

# Microbial shifts during dental biofilm re-development in the absence of oral hygiene in periodontal health and disease

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## Abstract

**Aim:** To monitor microbial shifts during dental biofilm re-development.

**Materials and methods:** Supra- and subgingival plaque samples were taken separately from 28 teeth in 38 healthy and 17 periodontitis subjects at baseline and immediately after tooth cleaning. Samples were taken again from seven teeth in randomly selected quadrants during 1, 2, 4 and 7 days of no oral hygiene. Samples were analysed using checkerboard DNA–DNA hybridization. Species counts were averaged within subjects at each time point. Significant differences in the counts between healthy and periodontitis subjects were determined using the Mann–Whitney test.

**Results:** The total supra- and subgingival counts were significantly higher in periodontitis on entry and reached or exceeded the baseline values after day 2. Supragingival counts of *Veillonella parvula*, *Fusobacterium nucleatum* ss *vincentii* and *Neisseria mucosa* increased from 2 to 7 days. Subgingival counts were greater for *Actinomyces*, green and orange complex species. Significant differences between groups in supragingival counts occurred for 17 of 41 species at entry, 0 at day 7; for subgingival plaque, these values were 39/41 taxa at entry, 17/41 at day 7.

**Conclusions:** Supragingival plaque re-development was similar in periodontitis and health, but subgingival species recolonization was more marked in periodontitis.

Key words: biofilms; health; oral bacteria; periodontal; periodontitis; subgingival; supragingival

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Periodontal diseases are associated with bacterial species present in biofilms that colonize dental surfaces. Most information that describes the microbial composition of dental biofilms is based on studies of samples from mature dental plaque. Light and electron microscopy studies (Listgarten et al. 1975, Listgar-

ten 1976, Listgarten 1999), in vitro adhesion and co-aggregation models (Gibbons & Nygaard 1970, Gibbons et al. 1988, Gibbons et al. 1991, Stromberg & Boren 1992, Kolenbrander & London 1993, Kolenbrander et al. 1993) and in vitro continuous culture studies (Stoodley et al. 1999, Bernimoulin 2003) have been helpful in describing the possible changes that might occur in species composition during biofilm formation. However, few studies have actually examined the shifts in microbial species that occur during in vivo supra- or subgingival plaque development. The body of knowledge describing the

microbial shifts that occur in microbial species during in vivo dental plaque development or re-development appears to result in large part from five studies that examined supragingival plaque samples taken from subjects who refrained from brushing during the period of plaque re-growth (Ritz 1967, Socransky et al. 1977, Zee et al. 1996a,b, Ramberg et al. 2003, Li et al. 2004). Although informative, those studies did not evaluate subgingival plaque development. Further, they assessed a limited number of samples, bacterial taxa and subjects and did not evaluate subjects with periodontitis. Thus, the

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purpose of the present investigation was to compare the changes in species' levels during early dental biofilm development in periodontally healthy and chronic periodontitis subjects who refrained from oral hygiene. The hypotheses to be tested were: (1) plaque development would be more rapid in periodontitis subjects than in periodontally healthy subjects; (2) the shift in the levels of individual taxa over time would differ from periodontally healthy to periodontally diseased subjects and (3) the re-growth of specific periodontal pathogens such as *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* would be slower than the re-growth of other species.

## Material and methods

### Subject population

The subject population consisted of 38 periodontally healthy and 17 chronic periodontitis subjects. The inclusion and exclusion criteria were as follows:

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 nursing, periodontal or antibiotic therapy in the previous 3 months, any systemic condition that might have influenced the course of periodontal disease or treatment (e.g. diabetes, AIDS), any systemic condition that required antibiotic coverage for routine periodontal procedures (e.g. heart conditions, joint replacements), any soft tissue lesions (e.g. leucoplakia, lichen planus) and current smokers.

Attempts were made to recruit approximately equal numbers of males and females. 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.

Table 1. Mean clinical parameters ( $\pm$  SD) of subject groups at baseline

	Healthy	Periodontitis	<i>p</i> (Mann–Whitney)
Number of subjects	38	17	
Age	32.32 $\pm$ 9.34	44.88 $\pm$ 11.94	<i>p</i> < 0.001
Number of missing teeth	0.87 $\pm$ 1.55	2.18 $\pm$ 2.40	<i>p</i> < 0.05
% of males	39	35	NS
% of past-smokers	29	29	NS
Pocket depth (mm)	1.94 $\pm$ 0.30	2.70 $\pm$ 0.29	<i>p</i> < 0.001
Attachment level (mm)	1.50 $\pm$ 0.63	2.98 $\pm$ 1.17	<i>p</i> < 0.001
Plaque index	1.22 $\pm$ 0.74	1.58 $\pm$ 0.73	NS
% of sites with			
Gingival redness	47.69 $\pm$ 30.71	62.63 $\pm$ 34.75	<i>p</i> < 0.001
BOP	7.33 $\pm$ 6.85	27.18 $\pm$ 12.79	<i>p</i> < 0.001
Suppuration	0.00 $\pm$ 0.00	0.16 $\pm$ 0.38	<i>p</i> < 0.01
Number of white	28	10	
Number of African–American	1	4	
Number of Asian	6	2	
Number of others	3	1	

Three white and two other subjects in the healthy group were Hispanic, while two white subjects in the periodontitis group were Hispanic.

