

Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis

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Ximénez-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. © Munksgaard, 2000.

Abstract

Background, aims: The purpose of the present study was to compare and relate the microbial composition of supra and subgingival plaque in 23 adult periodontitis subjects (mean age 51 ± 14 years).

Methods: A total of 1,170 samples of supra and subgingival plaque were collected from the mesial aspect of every tooth (up to 28 supra and 28 subgingival samples) from each subject and evaluated for the presence and levels of 40 bacterial taxa using whole genomic DNA probes and checkerboard DNA-DNA hybridization. Clinical assessments including dichotomous measures of gingival redness, bleeding on probing, plaque accumulation and suppuration, as well as duplicate measures of pocket depth and attachment level, were made at 6 sites per tooth. The counts (levels), % DNA probe count (proportion) and % of sites colonized (prevalence) of each species in supra and separately in subgingival plaque were computed for each subject. Significance of differences between supra and subgingival plaque for each species was sought using the Wilcoxon signed ranks test and adjusted for multiple comparisons.

Results: All 40 taxa were detected in both supra and subgingival plaque. *Actinomyces* species were the most prevalent taxa in both habitats. 75 to 100% of supra and 62 to 100% of subgingival sites were colonized by at least one of the 5 *Actinomyces* species. Supragingival samples exhibited significantly higher counts of *Actinomyces naeslundii* genospecies 1, *Actinomyces israelii*, *Actinomyces odontolyticus*, *Neisseria mucosa*, *Streptococcus gordonii*, *Capnocytophaga ochracea* and *Capnocytophaga sputigena* when compared with mean counts in subgingival samples taken from the same tooth surfaces. Subgingival plaque samples presented significantly higher counts of *Prevotella nigrescens*, *Prevotella intermedia*, *Bacteroides forsythus* and *Porphyromonas gingivalis*. Subgingival samples exhibited a significantly higher proportion of “red” and “orange complex” species, while supragingival plaque exhibited higher proportions of “green” and “purple” complex species as well as *Actinomyces* species. Suspected periodontal pathogens could be detected in supragingival plaque from sites where subgingival samples were negative for the same species.

Conclusions: The data indicate that supragingival plaque can harbor putative periodontal pathogens, suggesting a possible rôle of this environment as a reservoir of such species for the spread or reinfection of subgingival sites.

Key words: supragingival plaque; subgingival plaque; microbiology; bacteria periodontal diseases; DNA probes.

Accepted for publication 3 November 1999

To date, few comprehensive studies of the microbial composition of supragingival plaque have been carried out. Further, there appears to be no data which clearly describe the relationship between supragingival and subgingival

plaque composition on the same tooth surfaces. In reviewing the literature, the majority of recent studies have focused primarily on the composition of subgingival plaque suggesting a role for a number of subgingival microorganisms

in the initiation of periodontal infections (Moore & Moore 1994, Haffajee & Socransky 1994, Zambon 1996). The role of supragingival plaque in oral microbial ecology and the initiation of periodontal diseases is less clear.

Moore et al. (1983), examined 22 supra and 38 subgingival plaque samples taken from 22 subjects with moderate periodontitis. It was found that the taxa which predominated in supragingival plaque included 4 species of *Streptococcus*, 9 species of *Actinomyces*, 3 species of *Capnocytophaga*, *Veillonella parvula*, *Leptotrichia buccalis*, 2 species of *Selenomonas* and *Rothia dentocariosa*. In contrast, 3 *Fusobacterium* species, 2 *Peptostreptococcus* species, 7 *Eubacterium* species, *Campylobacter rectus*, *Porphyromonas gingivalis* and a number of *Prevotella* species were found to be the predominant taxa in subgingival plaque.

Using predominant cultivable techniques, Zee et al. (1996) examined the composition of 44 supragingival plaque samples taken from 11 subjects at 4 time points. During 14 days of plaque accumulation, supragingival plaque shifted from a microbiota predominated by Gram-positive cocci, primarily *Streptococcus* species, to one predominated by Gram-positive and negative rods including *Actinomyces*, *Fusobacterium*, *Veillonella* and *Capnocytophaga* species. At day 1 of plaque accumulation, the most frequently isolated organisms included *Staphylococcus epidermidis*, *Veillonella dispar*, *Actinomyces israelii*, *Propionibacterium granulosum*, *Gemella morbillorum*, *Streptococcus mitis* and *Streptococcus sanguis*. At 14 days, samples were predominated by multiple species including *Prevotella intermedia*, *Fusobacterium* and *Capnocytophaga* species.

Cao et al. (1990) used microscopy and cultural techniques to compare the microbiota of 10 supra and 10 subgingival plaque samples from the maxillary first molars of 10 Chinese subjects. Comparisons were made with data from 10 Caucasian subjects examined in previous studies. The cultivable supragingival microbiota of the Caucasian subjects was predominated by *Actinomyces* and *Streptococcus* species, while *Fusobacterium* and "black-pigmented *Bacteroides*" were the most predominant organisms in the Chinese subjects. These subjects also exhibited significantly higher percentages of spirochetes and motile rods in supragingival plaque than Caucasian subjects. "Black-pigmented *Bacteroides*", *Actinomyces* and *Streptococcus* species were similar in both supra and subgingival plaque samples of the Chinese subjects, but *Fusobacterium* and *Capno-*

cytophaga species were higher in supra-gingival samples.

Several investigators have sought specific "subgingival" species in supragingival plaque. Riviere et al. (1992), reported that spirochetes could be observed in both supra and subgingival plaque from periodontitis subjects with pathogen-related oral spirochetes (PROS) predominating in both supra and subgingival plaque samples. Other investigators have also observed spirochetes in both supra and subgingival samples (Armitage et al. 1982, Lindhe et al. 1980, Listgarten & Hellden 1978, Moore et al. 1982ab, Simonson et al. 1988). Gmür & Guggenheim (1994) analyzed 168 interdental supragingival plaque samples for the presence of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *C. rectus*, *P. gingivalis* and *P. intermedia*/*Prevotella nigrescens* using monoclonal antibodies. They detected all test species, except *P. gingivalis*, in a high percentage of sites (*A. actinomycetemcomitans* 33%, *B. forsythus* 38%, *C. rectus* 38%, *P. intermedia*/*P. nigrescens* 100%), suggesting that supragingival plaque may be a reservoir for such organisms.

Thus, available data suggest that similar species may be found in supragingival plaque to those described in subgingival plaque. However, the data are limited in terms of the number of species examined and the number of samples and subjects evaluated. In addition, few studies have attempted to compare and relate supra and subgingival plaque samples taken from the same tooth surface. For these reasons, the purpose of the present study was to compare and relate the microbial composition of supra and subgingival plaque samples obtained from subjects with adult periodontitis.

Material and Methods

Subject population

23 adult periodontitis subjects ranging in age from 24–82 years were selected for the study. All subjects had at least 20 teeth and at least 8 sites with pocket depth greater than 4 mm and/or more than 10% of sites with loss of attachment greater than 3 mm. Exclusion criteria included pregnancy, lactation, periodontal or antibiotic therapy in the previous 3 months, any systemic condition which could influence the course of periodontal disease, or which would require pre-medication for monitoring procedures, current smokers and subjects with localized juvenile periodontitis, rapidly progressive periodontitis or acute necrotizing ulcerative gingivitis.

