their return would be more robust by 14 to 21 d (51). The lowered proportions of the red-complex species, *T. forsythia*, *P. gingivalis* and *T. denticola*, might take even longer (months to years) to return to their pre-instrumentation levels (28,52). Thus, time is a critical factor affecting biofilm formation. Clearly, internal remodeling of tooth-associated biofilms takes place over time, enhancing the prominence of some species and the decline of others. The time constraints of this study could not follow these changes further.

The present paper focused on bacterial succession [i.e. it used the proportions of 41 bacterial species in samples to define the sequence of species blooms that occurred as biofilms redeveloped following a catastrophic event (tooth-cleaning)]. One limitation of the study was that the oral cavity harbors many more species than the 41 taxa examined (53–56). However, the species selected for study represent about 60% of dental biofilm isolates recovered by culture (57). Furthermore, eight of 11 of the most common taxa detected by clonal analysis of biofilm samples by Dewhirst *et al.* (55) were among the 41 taxa examined in the present study. In addition, prominent taxa in this study – *V. parvula* and *S. mitis* – accounted for the largest number of clones described in the survey of Dewhirst *et al.*

The design of the study precluded following changes in biofilm redevelopment over time on individual tooth surfaces. While this would have been an ideal goal, it would have necessitated one of two approaches. The first would have been to clean each surface, then take a sample immediately postcleaning, another sample from that surface at 1 d, then again at 2, 4 and 7 d. Unfortunately, when you take, for example, the 1-d sample, you have inadvertently altered the validity of the upcoming 2-d sample by removing a major portion of the developing biofilm. For this reason, this design was rejected. The alternative approach would be to start again from a recleaned tooth surface, each time creating a new time 0. The second design was precluded for two reasons. First, the repeated subgingival recleaning of each tooth surface (four times for each surface) might have affected the adjacent periodontal tissues and altered the typical pattern of recolonization. Furthermore, the subjects would have had to refrain from home care for 2 wk (0–1, 0–2, 0–4, or 0–7 d). Two weeks without oral hygiene was not acceptable to the Institutional Review Board, of concern to the clinical staff (particularly for the subjects with periodontitis) and would have severely compromised subject recruitment. Thus, the second design was also rejected and a compromised design, involving sampling randomly assigned quadrants only once, was utilized. This design lost the ability to follow the microbial changes on the same individual surfaces over time but it was better in that unknown effects of repeated sampling without cleaning a single tooth surface or repeat cleaning of each surface were eliminated. Variability in initial bacterial recolonization of oral surfaces has been demonstrated (58) and confirmed in this study. The use of randomly assigned quadrants did not allow us to follow changes in individual surfaces and therefore to fully assess such variability. However, it did permit us to follow the general pattern of species succession in the supragingival and subgingival biofilms present in periodontally healthy and diseased subjects.

One final limitation of the present study was our inability to describe the spatial relationships that occur among bacterial species during biofilm redevelopment. For example, Dige *et al.* (58) have demonstrated that streptococci are early biofilm colonizers on clean surfaces and that these organisms form 'chimney-like' structures in association with other taxa, often *Actinomyces naeslundii*, in the central portion of the chimney. Such findings suggest that a combination of approaches will be needed to fully appreciate biofilm development. Studies such as

the present one provide a broad quantitative assessment of microbial changes during biofilm development. However, finer details of intermicrobial association, particularly spatial relationships, are better measured by other techniques, including fluorescence *in situ* hybridization (FISH) and confocal microscopy.

In summary, there was clear evidence of bacterial succession in both supragingival and subgingival biofilm samples from periodontally healthy and diseased subjects. Species showing early 'blooms' included *S. mitis, S. oralis, N. mucosa, V. parvula, E. corrodens* and *C. gingivalis*. Species thought to play a role in periodontal pathogenesis, such as *P. gingivalis, T. forsythia, T. denticola* and *E. nodatum*, showed a decline in proportions during the early supragingival and subgingival recolonization period. Succession in supragingival plaque redevelopment was found to be similar in periodontal health and disease, but the redevelopment of subgingival plaque was different in the two clinical groups. This suggests that the bacterial species, the surfaces for colonization and the bulk fluid comprising the supragingival ecosystem, are generally more similar in health and disease than they are in the subgingival ecosystem. Understanding the sequence of bacterial succession that leads to blooms in pathogenic taxa could lead to new approaches to controlling their levels before they achieve sufficient numbers to elicit or contribute to damage to the periodontal tissues.

Acknowledgements

This work was supported, in part, by NIH/NIDCR R01 DE14368 (SS), R03 DE021742 (FT) and U01 DE021127 (RT) and the Eleanor and Miles Shore Fellowship Program for Scholars in Medicine (The Forsyth Institute/Harvard Medical School) (F.T.).