### Monitoring and treatment protocols

#### Clinical monitoring

After determination of suitability and obtaining informed consent, subjects entered the study. 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 described previously (Haffajee et al. 1983). The clinical parameters were measured in the following order: (1) gingival index (Loe & Silness 1963); (2) plaque index (Turesky et al. 1970); (3) pocket depth (mm); (4) attachment level (mm); (5) bleeding on probing (0 or 1) and (6) suppuration (0 or 1).

Pocket depth and attachment-level measurements were made to the nearest millimetre using a North Carolina periodontal probe. Pocket depth and attachment-level measurements were performed twice and the average of the pair of measurements was used for analysis. All clinical data were recorded on data sheets and entered into a computer using a prompted data entry program. The first set of supra- and subgingival plaque samples were taken before 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 days.

#### Microbiological sample taking and enumeration of organisms

Individual supra- and subgingival plaque samples were taken separately from the mesio-buccal 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 (baseline and immediately after SRP) from two locations (supragingival and subgingival) for a total of up to 6160 samples (55 subjects  $\times$  28 teeth  $\times$  2 visits  $\times$  2 locations). Quadrants in each subject were randomly assigned to be sampled at the 1-, 2-, 4- and 7-day time points. Seven supra- and separately 7 subgingival samples were taken at those time points, providing up to 3080 additional samples (55 subjects  $\times$  7 teeth  $\times$  4 visits  $\times$  2 locations).

Supragingival plaque samples were taken separately from each tooth using individual sterile Gracey curettes and evaluated for their content of 41 subgingival species using checkerboard DNA–DNA hybridization (Socransky et al. 1994, Socransky et al. 2004). After removing any remaining supragingival plaque, subgingival plaque samples

were taken separately from each tooth and evaluated as described above. Each sample was placed in individual tubes containing 0.15 ml TE (10 mM Tris-HCL, 0.1 mM EDTA, pH 7.6). 0.15 ml of freshly prepared 0.5 M NaOH was added. The samples were boiled for 5 min and neutralized using 0.8 ml 5 M ammonium acetate and placed in the extended slots of a Minislot (Immunelectrics, 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 (Socransky et al. 1994, Socransky et al. 2004).

#### Data evaluation

Clinical parameters including the plaque index, gingival index, % of sites with bleeding on probing and suppuration as well as the mean pocket depth and attachment level were computed for each subject, averaged within subjects and then averaged across subjects in the two groups at entry. Differences in the clinical parameters between groups were determined using the Mann-Whitney test.

The mean microbial counts of 41 test species in individual supragingival and subgingival biofilm samples were assessed separately. The counts for each species were averaged within each subject at each time point and then averaged across subjects in the two clinical groups separately. Up to 28 samples were averaged per subject pre-tooth cleaning and post-tooth cleaning, and seven samples at days 1, 2, 4 and 7. Significance of differences over time (pre-cleaning, post-cleaning and days 1, 2, 4 and 7) for each species was determined using the Friedman test. Pairwise comparisons between the post-cleaning and days 1, 2, 4 and 7 were determined individually using the Wilcoxon signed ranks test in the periodontally healthy and periodontitis groups separately for both the supra- and the subgingival biofilm samples. Differences between clinical groups for each species at each time point were determined using the Mann-Whitney test. The mean values for each species at each time point in the two clinical groups were depicted graphically as

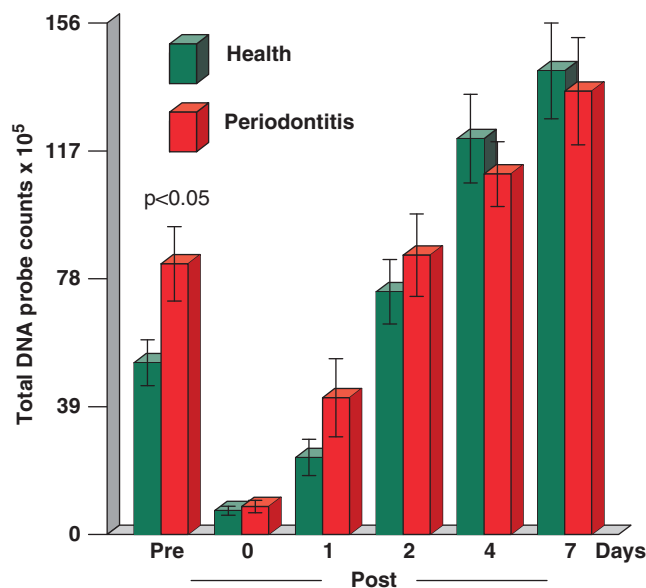


Fig. 1. Mean total DNA probe counts ( $\times 10^5$ ,  $\pm$  SEM) of supragingival biofilm samples taken at entry (pre-cleaning), immediately post-cleaning and after 1, 2, 4 and 7 days of biofilm development from 38 periodontally healthy subjects and 17 subjects with periodontitis. The total counts from each sample site were averaged in each subject at each time point and then averaged across subjects at each time point separately in each clinical group. Subjects did not perform oral hygiene procedures during the 7 days of the study. The significance of differences between clinical groups at each time point was determined using the Mann-Whitney test. The mean total DNA probe counts differed significantly over time in each group (Friedman's test,  $p < 0.001$ ). The bars represent the mean total DNA probe counts and the whiskers show the SEM at each time point.

“microbial profiles” ordered according to the microbial complexes described previously (Socransky et al. 1998).