Clinical monitoring

Subjects were screened for suitability and if accepted, signed informed consent. Clinical measurements were taken at 6 sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars (a maximum of 168 sites per subject) as previously described (Haffajee et al. 1983). Clinical assessment included plaque accumulation (0/1), overt gingivitis (0/1), bleeding on probing (0/1), suppuration (0/1), probing pocket depth and probing attachment level. Pocket depth and attachment level measurements were taken twice by the same examiner and the average of the pair of measurements was used for analysis. Measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL). The baseline clinical features of the 23 subjects are presented in Table 1.

Table 1. Baseline clinical characteristics of the subject group (n=23)

	Mean(±SD)	Range
age (years)	51±11	24–82
no. missing teeth	2.5±2.3	0–7
% males	61	
mean pocket depth (mm)	2.8±0.4	2.2±3.6
mean attachment level (mm)	2.7±0.8	1.5±4.3
% sites with:		
plaque	70±24	5–100
gingival erythema	56±33	0–100
bleeding on probing	29±19	4–76
suppuration	0.5±1.2	0–4.6

Microbial assessment*Bacterial strains and growth conditions*

The 40 bacterial strains used in the preparation of DNA probes are presented in Table 2. All strains were purchased as lyophilized stocks from the American Type Culture Collection (ATCC, Rockville, MD) (exceptions are noted in the Table). Bacterial stocks were rehydrated in Mycoplasma broth (Difco Laboratories, Detroit, MI) and grown on Trypticase soy agar with 5% defibrinated sheep blood (BBL, Baltimore Biological Laboratories, Cockeysville, MD) at 35°C under anaerobic conditions (80% N₂, 10% CO₂, 10% H₂). Several bacterial strains were grown on supplemented or enriched media: *B. forsythus* was grown on Trypticase soy agar supplemented with 5% defibrinated sheep blood and 10 µg/ml N-acetyl muramic acid (Sigma Chemical Co., St. Louis, MO). *P. gingivalis* was grown on a similar medium supplemented with 5% defibrinated sheep blood, 0.3 µg/ml menadione (Sigma) and 5 µg/ml hemin (Sigma). *Eubacterium* and *Neisseria* species were grown on Fastidious Anaerobic Agar (BBL) with 5% defibrinated sheep blood. *Treponema denticola* and *Treponema socranskii* were grown in Mycoplasma broth supplemented with 1 mg/ml glucose, 400 µg/ml niacinamide, 150 µg/ml

spermine tetrahydrochloride, 20 µg/ml Na isobutyrate, 1 mg/ml L-cysteine, 5 µg/ml thiamine pyrophosphate and 0.5% bovine serum.

DNA isolation and preparation of DNA probes

Bacterial strains were grown anaerobically on the surface of blood agar plates (except the 2 spirochetes which were grown in broth) for 3 to 7 days. The growth was harvested and placed in 1.5 ml microcentrifuge tubes containing 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed 2× by centrifugation in TE buffer at 3,500 rpm for 10 min. The cells were resuspended and lysed either with 10% SDS and Proteinase K (20 mg/ml) (Sigma) for Gram-negative strains or in 150 µl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma) and 5 mg/ml achromopeptidase (Sigma) in TE buffer (pH 8.0) for Gram-positive strains. The pelleted cells were resuspended by 15 s sonication and incubated at 37°C for 1 h. DNA was isolated and purified using the method described by Smith et al. (1989). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed by the ratio of DNA to protein as measured by the ratio of the absorb-

ances at 260 nm and 280 nm. Whole-genomic DNA probes were prepared from each of the 40 test species by labeling 1 µg of DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN) using a random primer technique (Feinberg & Vogelstein 1983).

Sample collection and DNA-DNA hybridization

Samples of supra and subgingival plaque were individually analyzed by checkerboard DNA-DNA hybridization (Socransky et al. 1994). After drying and isolation with cotton rolls, supragingival plaque was sampled from the mesial-buccal aspect of each tooth using sterile Gracey curettes. Each plaque sample was placed in a separate tube containing 150 µl of TE buffer (pH 7.6). After removal of the supragingival sample and any remaining supragingival plaque, subgingival plaque samples were taken from the same sites (i.e., the mesial-buccal aspect of each tooth) using sterile Gracey curettes and placed in a second set of individual tubes. 100 µl of 0.5 M NaOH were added to each tube and the samples were dispersed using a vortex mixer. The samples were boiled for 10 min and neutralized using 800 µl of 5 M ammonium acetate. The released DNA was then placed into the extended slots of a Minislot-30 apparatus (Im-

Table 2. Stains employed for the development of DNA probes

Actinomyces species		"Orange" complex		Other species and new DNA probes	
<i>Actinomyces gerencseriae</i>	23840	<i>Campylobacter gracilis</i>	33236	<i>Eubacterium sabureum</i>	33271
<i>Actinomyces israelii</i>	12102	<i>Campylobacter rectus</i>	33238	<i>Gemella morbillorum</i>	27824
<i>Actinomyces naeslundii</i> genospecies 1	12104	<i>Campylobacter showae</i>	51146	<i>Leptotrichia buccalis</i>	14201
<i>Actinomyces naeslundii</i> genospecies 2	43146	<i>Eubacterium nodatum</i>	33099	<i>Neisseria mucosa</i>	19696
		<i>Fusobacterium nucleatum ss nucleatum</i>	25586	<i>Prevotella melaninogenica</i>	25845
"Purple" complex		<i>Fusobacterium nucleatum ss polymorphum</i>	10953	<i>Propionibacterium acnes</i>	**
<i>Actinomyces odontolyticus</i>	17929	<i>Fusobacterium nucleatum ss vincentii</i>	49256	<i>Selenomonas noxia</i>	43541
<i>Veillonella parvula</i>	10790	<i>Fusobacterium periodonticum</i>	33693	<i>Streptococcus anginosus</i>	33397
		<i>Peptostreptococcus micros</i>	33270	<i>Treponema socranskii</i>	S1
"Yellow" complex		<i>Prevotella intermedia</i>	25611		
<i>Streptococcus gordonii</i>	10558	<i>Prevotella nigrescens</i>	33563		
<i>Streptococcus intermedius</i>	27335	<i>Streptococcus constellatus</i>	27823		
<i>Streptococcus mitis</i>	49456				
<i>Streptococcus oralis</i>	35037	"Red" complex			
<i>Streptococcus sanguis</i>	10556	<i>Bacteroides forsythus</i>	43037		
		<i>Porphyromonas gingivalis</i>	33277		
"Green" complex		<i>Treponema denticola</i>	B1		
<i>Actinobacillus actinomycetemcomitans</i>	*				
<i>Capnocytophaga gingivalis</i>	33624				
<i>Capnocytophaga ochracea</i>	33596				
<i>Capnocytophaga sputigena</i>	33612				
<i>Eikenella corrodens</i>	23834				

All strains were obtained from the American Type Culture Collection (ATCC) except *Treponema denticola* B1 and *Treponema socranskii* S1 which were obtained from Forsyth Dental Center. Microbial "complexes" were described by Socransky et al. (1998). *ATCC strains 43718 and 29523; **ATCC strains 11827 and 11828.

munetics, Cambridge MA), concentrated onto a 15×15 cm positively-charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) and fixed to the membrane by cross-linking under ultraviolet light followed by baking at 120°C for 20 min. Two lanes on each membrane had standards that consisted of a mixture at 10⁵ and 10⁶ cells of each bacterial species tested. The membranes were prehybridized, then hybridized in the checkerboard format and signals detected using chemifluorescence and a fluorimager as described previously (Feres et al. 1999).