References

1. Li J, Helmerhorst EJ, Leone CW *et al.* Identification of early microbial colonizers in human dental biofilm. *J Appl Microbiol* 2004;**97:**1311–1318.
2. Sharawy AM, Sabharwal K, Socransky SS, Lobene RR. A quantitative study of plaque and calculus formation in normal and periodontally involved mouths. *J Periodontol* 1966;**37:**495–501.
3. Socransky SS, Manganiello AD, Propas D, Oram V, van Houte J. Bacteriological studies of developing supragingival dental plaque. *J Periodontal Res* 1977;**12:**90–106.
4. Zee KY, Samaranayake LP, Attstrom R. Predominant cultivable supragingival plaque in Chinese "rapid" and "slow" plaque formers. *J Clin Periodontol* 1996;**23:**1025–1031.
5. Ramberg P, Sekino S, Uzel NG, Socransky S, Lindhe J. Bacterial colonization during de novo plaque formation. *J Clin Periodontol* 2003;**30:**990–995.
6. Ritz HL. Microbial population shifts in developing human dental plaque. *Arch Oral Biol* 1967;**12:**1561–1568.
7. Nyvad B, Kilian M. Microbiology of the early colonization of human enamel and root surfaces *in vivo*. *Scand J Dent Res* 1987;**95:**369–380.
8. Nyvad B, Kilian M. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. *Caries Res* 1990;**24:**267–272.
9. Quirynen M, Vogels R, Pauwels M *et al.* Initial subgingival colonization of 'pristine' pockets. *J Dent Res* 2005;**84:**340–344.
10. Quirynen M, Vogels R, Peeters W, van Steenberghe D, Naert I, Haffajee A. Dynamics of initial subgingival colonization of 'pristine' peri-implant pockets. *Clin Oral Implants Res* 2006;**17:**25–37.
11. Uzel NG, Teles FR, Teles RP *et al.* Microbial shifts during dental biofilm re-development in the absence of oral hygiene in periodontal health and disease. *J Clin Periodontol* 2011;**38:**612–620.
12. Fierer N, Nemergut D, Knight R, Craine JM. Changes through time: integrating microorganisms into the study of succession. *Res Microbiol* 2010;**161:**635–642.
13. Haffajee AD, Socransky SS, Goodson JM. Comparison of different data analyses for detecting changes in attachment level. *J Clin Periodontol* 1983;**10:**298–310.
14. Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* 1963;**21:**533–551.
15. Turesky S, Gilmore ND, Glickman I. Reduced plaque formation by the chloromethyl analogue of victamine C. *J Periodontol* 1970;**41:**41–43.
16. Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE. "Checkerboard" DNA-DNA hybridization. *BioTechniques* 1994;**17:**788–792.
17. Socransky SS, Haffajee AD, Smith C *et al.* Use of checkerboard DNA-DNA hybridization to study complex microbial ecosystems. *Oral Microbiol Immunol* 2004;**19:**352–362.
18. Legendre PLL. *Numerical Ecology*. Amsterdam: Elsevier, 2003.
19. Dale MRT, Dixon P, Fortin MJ, Legendre P, Myers DE, Rosenberg MS. Conceptual and mathematical relationships among methods for spatial analysis. *Ecography* 2002;**25:**558–577.
20. Carlson ML, Flagstad LA, Gillet F, Mitchell EAD. Community development along a proglacial chronosequence: are above-ground and below-ground community structure controlled more by biotic than abiotic factors? *J Ecol* 2010;**98:**1084–1095.
21. Parn J, Remm K, Mander U. Correspondence of vegetation boundaries to redox barriers in a Northern European moraine plain. *Basic Appl Ecol* 2010;**11:**54–64.
22. Redford AJ, Fierer N. Bacterial succession on the leaf surface: a novel system for studying successional dynamics. *Microb Ecol* 2009;**58:**189–198.
23. Wittlebolle L, Verstraete W, Boon N. The inoculum effect on the amonia-oxidizing bacterial communities in parallel sequential batch reactors. *Water Res* 2009;**43:**4149–4158.
24. Kent M, Gill WJ, Weaver R, Armitage R. Landscape and plant community boundaries in biogeography. *Prog Phys Geogr* 1997;**21:**315–353.
25. Diaz PI, Chalmers NI, Rickard AH *et al.* Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Appl Environ Microbiol* 2006;**72:**2837–2848.
26. Kamma JJ, Nakou M, Baehni PC. Clinical and microbiological characteristics of smokers with early onset periodontitis. *J Periodontal Res* 1999;**34:**25–33.
27. Haffajee AD, Uzel NG, Arguello EI, Torresyap G, Guerrero DM, Socransky SS. Clinical and microbiological changes associated with the use of combined antimicrobial therapies to treat "refractory" periodontitis. *J Clin Periodontol* 2004;**31:**869–877.
28. Cugini MA, Haffajee AD, Smith C, Kent RL Jr, Socransky SS. The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results. *J Clin Periodontol* 2000;**27:**30–36.
29. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
30. Haffajee AD, Teles RP, Socransky SS. The effect of periodontal therapy on the composition of the subgingival microbiota. *Periodontol 2000* 2006;**42:**219–258.
31. Gmur R, Wyss C, Xue Y, Thurnheer T, Guggenheim B. Gingival crevice microbiota from Chinese patients with gingivitis