#### Results

##### Changes in supragingival microbial species counts during biofilm re-development

The changes in the mean total DNA probe counts of the supragingival plaque samples obtained from periodontitis and periodontally healthy subjects are presented in Fig. 1. There was a significant difference in the mean total DNA probe counts at baseline between periodontitis and periodontally healthy subjects. The mean ( $\times 10^5$ ,  $\pm$  SEM) total supragingival DNA probe counts were  $50.2 \pm 6.6$  and  $84.1 \pm 12.4$  in healthy and periodontitis subjects, respectively ( $p < 0.05$ ). The mean total DNA probe counts exceeded baseline levels at day 2 in both periodontally healthy subjects and periodontitis subjects and a steady increase in the total counts was observed until day 7. Differences between clinical groups in the mean total microbial counts immediately after prophylaxis and at 1, 2, 4 and 7 days were not

statistically significant. Interestingly, the mean total counts in healthy subjects were greater than those in periodontitis subjects at days 4 and 7. Cumulative plots of the mean counts of the individual species in periodontally healthy and diseased subjects were remarkably similar other than higher total mean species counts in the periodontitis group at baseline (Fig. 2). Most species surpassed entry levels by day 2. A drastic increase in the mean counts of species was observed in periodontally healthy subjects from 1 to 4 days and in periodontally diseased subjects from immediately post-cleaning to 2 days. The mean increases in the species counts in both groups slowed thereafter.

The mean counts ( $\times 10^5$ ) for 41 individual bacterial species in supragingival plaque samples at different time points in the periodontally healthy and diseased subjects are shown in Fig. 3. There was a significant difference over time in the mean counts of all the test species, from pre-therapy to 7 days (Friedman's test,  $p < 0.001$ ). In addition, there was a significant difference over time for all species from the post-cleaning samples to 7 days (Friedman's test,  $p < 0.001$ ) in both clinical groups.

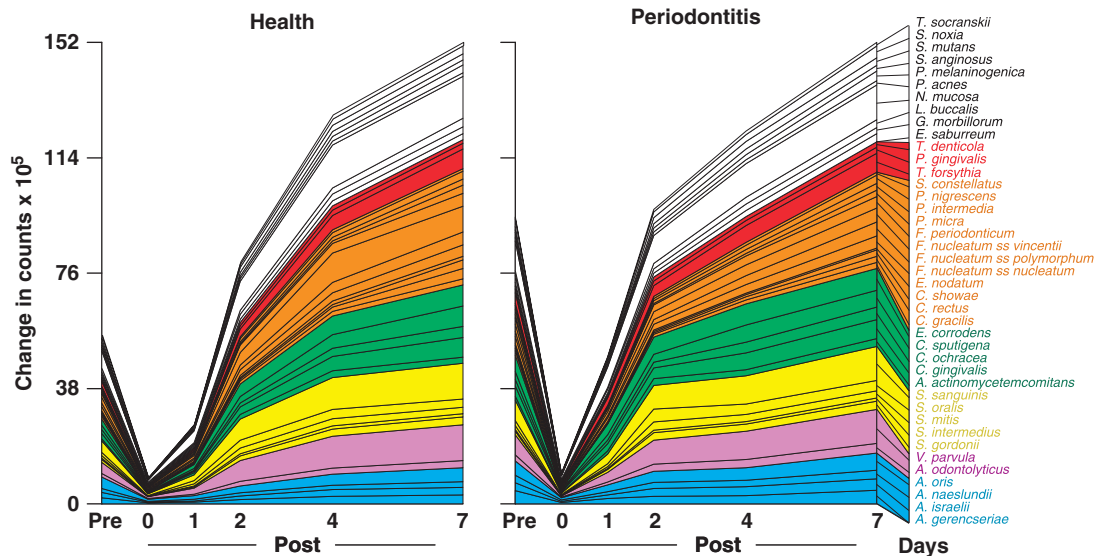


Fig. 2. Cumulative mean counts ( $\times 10^5$ ) of 41 bacterial species in supragingival samples taken from 38 periodontally healthy and 17 subjects with periodontitis before professional removal of the dental biofilms, immediately after cleaning and after 1, 2, 4 and 7 days of re-development. The subjects refrained from oral hygiene procedures for the 7-day test period. Samples were removed from the mesio-buccal aspect of each tooth (excluding third molars) pre-cleaning and immediately post-cleaning. In addition, supragingival samples were obtained from up to 7 teeth in randomly selected quadrants at 1, 2, 4 and 7 days after tooth cleaning. All samples were individually analysed for their content of 41 taxa using checkerboard DNA–DNA hybridization. Species counts in the samples were averaged within each subject at each time point and then averaged across subjects in the two clinical groups. The plots present the cumulative mean values at each time point in each clinical group. The species were ordered and colour-coded according to previously described microbial complexes (Socransky et al. 1998).

Pairwise comparisons between post-cleaning samples and each of the time points (i.e., 1, 2, 4, 7 days) indicated significant differences for all species when comparing the post-cleaning samples with those taken at day 2, day 4 or day 7 (Wilcoxon's signed ranks test,  $p < 0.001$ ) in both clinical groups. When the post-cleaning samples were compared with the samples collected at day 1 for the periodontally healthy subjects, *Actinomyces gerencseriae*, *Actinomyces israelii*, *Actinomyces odontolyticus*, *Capnocytophaga sputigena*, *Eubacterium nodatum*, *Fusobacterium nucleatum ss polymorphum*, *Fusobacterium nucleatum ss vincentii*, *Fusobacterium periodonticum*, *Prevotella nigrescens*, *T. denticola* and *Treponema socranskii* did not differ significantly ( $p > 0.05$ ). For the periodontitis group, *Campylobacter rectus*, *E. nodatum*, *Prevotella intermedia* and *Streptococcus constellatus* did not differ significantly from the post-cleaning visit to 1 day (Wilcoxon's signed ranks test,  $p > 0.05$ ). At entry, the counts of 17/41 taxa were significantly elevated in the periodontitis group, but few significant differences were observed during the observation period and none were present at day 7. The mean counts of individual species were quite similar