Data analysis

Microbiological data available for each subject were the counts of each of the 40 test species from up to 28 supragingival and, separately, up to 28 subgingival plaque samples per subject. The analyses compared microbial data expressed in 3 ways: counts (levels), % DNA probe count (proportions) and prevalence (% of sites colonized). In order to compare the counts of each of the bacterial species, the data were expressed as counts ×10⁵ at each site, averaged within a subject and then averaged across subjects. In a similar fashion, the % DNA probe count and prevalence of each species were computed at each site, averaged across sites within each subject and then across subjects. Significance of differences between supra and subgingival plaque for each species was sought using the Wilcoxon signed ranks test. Adjustments were made for multiple comparisons as described by Socransky et al. (1991).

To examine the associations between individual species in supragingival and subgingival plaque samples on the same tooth surface, 2×2 contingency tables were constructed for each species that summarized the presence or absence of that species at all sampled sites for the 23 subjects. Because there were multiple sites examined in each subject, the significance of the associations was examined using Mantel-Haenszel procedures in which each subject was a stratum in the analysis.

To examine the relationship between microbial species in supra and subgingival plaque samples and pocket depth, sampled sites were subset at the quartiles into categories of <3, 3–4, and >4 mm pocket depth. Counts and proportions of each species were averaged

for each pocket depth category within a subject and averaged across subjects in the 3 categories for supra and subgingival plaque samples separately. The significance of differences between counts or proportions of bacterial species in supra, and separately, subgingival plaque samples for each pocket depth category was tested using the Quade test (Conover 1980). The significance of differences between counts of the same microbial species in supra and subgingival plaque samples in the 3 pocket depth categories was tested using the Wilcoxon signed ranks test. Adjustments were made for multiple comparisons as described above.

Results

Mean counts

The mean total count (×10⁵±SEM) was somewhat higher in supragingival plaque samples than subgingival plaque samples; 133±18 and 100±18 respectively. The difference was not statistically significant. The mean counts (×10⁵±SEM) of the 40 individual species in supra and subgingival plaque samples are presented in Fig. 1. Supragingival samples exhibited significantly higher counts of *Actinomyces naeslundii* genospecies 1, *A. israelii*, *Actinomyces odontolyticus*, *Neisseria mucosa*, *Streptococcus gordonii*, *Capnocytophaga och-*

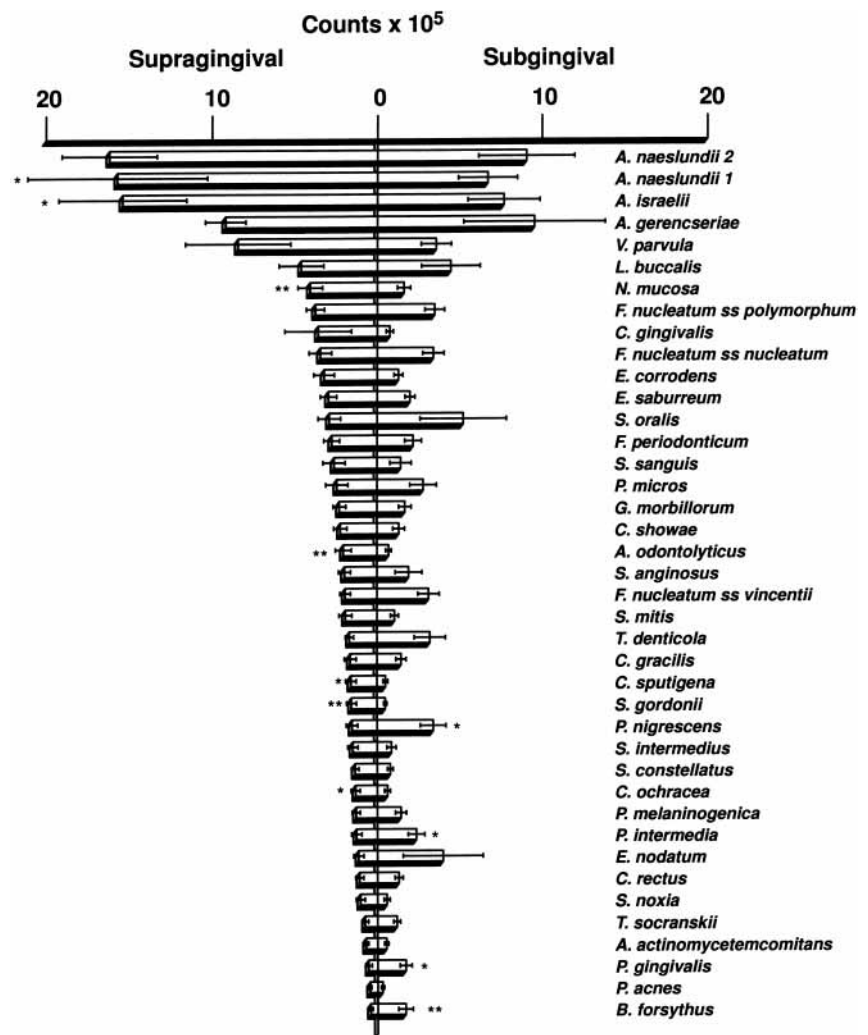


Fig. 1. Bi-lateral bar chart of the mean counts (×10⁵±SEM) of individual species in supra and subgingival plaque samples from 23 periodontitis subjects. The mean count was computed for each species in supra and subgingival plaque for each subject, and then averaged across subjects. The data are ordered on the basis of mean counts in the supragingival plaque samples. The significance of differences between supra and subgingival counts was tested using the Wilcoxon signed ranks test. **p*<0.05, ***p*<0.01 after adjusting for multiple comparisons.