or necrotizing ulcerative gingivitis. *Eur J Oral Sci* 2004;**112:**33–41.
32. Gmur R, Thurnheer T. Direct quantitative differentiation between *Prevotella intermedia* and *Prevotella nigrescens* in clinical specimens. *Microbiology* 2002;**148:**1379–1387.
33. Kolenbrander PE, Palmer RJ Jr, Rickard AH, Jakubovics NS, Chalmers NI, Diaz PI. Bacterial interactions and successions during plaque development. *Periodontol 2000* 2006;**42:**47–79.
34. Dalwai F, Spratt DA, Pratten J. Modeling shifts in microbial populations associated with health or disease. *Appl Environ Microbiol* 2006;**72:**3678–3684.
35. Palmer RJ Jr, Diaz PI, Kolenbrander PE. Rapid succession within the *Veillonella* population of a developing human oral biofilm *in situ*. *J Bacteriol* 2006;**188:**4117–4124.
36. Periasamy S, Kolenbrander PE. Aggregatibacter actinomycetemcomitans builds mutualistic biofilm communities with *Fusobacterium nucleatum* and *Veillonella* species in saliva. *Infect Immun* 2009;**77:**3542–3551.
37. Periasamy S, Kolenbrander PE. Mutualistic biofilm communities develop with *Porphyromonas gingivalis* and initial, early, and late colonizers of enamel. *J Bacteriol* 2009;**191:**6804–6811.
38. Haffajee AD, Teles RP, Patel MR, Song X, Veiga N, Socransky SS. Factors affecting human supragingival biofilm composition. I. Plaque mass. *J Periodontal Res* 2009;**44:**511–519.
39. Periasamy S, Kolenbrander PE. Central role of the early colonizer *Veillonella* sp. in establishing multispecies biofilm communities with initial, middle, and late colonizers of enamel. *J Bacteriol* 2010;**192:**2965–2972.
40. Kolenbrander PE. Multispecies communities: interspecies interactions influence growth on saliva as sole nutritional source. *Int J Oral Sci* 2011;**3:**49–54.
41. Teles R, Sakellari D, Teles F *et al*. Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. *J Periodontol* 2010;**81:**89–98.
42. Salvi GE, Franco LM, Braun TM *et al*. Pro-inflammatory biomarkers during experimental gingivitis in patients with type 1 diabetes mellitus: a proof-of-concept study. *J Clin Periodontol* 2010;**37:**9–16.
43. Rudiger SG, Carlen A, Meurman JH, Kari K, Olsson J. Dental biofilms at healthy and inflamed gingival margins. *J Clin Periodontol* 2002;**29:**524–530.
44. Offenbacher S, Barros SP, Singer RE, Moss K, Williams RC, Beck JD. Periodontal disease at the biofilm-gingival interface. *J Periodontol* 2007;**78:**1911–1925.
45. Beikler T, Abdeen G, Schnitzer S *et al*. Microbiological shifts in intra- and extra-oral habitats following mechanical periodontal therapy. *J Clin Periodontol* 2004;**31:**777–783.
46. Madianos PN, Bobetsis YA, Kinane DF. Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. *J Clin Periodontol* 2005;**32**(suppl 6)**:**57–71.
47. Salvi GE, Ramseier CA, Kandylaki M, Sigrist L, Awedowa E, Lang NP. Experimental gingivitis in cigarette smokers: a clinical and microbiological study. *J Clin Periodontol* 2005;**32:**441–447.
48. Filoche S, Wong L, Sissons CH. Oral biofilms: emerging concepts in microbial ecology. *J Dent Res* 2010;**89:**8–18.
49. Marsh PD, Devine DA. How is the development of dental biofilms influenced by the host? *J Clin Periodontol* 2011;**38**(suppl 11)**:**28–35.
50. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. *J Clin Periodontol* 2000;**27:**722–732.
51. Lie MA, van der Weijden GA, Timmerman MF, Loos BG, van Steenbergen TJ, van der Velden U. Oral microbiota in smokers and non-smokers in natural and experimentally-induced gingivitis. *J Clin Periodontol* 1998;**25:**677–686.
52. Haffajee AD, Cugini MA, Dibart S, Smith C, Kent RL Jr, Socransky SS. The effect of SRP on the clinical and microbiological parameters of periodontal diseases. *J Clin Periodontol* 1997;**24:**324–334.
53. Paster BJ, Boches SK, Galvin JL *et al*. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;**183:**3770–3783.
54. Zaura E, Keijser BJ, Huse SM, Crielaard W. Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiol* 2009;**9:**259.
55. Dewhirst FE, Chen T, Izard J *et al*. The human oral microbiome. *J Bacteriol* 2010;**192:**5002–5017.
56. Keijser BJ, Zaura E, Huse SM *et al*. Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res* 2008;**87:**1016–1020.
57. Socransky SS, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000* 2005;**38:**135–187.
58. Dige I, Raarup MK, Nyengaard JR, Kilian M, Nyvad B. Actinomyces naeslundii in initial dental biofilm formation. *Microbiology* 2009;**155:**2116–2126.