post-prophylaxis and at 1, 2, 4 and 7 days in both groups. On day 1, "early colonizers" could be observed, including *Veillonella parvula*, *Neisseria mucosa* and *Actinomyces oris* in both groups. At the same time point, the periodontitis group exhibited somewhat increased counts of *Capnocytophaga gingivalis*, *C. sputigena* and *Eikenella corrodens*. On day 2, the increase observed at day 1 became more evident. Further, the counts of *F. nucleatum ss vincentii* (particularly in the periodontitis group) increased in both groups. On days 4 and 7, *Actinomyces* species reached entry levels and the trends observed earlier became even more pronounced.

#### Changes in subgingival microbial species counts during biofilm re-development

The mean ( $\times 10^5$ ,  $\pm$  SEM) total subgingival DNA probe counts in healthy and periodontitis subjects, respectively, were  $19.4 \pm 3.7$  and  $40.9 \pm 7.1$  ( $p < 0.001$ ) on entry and  $4.6 \pm 0.8$  and  $5.9 \pm 0.7$  ( $p < 0.05$ ) immediately post-cleaning (Fig. 4). Although the mean total DNA probe counts were consistently lower in samples from the periodontally healthy subjects than the periodontitis subjects at subsequent

time points, the differences were statistically significant only at 7 days ( $39.8 \pm 7.3$  versus  $63.4 \pm 12.3$ ,  $p < 0.05$ ). In the periodontally healthy group, the mean total counts surpassed entry levels at day 2 and exhibited gradual increases until day 7. In the periodontitis patients, a slower increase was observed, as the levels seemed to plateau at days 2 and 4 and increase at day 7, when they surpassed entry levels.

The cumulative mean species counts (Fig. 5) showed a rapid increase in the individual mean species counts particularly for purple, green and orange complex taxa as well as *N. mucosa* in periodontally healthy subjects. In this group, the mean total species counts reached or exceeded pre-therapy levels by 2 days. In the periodontitis group, this occurred only at day 7 (Figs 4 and 5). These increases were more marked between days 1 and 2 and between days 4 and 7, suggesting that there were 2 waves of colonization. There was a drastic increase in the counts of all taxa from day 1 to 2, then a plateau or a decrease for a number of species, followed by a drastic increase in the counts of all taxa, except for *Actinomyces naeslundii*, *A. gerencseriae* and *A. israelii*.