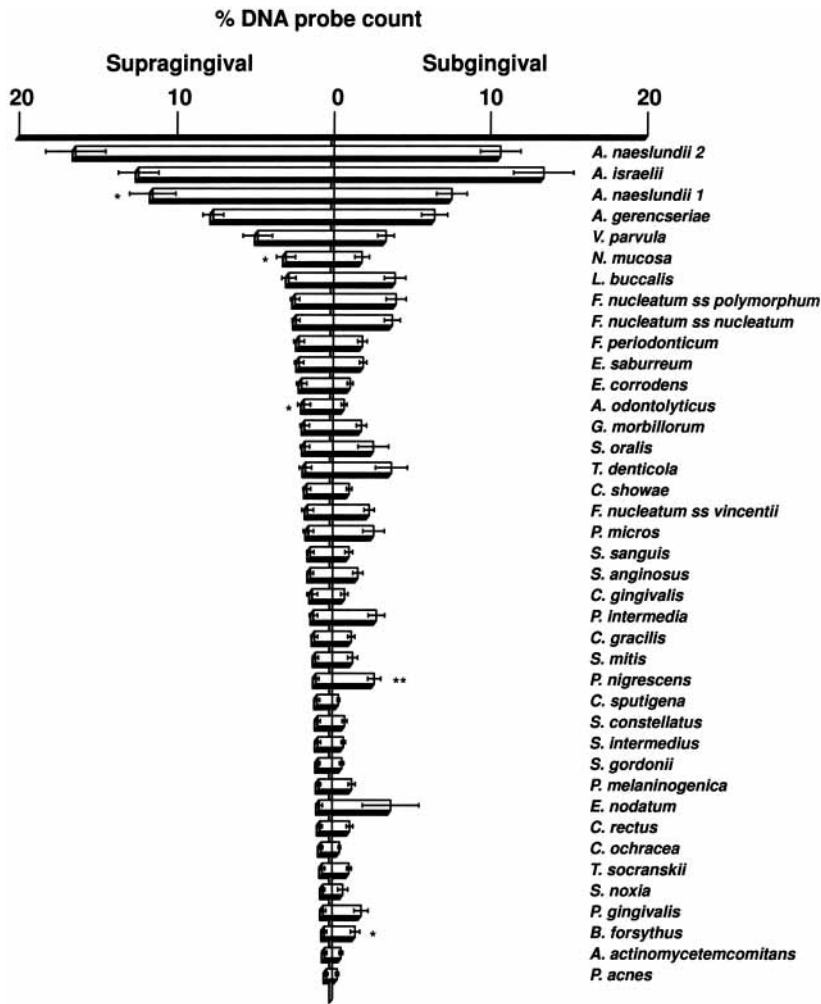


Fig. 2. Bi-lateral bar chart of the mean % DNA probe count (\pm SEM) comprised by each species in supra and subgingival plaque samples from 23 periodontitis subjects. The % DNA probe count was computed for each species in each plaque sample, averaged within a subject and then averaged across subjects for the supra and subgingival plaque samples separately. The data are ordered on the basis of the proportions in the supragingival plaque samples. The significance of differences between supra and subgingival percentages was tested using the Wilcoxon signed ranks test. * $p < 0.05$, ** $p < 0.01$ after adjusting for multiple comparisons.

racea and *Capnocytophaga sputigena* when compared with mean counts in subgingival samples taken from the same tooth surfaces. Subgingival plaque samples presented significantly higher counts of *P. nigrescens*, *P. intermedia*, *B. forsythus* and *P. gingivalis*. None of the species that were detected in significantly higher counts in supragingival plaque samples were species thought to be periodontal pathogens.

Mean % DNA probe count

The mean proportions that each species comprised in the supra and subgingival plaque samples are presented in Fig. 2. *A. naeslundii* genospecies 1, *N. mucosa* and *A. odontolyticus* were in significantly greater proportions in supragingival plaque while *P. nigrescens* and *B. forsythus* were at higher proportions in the subgingival samples. The 40 test species were grouped into microbial complexes (Table 2) as described by Socransky et al. (1998) (Fig. 3). Supra and subgingival plaque differed primarily in that subgingival samples exhibited a significantly higher proportion of “red” and “orange” complex species, while supragingival plaque exhibited higher proportions of “green” and “purple” complex species as well as *Actinomyces* species.

Mean % of sites colonized

Fig. 4 presents the mean prevalence (mean % of sites colonized) of the 40 species in supra and subgingival plaque samples. All species were detected in both habitats. *Actinomyces* species such

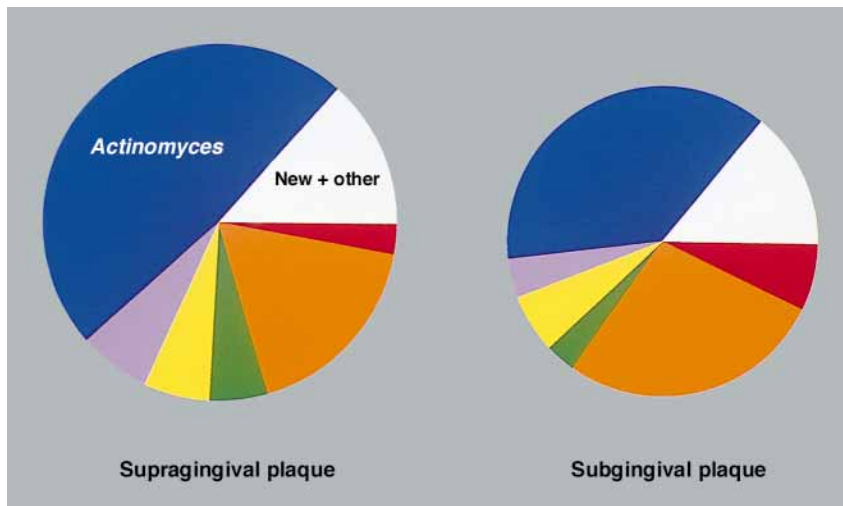


Fig. 3. Pie charts of the mean % DNA probe count of microbial groups in supra and subgingival plaque samples from 23 periodontitis subjects. The species were grouped into the 7 microbial groups presented in Table 2 based on the description of Socransky et al. (1998). The areas of the pies were adjusted to reflect the mean total DNA probe count at the 2 sample locations. The significance of differences in mean percentages of the supra and subgingival complexes was tested using the Wilcoxon signed ranks test. The “red”, “orange”, “green”, “purple” and *Actinomyces* complexes differed at $p < 0.05$ after adjusting for 7 comparisons.

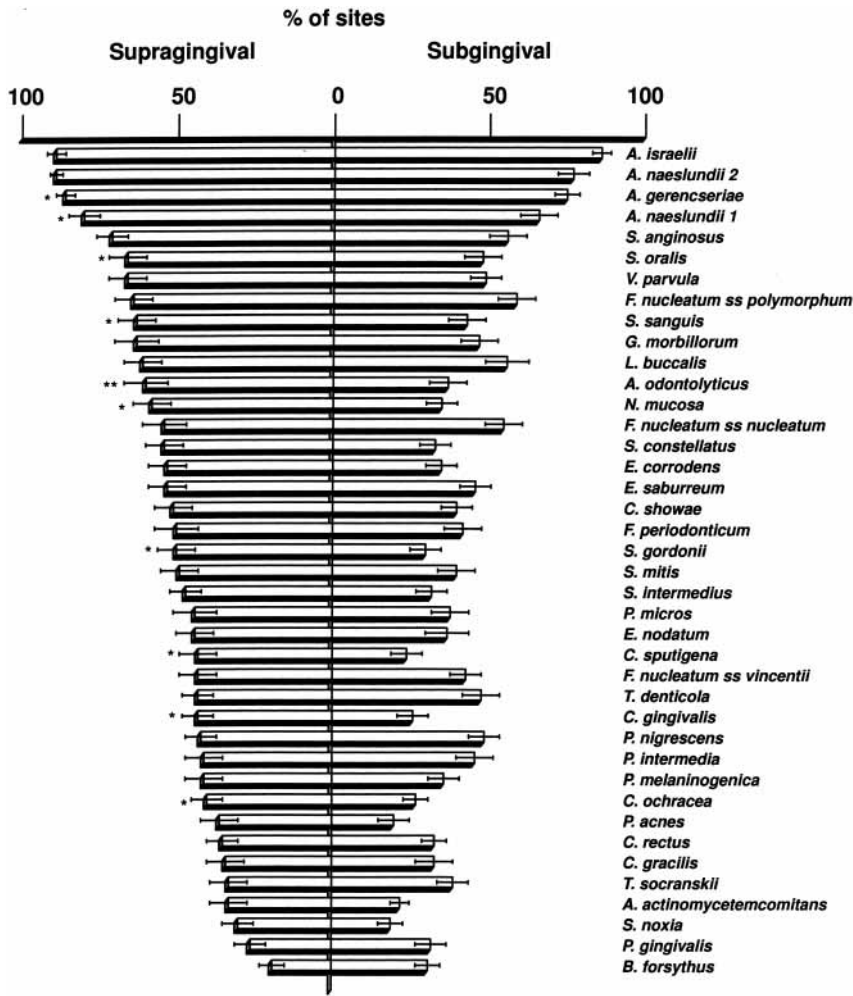


Fig. 4. Bi-lateral bar chart of the mean prevalence (% of sites colonized, ±SEM) of individual species in supra and subgingival plaque samples from 23 periodontitis subjects. The prevalence was computed for each species in supra and subgingival plaque individually for each subject and then averaged across subjects. The data are ordered on the basis of the prevalence in the supragingival plaque samples. The significance of differences between supra and subgingival mean values was tested using the Wilcoxon signed ranks test. * $p < 0.05$, ** $p < 0.01$ after adjusting for multiple comparisons.

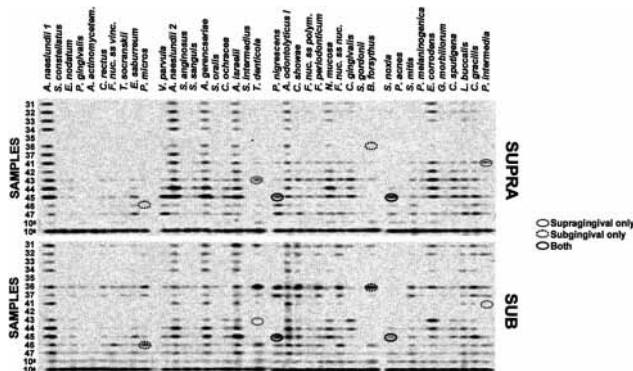


Fig. 5. Checkerboard DNA-DNA hybridization membranes showing reactions of 40 DNA probes with 14 supra and 14 subgingival plaque samples from a single periodontitis subject. Mixed standards at 10^5 and 10^6 cells are shown in the last 2 lanes of each membrane. Signals for 6 putative periodontal pathogens are indicated according to the detection of each species in either supra or subgingival plaque samples only, or in both.

as *A. naeslundii* genospecies 1 and 2, *Actinomyces gerencseriae* and *A. israelii* were the most prevalent taxa in both supra and subgingival plaque. In individual subjects, 75–100% of supragingival samples and 62–100% of subgingival samples harbored at least one of the 5 *Actinomyces* species. Ten species including 3 species each of *Actinomyces*, *Streptococcus* and *Capnocytophaga* as well as *N. mucosa* were significantly more prevalent in supragingival plaque. No species was significantly more prevalent in the subgingival plaque samples.

Relationship of species in supra and subgingival plaque samples

Fig. 5 presents images of sections of 2 “checkerboard” membranes with supra and subgingival plaque samples taken from the same sites in one subject. Examination of supra and subgingival plaque samples from the same tooth surface revealed that a given species could be detected in supra or subgingival plaque only, in both or in neither. The frequency of detection of each species in supra and subgingival plaque samples was examined using 2×2 contingency tables. Table 3 is an example of data for *P. gingivalis*, *A. naeslundii* genospecies 2, *P. intermedia* and *V. parvula*. Data for all 40 species are summarized in Fig. 6. While the presence or absence of many species in supragingival samples was significantly associated with their presence or absence in subgingival samples (Table 3, Fig. 6), the association was not significant for many species including *A. actinomycescomitans*, *B. forsythus* and *T. denticola*. Species were frequently detected in supragingival plaque or subgingival plaque samples only.

Fig. 7 presents the ratio of the mean counts for each species in supragingival plaque compared to subgingival plaque in the 23 periodontitis subjects. The circles to the left of the mid-line indicate species whose mean counts were higher in supragingival than subgingival plaque while species to the right of the mid-line had higher mean counts in sub than supragingival plaque. For example, mean counts ($\times 10^5$) for *Capnocytophaga gingivalis* were 3.59 and 0.75 in supra and subgingival plaque samples respectively providing a ratio of 4.79. Similarly, mean counts ($\times 10^5$) for *B. forsythus* were 1.69 and 0.43 in sub and supragingival samples respec-

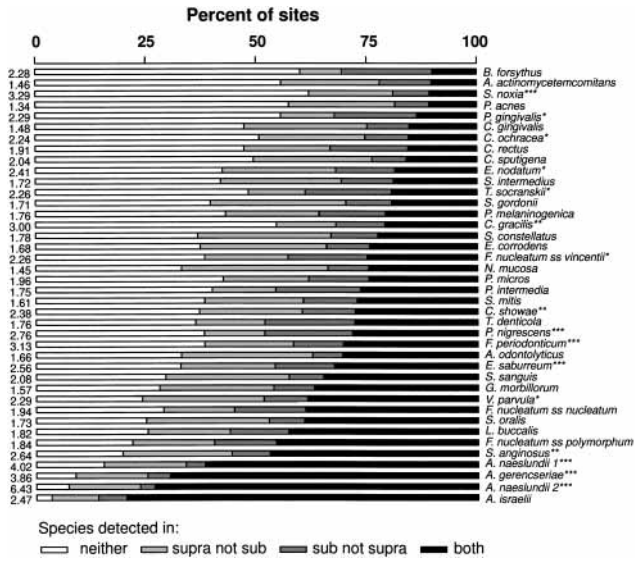


Fig. 6. Stacked bar chart of the % of sites at which each species was detected in either supra or subgingival plaque samples, both or neither. Plaque samples were taken from the same tooth surface at 585 sites in 23 subjects with adult periodontitis. 2x2 contingency tables were prepared for each species that indicated for each surface whether the species was detected at supragingival, subgingival, both or neither (Table 3). The values were converted to percents prior to plotting. The species are ordered on the basis of the % of sites at which a species was detected in both supra and subgingival samples. Mantel-Haenszel adjusted odds ratios are presented to the left of the bars. Mantel-Haenszel *p* values were adjusted for multiple comparisons. **p*<0.05, ***p*<0.01, ****p*<0.001.