The mean counts of the 41 enumerated bacterial species in subgingival plaque

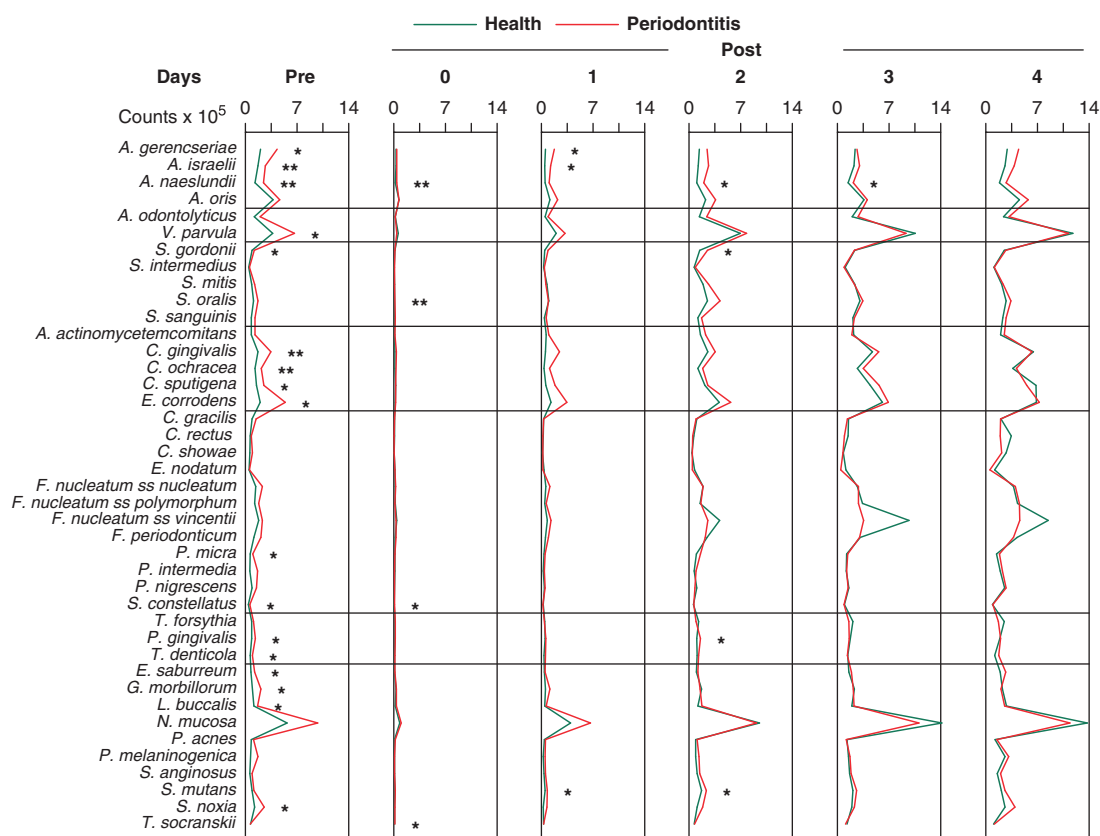


Fig. 3. Mean counts ( $\times 10^5$ ) of 41 bacterial species in supragingival samples taken from 38 periodontally healthy and 17 subjects with periodontitis before professional removal of the dental biofilms, immediately after cleaning and after 1, 2, 4 and 7 days of biofilm redevelopment. The subjects refrained from oral hygiene procedures for the 7-day test period. Supragingival biofilm samples were removed from the mesio-buccal aspect of all teeth (excluding third molars) pre-cleaning and immediately post-cleaning. Supragingival samples were also obtained from up to seven teeth in randomly selected quadrants at 1, 2, 4 and 7 days after tooth cleaning. All samples were individually analysed for their content of 41 taxa using checkerboard DNA–DNA hybridization. For analysis, species counts in the samples were averaged within each subject at each time point and then averaged across subjects in the two clinical groups. The significance of differences in the mean species counts between groups at each time point was determined using the Mann–Whitney test; \* $p < 0.05$ , \*\* $p < 0.01$ . The species were ordered according to previously described microbial complexes (Socransky et al. 1998).

samples at the different time points in the periodontally healthy and diseased subjects are shown in Fig. 6. There was a significant difference over time in the mean counts of all the test species in both clinical groups, from pre-therapy to 7 days (Friedman's test,  $p < 0.001$ ) and from the post-cleaning samples to 7 days (Friedman's test,  $p < 0.001$ ). Pairwise comparisons between post-cleaning samples and each time point (i.e., 1, 2, 4 and 7 days) were performed. Multiple species did not differ significantly in the subgingival samples from the periodontally healthy group when the post-cleaning samples were compared with the samples from day 1. Species that did not differ significantly included *A. gerencseriae*, *A. israelii*, *A. naeslundii*, *A. odontolyticus*, *C. gingivalis*, *Campylobacter gracilis*, *E. nodatum*, *Fusobacterium nucleatum ss nucleatum*, *P. intermedia*, *P. nigrescens*, *T. forsythia*, *T. denticola*, *Eubacterium*

*saburreum*, *Streptococcus mutans*, *Selexomonas noxia* and *T. socranskii* (Wilcoxon's signed ranks test,  $p > 0.05$ ). For the periodontitis group, the only significant differences occurred for *V. parvula* and *Streptococcus mitis* ( $p < 0.05$ ). Most species in both clinical groups differed significantly from the post-cleaning samples to the day 2 samples. The exceptions were *A. israelii* in the periodontally healthy group and *C. gracilis*, *C. rectus*, *E. nodatum*, *T. forsythia*, *T. denticola*, *Prevotella melaninogenica* and *T. socranskii* in the periodontitis group ( $p > 0.05$ ). From the post-cleaning samples to day 4, the mean counts of both *E. nodatum* and *P. gingivalis* did not differ significantly in the periodontitis subjects. The same comparison in the periodontally healthy group revealed that the mean counts of all species were significantly different. Pairwise comparisons between post-cleaning samples and day

7 samples indicated significant changes for all the species tested in both clinical groups ( $p < 0.001$ ).

The mean counts of 39/41 species were significantly higher in the periodontitis than in the periodontally healthy subjects at entry. The mean counts of *Actinomyces* species were significantly higher in samples from the periodontitis group before and at most time points post-cleaning (Fig. 6). Interestingly, even at '0' day, i.e., immediately after cleaning, the test species could be quantified and the levels of 16/41 taxa were significantly higher in the periodontitis group. At day 1, the most prominent taxa were *V. parvula* and *N. mucosa* in both groups, and *A. oris* in the periodontitis group. These trends became more evident on day 2, when the counts of yellow and green complex species became elevated, as well as *F. nucleatum ss*



*nucleatum* and *F. nucleatum* ss. *vincentii*, in both groups. The major differences observed between groups were in the higher levels of *Actinomyces* species, *Capnocytophaga ochracea*, *C. gingivalis* and *E. corrodens* in the periodontitis group.

By day 7, all taxa surpassed entry levels in both groups and 17/41 taxa exhibited significantly higher mean counts in samples from the periodontitis than the periodontally healthy subjects. Notably increased over time in both

clinical groups were the mean counts of *V. parvula*, *C. gingivalis*, *E. corrodens*, *F. nucleatum* subspecies, *P. melaninogenica* and *N. mucosa*. The significant differences detected in the mean counts between groups included *Actinomyces* species, green and orange complexes, as well as *P. gingivalis*. These increases started at day 2 and were clear at 7, when they were more evident in the periodontitis than in the periodontally healthy group. Red complex species were reduced immediately

after cleaning in both groups and remained at low levels until day 7, when the counts of *P. gingivalis* increased slightly in the periodontitis group, although they remained at lower mean levels than at entry.

## Discussion

The purpose of the present investigation was to examine the microbial shifts during the re-colonization of supra- and subgingival tooth surfaces after professional tooth cleaning in the absence of oral hygiene. As plaque re-colonization might have been affected by sampling, quadrants were randomly assigned to be sampled at the 1-, 2-, 4- and 7-day test points. For this reason, re-development could be determined only once per tooth, and only 7 of 28 teeth were sampled at each time point in each subject.