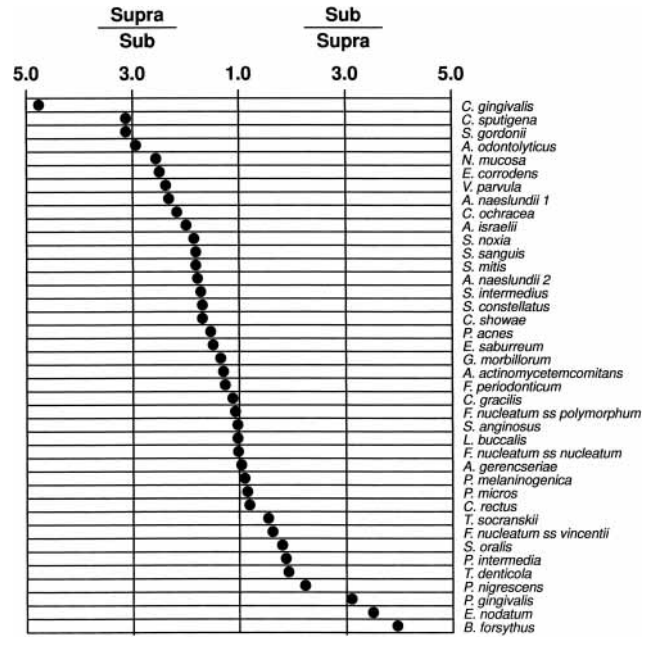


Fig. 7. Plot of the ratios of mean counts of each species in supra and subgingival plaque samples. The counts of each species were averaged in supra and subgingival plaque samples separately and then averaged across subjects. The circles to the left of the mid-line represent the ratios for species which were found at higher levels in the supra-gingival samples while circles to the right of the mid-line represent ratios for species found at higher levels in subgingival samples.

tively providing a ratio of 3.93. These data suggest that species such as *B. forsythus*, *Eubacterium nodatum* and *P. gingivalis* achieved higher levels in the subgingival environment in adult periodontitis subjects while *C. gingivalis*, *C. sputigena*, *S. gordonii* and *A. odontolyticus* achieved higher levels in the supra-gingival plaque.

Relationship of microbial composition of supra and subgingival plaque to pocket depth

Fig. 8 presents mean counts of each bacterial species tested in supra and subgingival plaque samples in pockets <3, 3–4 and >4 mm. Mean counts were generally high at sites with greater pocket depth for both the supra-gingival and subgingival samples. After adjusting for multiple comparisons, only *T. socranskii* and *Eubacterium saburreum* were significantly related to increased pocket depth in supra-gingival plaque. In contrast, mean counts of 17 species were significantly higher at deeper periodontal pockets for the subgingival plaque samples. In particular, members of the “red” and “orange”

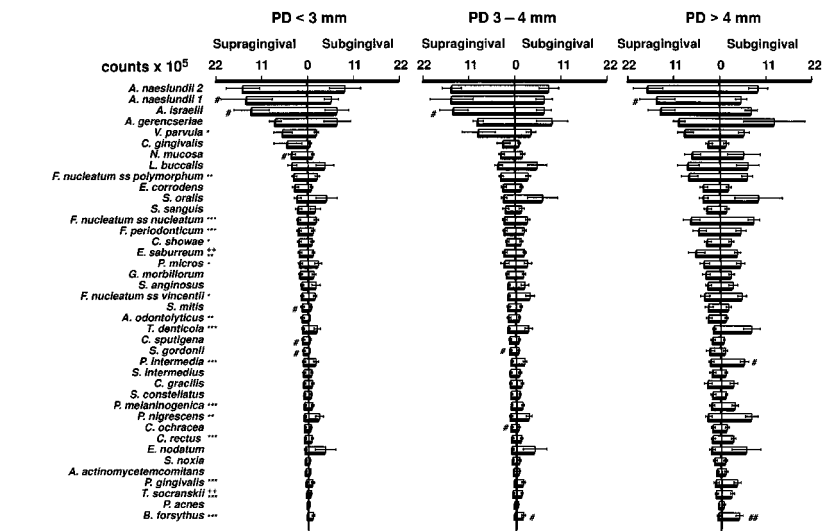


Fig. 8. Bi-lateral bar charts of the mean counts (x 10⁵, ± SEM) of individual species in supra and subgingival plaque samples from 23 periodontitis subjects at sites with pocket depths <3, 3–4 and >4 mm. The mean count was computed for each species in supra and subgingival plaque in each pocket depth category for each subject, and then averaged across subjects in the 3 pocket depth groups. The data are ordered on the basis of mean counts in the supra-gingival plaque samples from sites with pocket depths <3 mm. The significance of differences between supra and subgingival counts in each pocket depth category was tested using the Wilcoxon signed ranks test. #*p*<0.05, ##*p*<0.01 after adjusting for multiple comparisons. The significance of differences of mean counts of each species in supra and separately subgingival plaque samples among pocket depth categories was tested using the Quade test. Supra-gingival plaque: +*p*<0.01; Subgingival plaque: **p*<0.05, ***p*<0.01, ****p*<0.001 after adjusting for multiple comparisons.

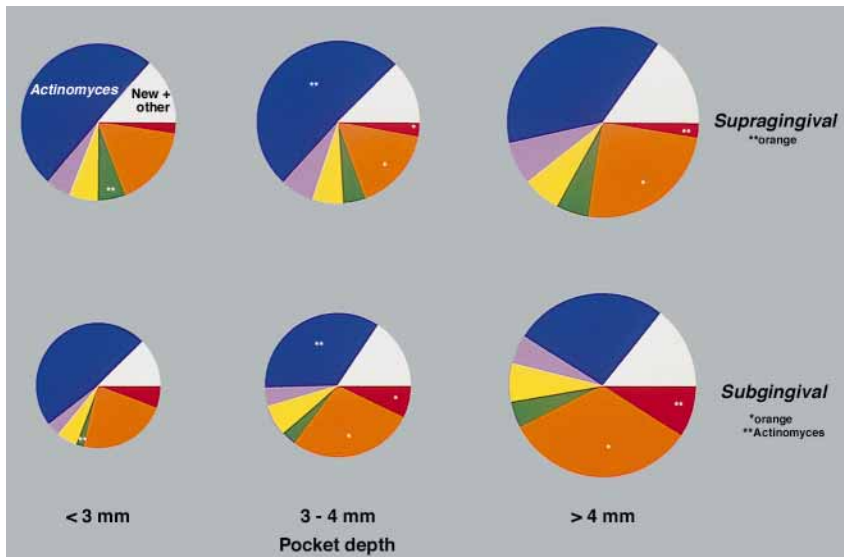


Fig. 9. Pie charts of the mean % DNA probe count of microbial groups in supra and subgingival plaque samples from 23 periodontitis subjects at sampled sites subset at the quartiles into pocket depth categories of <3, 3–4 and >4 mm. The species were grouped into the 7 microbial groups presented in Table 2 based on the description of Socransky et al. (1998). The areas of the pies were adjusted to reflect the mean total DNA probe count at each sample location. The significance of differences in mean percentages of the supra and subgingival complexes for individual complexes in each pocket depth category are indicated as follows: * $p < 0.05$, ** $p < 0.01$ after adjusting for 7 comparisons. Only the “orange” complex differed significantly among pocket depth categories both for supra and subgingival samples, while the *Actinomyces* differed among pocket depth categories for the subgingival plaque samples.

complexes, including *Fusobacterium* species, *P. intermedia*, *P. nigrescens*, *C. rectus*, *B. forsythus*, *P. gingivalis* and *T. denticola*, were detected at higher numbers at pockets ≥ 3 mm. At sampled sites < 3 mm, 6 species were significantly higher in supragingival samples compared with subgingival samples. These included 2 species each of the genera *Actinomyces* and *Streptococcus* as well as *C. sputigena* and *N. mucosa*. Fewer taxa differed between supra and subgingival plaque samples at pockets > 3 mm. Notable, were the higher mean counts of *B. forsythus* and *P. intermedia* at the deep periodontal sites.

The proportion of microbial complexes in supra and subgingival plaque samples at the different pocket depth categories is presented in Fig. 9. The areas of the pies were adjusted to reflect the mean total DNA probe count at each of the sample pocket depth categories. Total counts were highest in supragingival plaque from pockets >4 mm and lowest in subgingival samples from pockets <3 mm. The proportions of “orange” complex species were significantly increased in both supra and subgingival samples from sites with

deep pockets. *Actinomyces* species were significantly lower in the subgingival samples of the deep sites. At shallow sites, “green” complex species were in higher mean proportions in supragingival samples, whereas, “orange” and “red” complex species were in higher proportions in subgingival samples at the deeper sites.

Discussion

The purpose of the current investigation was to examine the microbial composition of supra and subgingival plaque samples from adult periodontitis subjects in order to compare and relate the species found in the 2 environments. While many studies have evaluated the composition of subgingival plaque, studies of supragingival plaque composition have been comparatively few. Further, these studies were limited in terms of the number of samples, subjects and species evaluated. The use of checkerboard DNA-DNA hybridization allowed the examination of 1170 supra and subgingival plaque samples obtained from 23 adult periodontitis subjects for their content of 40 species.

One unique aspect of this study was the sampling of supra and subgingival plaque from the same tooth surface permitting the direct comparison of species at the same tooth site.

Another important aspect of the current investigation was the use of 3 methods of data evaluation in order to describe more comprehensively the composition of supra and subgingival plaque. The prevalence or % of sites colonized provided an estimate of the extent of colonization of the individual species. Counts provided the level of colonization of each species at individual sites while the % DNA probe count indicated the proportion that each species comprised of the total DNA probe count in each sample. Each method provided different information. While there was some relatedness among these measures, detection of a species at a large proportion of sites did not necessarily imply that this species was present in high numbers or represented a large proportion of the total count. For example, *A. israelii* was found at a large proportion of both supra and subgingival sites; however, levels of this species were significantly higher in the supra than the subgingival plaque samples. In contrast, *B. forsythus* was found less commonly both supra and subgingivally but counts were significantly higher in subgingival plaque samples.

One of the most striking findings of the current investigation was the predominance of the *Actinomyces* species in both supra and subgingival plaque samples. Irrespective of the analysis employed, 4 of the 5 *Actinomyces* species examined, *A. naeslundii* genospecies 1 and 2, *A. gerencseriae* and *A. israelii*, were consistently the most prevalent, exhibited the highest mean counts and proportions in both supra and subgingival plaque samples. The ubiquitous colonization of these species was further emphasized in that individual subjects harbored at least one of the *Actinomyces* species in 75 to 100% of supragingival samples and 62 to 100% of subgingival samples. However, although predominant in both supra and subgingival plaque, the mean total proportion of *Actinomyces* species was significantly lower (38%) in subgingival plaque compared with 48% in supragingival plaque samples.

Not surprisingly, species thought to play a major rôle in the pathogenesis of periodontal infections were detected less frequently overall compared with

the *Actinomyces* and other host compatible species. The prevalence of suspected periodontal pathogens such as *B. forsythus*, *P. gingivalis* and *T. denticola* did not differ significantly between supra and subgingival plaque and one or more of these species were detected on average at greater than 50% of sites. However, counts of *B. forsythus* and *P. gingivalis* were significantly higher in subgingival plaque and proportions of these 2 species and *T. denticola* appeared to be higher subgingivally. Other species thought to be important in periodontal diseases, such as members of the genera *Fusobacterium* and *Prevotella* showed similar patterns of colonization.

The data in the present investigation are in accord with previous studies (Gmur et al. 1989, Haffajee et al. 1998, Socransky et al. 1998) that indicate that members of the "red" and "orange" complexes are in higher numbers and proportions in subgingival plaque samples from deeper periodontal pockets. However, unique to this investigation was the recognition that certain species, such as *T. socranskii* and *E. saburreum* were at higher mean counts in supragingival plaque samples taken from sites with deeper pocket depths than sites with shallower pockets. Members of the "orange" complex were also at greater mean proportions in supragingival samples from deep pocket sites than sites with shallow pockets. Such data imply an association of supragingival plaque composition with pocket depth at the adjacent site although cause or effect cannot be discriminated.

One of the prerequisites for colonization of a species is its ability to adhere to one or more oral surfaces in order to overcome the potent fluid flow present

in the oral cavity (Gibbons 1989, Gibbons et al. 1990). The habitats above and below the gingival margin both provide a tooth surface for colonization but differ in the nature of the boundary away from the tooth surface. Supragingivally, the surface of the plaque is not physically constrained but is in direct contact with saliva. Subgingivally, plaque is confined within the gingival sulcus or periodontal pocket which provides a second, quite different epithelial surface for colonization. The soft tissue wall also provides a source of somewhat different nutrients than those provided in the supragingival environment. Given these differences in habitat, one might expect major differences in the composition of plaque in the 2 domains. However, there did not appear to be an abrupt change in plaque composition at the gingival margin. The nature of the species colonizing above and below the gum was not strikingly different when only presence or absence was evaluated. The major differences were in counts and proportions of many of the species examined. In particular, the proportions of "red" and "orange" complex species were significantly higher in subgingival plaque. The proportions of the "red" complex species (*B. forsythus*, *P. gingivalis*, *T. denticola*) in subgingival plaque was twice that observed supragingivally (7.0 versus 2.8%) and "orange" complex species comprised about 18 and 28% of the total DNA probe count supra and subgingivally respectively. This finding was not unexpected, since several members of the "red" and "orange" complexes are either designated or suspected periodontal pathogens whose most advantageous habitat might be expected to be the subgingival space. Several investi-

gators have shown that species such as *B. forsythus* and *P. gingivalis* thrive in deep periodontal pockets (Gmur et al. 1989, Haffajee et al. 1998, Socransky et al. 1998) and have the potential to colonize the epithelial lining of the periodontal pocket (Childs & Gibbons 1988,1990). In contrast, it appears that the *Actinomyces* attach well to the tooth surface (Gibbons et al. 1990) above and below the gum and are a major component of plaque in both ecosystems. Preliminary data from ongoing studies indicate that *Actinomyces* species are found in lower proportions in samples from oral soft tissues such as the tongue, cheeks and palate, suggesting that the primary and most important area for colonization of members of this genus may be the tooth surface.

The finding of suspected periodontal pathogens in supragingival plaque samples has been described by a number of investigators (Cao et al. 1990, Riviere et al. 1992, Gmur & Guggenheim 1994, Zee et al. 1996) and has important implications. In accord with those studies, the current investigation detected suspected periodontal pathogens in supragingival plaque samples at a significant number of sites, although counts and proportions were relatively low. Further, these species were found at supragingival sites where the corresponding subgingival site did not harbor the species. For example, *P. gingivalis* was found at 18% (69/385) of supragingival sites where it was not detected subgingivally (Table 3). Thus, the results of the present investigation suggested that supragingival colonization by such species is more than merely a transient phenomenon and do not support the notion of subgingival overgrowth.

Table 3. Relationship of species in supra and subgingival plaque samples at individual sites.