## Development of supragingival biofilms

The significantly higher baseline mean bacterial counts of supragingival bacteria in periodontitis subjects than in periodontally healthy subjects ( $50.2 \pm 6.6$  and  $84.1 \pm 12.4 \times 10^5$ ) were similar to the results reported previously (Ximenez-Fyvie et al. 2000) ( $72.1 \pm 11.1$  and  $132.7 \pm 17.5 \times 10^5$ , respectively) and it was in accord with the notion that more plaque would be present on coronal surfaces of teeth in subjects with periodontitis than in periodontally healthy

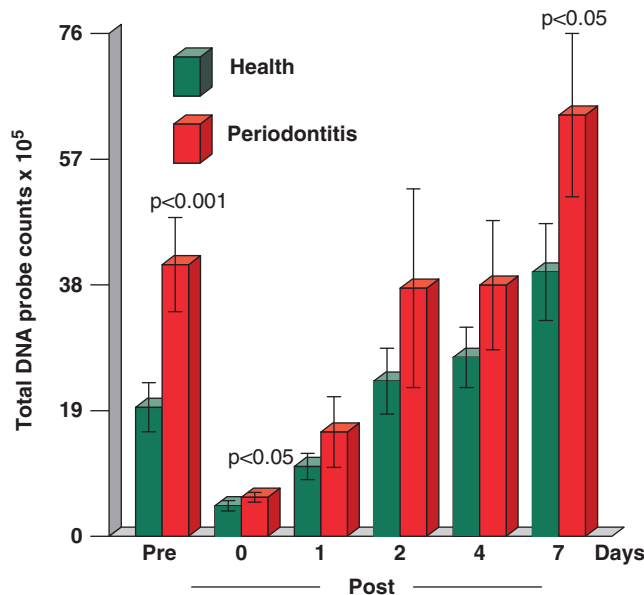


Fig. 4. Mean total DNA probe counts ( $\times 10^5$ ,  $\pm$  SEM) of subgingival biofilm samples taken pre-cleaning, immediately post-cleaning and after 1, 2, 4 and 7 days of biofilm development. Computation of counts and data analysis was performed as described in Fig. 1.

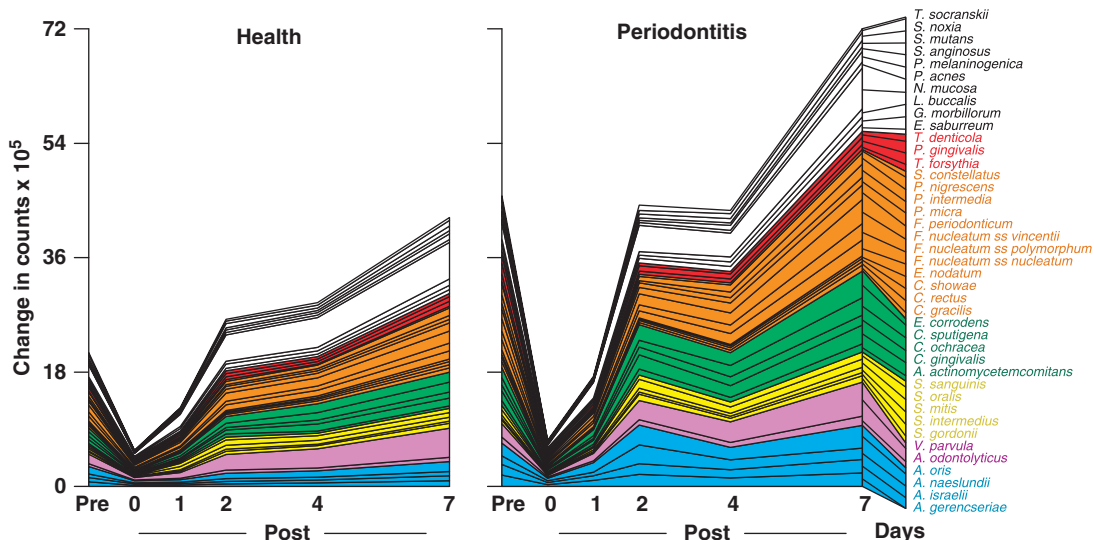


Fig. 5. Cumulative mean counts ( $\times 10^5$ ) of 41 bacterial species in subgingival samples taken from 38 periodontally healthy and 17 subjects with periodontitis before professional removal of the dental biofilms, immediately after cleaning and after 1, 2, 4 and 7 days of re-development. Calculation of species counts and description of the plot were as described in Fig. 2.

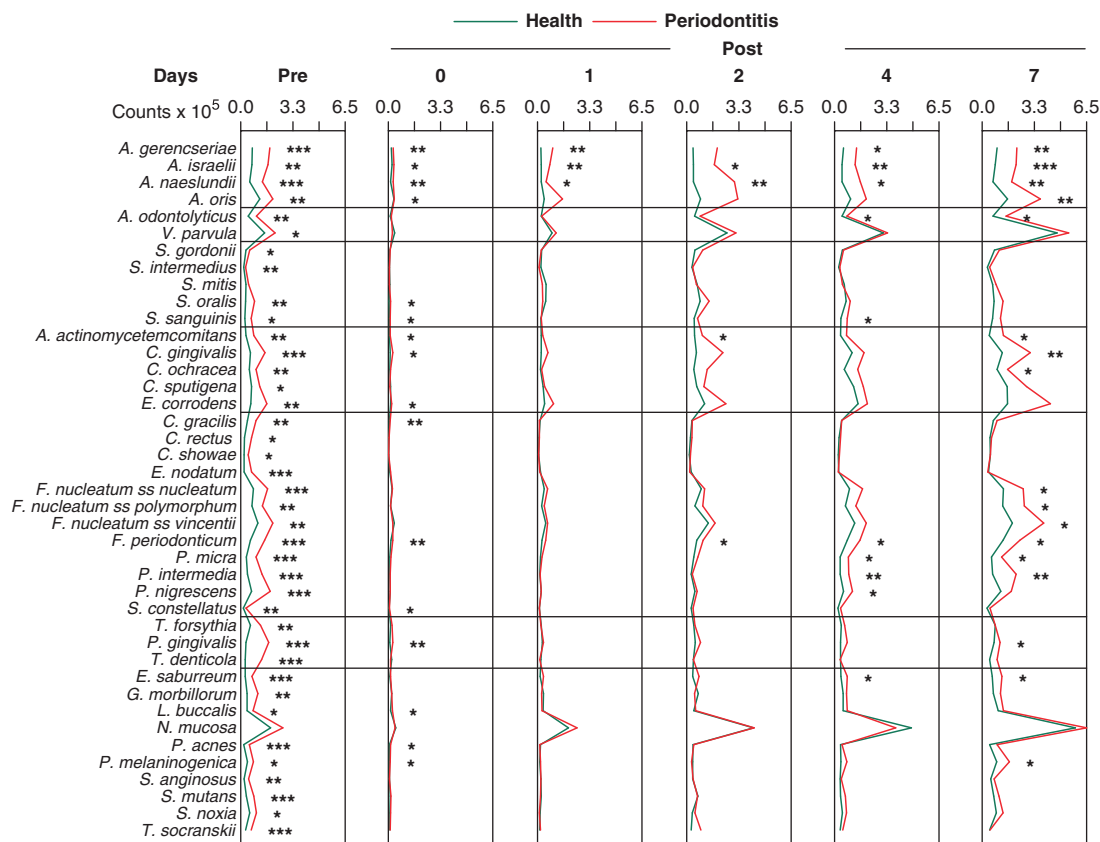


Fig. 6. Mean counts ( $\times 10^5$ ) of 41 bacterial species in subgingival samples taken from 38 periodontally healthy and 17 subjects with periodontitis before professional removal of the dental biofilms, immediately after cleaning and after 1, 2, 4 and 7 days of biofilm re-development. Computation of counts and data analysis was performed as described in Fig. 3.

individuals. The prophylaxis and SRP procedures could not completely eliminate the test species. Quantifiable levels of the taxa assessed could be observed at day '0'. This finding is in accord with results from previous studies (Ramberg et al. 2003, Li et al. 2004) and suggest that cleaning measures can be further improved.

Our data demonstrated that re-colonization of species on the supragingival surfaces was quite similar in subjects with periodontitis or periodontal health. As demonstrated in previous investigations (Simonsson et al. 1987a,b, Ramberg et al. 2003), the total supragingival counts reached pre-cleaning levels in 2 days, indicating that re-colonization is an extremely rapid process. Certain species appeared to flourish during the 7-day recolonization phase in both clinical groups in supragingival biofilm. These included *V. parvula*, *C. gingivalis*, *C. sputigena*, *E. corrodens*, *N. mucosa* and *F. nucleatum* ss *nucleatum* (particularly in the periodontitis group) (Fig. 3). The increases in these species are in accord with

previous reports (Ritz 1967, Zee et al. 1996a, Ramberg et al. 2003). Li et al. (2004) also showed similar results in a study that examined the very early stages of supragingival plaque development in 15 periodontally healthy subjects using the checkerboard DNA-DNA hybridization technique. Supragingival plaque samples were taken at 0, 2, 4 and 6 h after cleaning. In that study, it was demonstrated that even though there was selectivity in the initial adhesion of different species, some increased rapidly after adhesion. *A. oris* was observed to increase from 0 to 2 h but remained essentially constant to 6 h. In our study, *Actinomyces* levels surpassed the baseline values at day 2 and showed a modest increase at the remaining time points, which was also in accord with Ramberg et al. (2003). Li et al. (2004) observed that the major early increases that were observed during biofilm development were for *S. mitis* and *Streptococcus oralis*, which increased markedly up to 6 h. Other species remained at negligible or low levels during the 6-h time period. *S.*

*mitis* and *S. oralis* also increased at the early time points of the present study. However, other taxa increased at the earliest post-cleaning time points in the present study. Major early increases occurred at day 1 in the counts of *V. parvula* and *N. mucosa*, in both clinical groups. The major increase in *Neisseria* species that was observed in the present investigation has also been observed using cultural techniques (Ritz 1967). The difference in the results from the Li et al. (2004) study may have been due to the time of sampling (24 rather than 6 h) and sample location (mesial rather than facial surfaces of the teeth).

#### Development of subgingival biofilms

The significantly higher baseline mean bacterial counts of subgingival bacteria in periodontitis subjects than in periodontally healthy subjects ( $19.4 \pm 3.7$  and  $40.9 \pm 7.1 \times 10^5$ ) were in accord with the results reported by Sharawy et al. (1966) (16 and 84 mg wet weight) and Ximenez-Fyvie et al. (2000) ( $22.1 \pm 6.6$  and  $100.3 \pm 18.4 \times 10^5$ ).

This was likely due to the greater number of deeper periodontal pockets in the periodontitis subjects than in the periodontally healthy subjects.

Both supra- and subgingival samples exhibited a marked increase in biofilm species counts from 1 to 2 days; a plateau in the counts between 2 and 4 days; and then sharp increase in the mean species counts between 4 and 7 days. It is not clear why there appeared to be these two phases in subgingival biofilm re-development. It might be speculated that the two phases represented a change in the environment brought about by the species that increased in the first wave. The proliferation of certain taxa might have led to changes in the levels of oxygen, availability of nutrients or pH, which might no longer be conducive to the growth of those species, but facilitated the increase of others. This pattern suggests the occurrence of autogenic microbial species succession (Socransky & Haffajee 2005).

While re-development of biofilms was quite similar in periodontitis and periodontally healthy individuals for the supragingival plaque samples, there were quite marked differences in the re-development of subgingival biofilms. As expected, at baseline, the mean total DNA probe counts and counts of 39 of 41 species were significantly higher in the subgingival samples obtained from periodontitis subjects than in the samples obtained from periodontally healthy subjects. Unlike the supragingival environment, the re-development of biofilm in the subgingival area was more rapid in the periodontitis than in the periodontally healthy subjects. Notable was the modest increase in *Actinomyces* species particularly in the subjects with periodontitis. In addition, green and orange complex species such as *C. gingivalis*, *E. corrodens*, *Fusobacterium* subspecies and *P. intermedia* increased more profoundly in the periodontitis than in the periodontally healthy subjects. This difference could be observed as early as day 2. To a lesser extent, the counts of *P. gingivalis* also showed an increase over time in this group.

#### Differences in the development of supra- and subgingival biofilms

The present investigation showed that *Streptococcus* (yellow complex) species returned to baseline levels by 2 days in periodontitis and healthy subjects in both supra- and subgingival biofilms. In supragingival samples, their levels

showed a modest increase until day 7. In subgingival biofilms, these showed a plateau and a decrease in periodontally healthy and periodontitis subjects, respectively, from day 2 to 4, followed by an increase at day 7. The early increase may have been due to the re-adsorption of detached species in the oral cavity after cleaning procedures (Li et al. 2004).

The data of the present investigation also showed that initial colonization appeared to involve members of the yellow, green and purple complexes along with *Actinomyces* species (Figs 3 and 6). The counts of *Actinomyces* species were close to the pre-cleaning levels at day 7 in supragingival, whereas they increased to about or slightly above entry levels in subgingival plaque in both clinical groups. These findings are similar to those reported by Ramberg et al. (2003) but in contrast to those reported by Zee et al. (1996a). The latter investigators used predominantly cultivable microbiota techniques to examine one pooled plaque sample from each of five "rapid" plaque-forming subjects and six "slow" plaque-forming subjects at 1, 2, 7 and 14 days. They indicated that *Actinomyces* species increased from a mean of 10% and 5% in "slow" and "rapid" groups, respectively, to 30% and 15% at 14 days. They also showed that *Capnocytophaga* species were low in proportion at 1 day but increased at 3 to 14 days. This was in accord with the findings of the present investigation in which *C. gingivalis*, *C. ochracea* and *C. sputigena* were low in counts at day 1 but increased significantly in levels, exceeding entry levels at day 4.

Even though the total DNA probe counts of subgingival biofilm samples exceeded baseline values at day 2 in both healthy and periodontitis subjects, there were striking differences between health and periodontitis in the patterns of re-colonization. In periodontally healthy subjects, the counts of most species increased slowly over time, reaching or exceeding baseline values by 2 days. The reason for the difference between health and disease is not known, but one might speculate that the greater gingival crevice fluid flow in the periodontitis subjects may have favoured subgingival biofilm development. Alternatively, there may have been a greater reservoir of bacterial cells that were missed in the scaling of deep pockets or remaining in reservoirs within host cells or dentinal tubules.

The findings presented in this manuscript should be seen in light of the limitations of the study. The necessity of taking samples from randomly selected quadrants at days 1, 2, 4 and 7 prevented following biofilm changes at individual teeth. The 7-day duration of the study did not allow us to follow biofilm changes to a microbial composition that was similar to that of mature plaque. Also, the lack of a comparison group that continued self-performed oral hygiene prevented the determination of the effect of oral hygiene procedures on microbial shifts during re-colonization.

In summary, the hypothesis that biofilm re-development would be more rapid in periodontitis than in periodontally healthy subjects was rejected for supragingival biofilms but could not be rejected for subgingival biofilm re-development. The hypothesis that the shift in the levels of individual taxa over time would differ from periodontally healthy to periodontally diseased subjects could not be rejected in that *Actinomyces*, green and orange complex species returned much more rapidly in subgingival biofilm samples from periodontitis than periodontally healthy individuals. The hypothesis that the re-growth of specific periodontal pathogens such as *P. gingivalis*, *T. forsythia* and *T. denticola* would be slower than the re-growth of other species was not rejected. Finally, the re-development of oral biofilm on dental tissues was so rapid that the total DNA probe counts of many species exceeded the baseline values at day 2.

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**Clinical Relevance**

*Scientific rationale for the study:* The study of dental biofilm re-development in the absence of oral hygiene in periodontal health and disease can show the dynamics of colonization by pathogens and host-compatible bacteria.

*Principal findings:* Supra- and subgingival counts reached or exceeded the baseline values after day 2. Differences between groups in the supragingival counts occurred for 17/41 species at entry, 0 at day 7 and in subgingival plaque for 39/41 taxa at entry and 17/41 at day 7.

Subgingival counts were greater for Actinomyces, green and orange complex species.

*Practical implications:* Microbial shifts during biofilm re-development can indicate critical periods for prevention and intervention.

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