		<i>P. gingivalis</i>				<i>A. naeshundii 2</i>	
		supragingival				supragingival	
		0	+			0	+
subgingival	0	316	69	subgingival	0	42	93
	+	106	77		+	19	420
		OR=2.29, $p<0.05$				OR=6.43, $p<0.001$	
		<i>P. intermedia</i>				<i>V. paravula</i>	
		supragingival				supragingival	
		0	+			0	+
subgingival	0	225	81	subgingival	0	134	153
	+	108	149		+	56	214
		OR=1.75, N.S.				OR=2.29, $p<0.05$	

The "0" and "+" row and column headings represent "not detected" and "detected" respectively. The odds ratios and p values were computed using Mantel-Haenszel procedures with the subject as the stratum. The p -values were adjusted for 40 multiple comparisons.

The detection of "subgingival" species in supragingival sites indicates that such species can colonize supragingival plaque even though the majority of them are thought to be oxygen sensitive and/or require a low oxidation-reduction potential for growth. In earlier years, colonization of anaerobic species in the supposedly "aerobic" supragingival plaque was thought to be unlikely. Recent studies of the micro-organization of the bacterial constituents of dental plaque offer reasonable explanations for detection of anaerobes in supragingival plaque. Such studies indicate that in multi-species biofilms, such as dental plaque, mixed-species microcolonies are formed (Costerton et al. 1994, 1995). An individual cell within a mature multispecies biofilm typically lives in a unique microhabitat where nutrients are provided by neighboring cells and by diffusion, where products are removed by the same processes, and where antagonists may be kept at a distance by diffusion barriers (Costerton et al. 1987). In situ biofilm measurements of pH, dissolved oxygen, sulfide and other components have provided critical information about the properties of these bacterial structures. When a microelectrode was inserted into a bacterial microcolony within a biofilm, the values of dissolved oxygen decreased as the electrode was inserted further into the microcolony, reaching almost totally anaerobic levels in the center. These direct observations of living biofilms may explain the existence, and even the physiological activity, of fastidious anaerobes within mixed biofilms in supposedly aerobic environments such as supragingival plaque.

The frequent detection of potential periodontal pathogens in supragingival plaque has important clinical and ecological ramifications. Until now, the main reason to control supragingival plaque has been to prevent gingivitis. The data of the present investigation suggest a second, perhaps more compelling reason for controlling supragingival plaque; i.e., supragingival plaque may be a major reservoir for species that ultimately initiate periodontitis. From this reservoir, species could spread to uninfected sites or recolonize subgingival sites which have been periodontally treated. Further, transmission of species from one subject to another would be more readily achieved if the species were located in the supragingival plaque. In both instances,

meticulous supragingival plaque removal may be crucial in preventing the initiation and recurrence of periodontal infections, confirming the importance placed on the use of proper oral hygiene regimes.

Acknowledgments

This work was supported in part by research grants DE-12108, DE-10977 and DE-04881 from the National Institute of Dental and Craniofacial Research and by DGAPA, National Autonomous University of Mexico (UNAM).

Zusammenfassung

Mikrobielle Zusammensetzung supra- und subgingivaler Plaque bei Patienten mit Erwachsenenparodontitis

Einleitung: Diese Studie sollte die mikrobielle Zusammensetzung supra- und subgingivaler Plaque von 23 Patienten mit Erwachsenenparodontitis (mittleres Alter 51 ± 14 Jahre) vergleichen und in Beziehung setzen.

Methoden: Insgesamt wurden 1170 Proben supra- und subgingivaler Plaque von den mesialen Stellen eines jeden Zahnes (bis zu 28 supra- und 28 subgingivale Proben pro Patient) gewonnen und auf das Vorhandensein sowie die Konzentration von 40 verschiedenen Bakterienarten mittels Gesamtgenom-DNS-Sonden und "Schachbrett"-DNS-DNS-Hybridisierung untersucht. Die klinischen Parameter umfaßten dichotome Beurteilungen der Rötung der Gingiva, des Blutens auf Sondieren, Plaqueansammlung und Eiterung sowie Doppelmessungen der Sondierungstiefen und klinischen Attachmentlevel an 6 Stellen pro Zahn. Die Anzahl, der prozentuale Anteil der mit DNS-Sonden nachgewiesenen Zahlen (Proportion) und der prozentuale Anteil kolonisierter Stellen (Prävalenz) jeder Spezies in supra- und separat in subgingivaler Plaque wurden für jeden Patienten berechnet. Die Signifikanz von Unterschieden zwischen supra- und subgingivaler Plaque für jede Spezies wurden mittels des Wilcoxon-Tests bestimmt und für multiple Vergleiche korrigiert.

Ergebnisse: Alle 40 Spezies wurden in supra- und subgingivaler Plaque identifiziert. *Actinomyces*-Spezies waren die Arten, die in beiden Lebensräumen am häufigsten vorkamen. 75 bis 100% der supra- und 62 bis 100% der subgingivalen Stellen waren zumindest von einer von 5 *Actinomyces*-Arten besiedelt. Supragingivale Proben zeigten signifikant höhere Zahlen von *Actinomyces naeslundii* Genospezies 1, *Actinomyces israelii*, *Actinomyces odontolyticus*, *Neisseria mucosa*, *Streptococcus gordonii*, *Capnocytophaga ochracea* und *Capnocytophaga sputigena* im Vergleich mit subgingivalen Proben der gleichen Stellen. Proben subgingivaler Plaqueproben zeigten signifikant höhere Zahlen von *Prevotella nigrescens*, *Prevotella intermedia*, *Bacteroides*

forsythus und *Porphyromonas gingivalis*. Subgingivale Proben zeigten einen signifikant höheren Anteil der Arten des "roten" und "orangen" Komplexes, während in supra- und subgingivaler Plaque höhere Anteile der Spezies des "grünen" und "rosa" Komplexes sowie *Actinomyces*-Arten beobachtet wurden. Putative Parodontalpathogene konnten in supra- und subgingivaler Plaque von Stellen gefunden werden, bei denen diese Arten subgingival nicht gefunden wurden.

Schlussfolgerungen: Diese Ergebnisse weisen darauf hin, daß supragingivale Plaque putative Parodontalpathogene beherbergen kann, was auf eine mögliche Rolle dieser Umgebung als Reservoir dieser Keime für die Wiederbesiedlung subgingivaler Stellen hindeutet.

Résumé

Composition microbienne de la plaque supra et sous gingivale chez des sujets atteints de parodontite de l'adulte.

Cette étude se propose de comparer et de mettre en relation la composition microbienne de la plaque supra et sous gingivale chez 23 patients atteints de parodontite de l'adulte (âge moyen 51 ± 14 ans). Un total de 1170 échantillons de plaque supra et sous et 28 échantillons sous gingivaux), chez chaque sujet et la présence et le taux de 40 taxons bactériens, à l'aide de sonde génomique complète et d'hybridation DNA-DNA en damier, furent évalués. Les relevés cliniques suivants, appréciation dichotomique de la rougeur gingivale, saignement au sondage, accumulation de plaque et suppuration, ainsi que la mesure dupliquée de la profondeur de poche et du niveau d'attache ont été réalisés sur 6 sites par dent. Le nombre (niveau), le % du total des sondes DNA (proportion) et le % de sites colonisés (prévalence) de chaque espèce, dans la plaque supragingivale, et séparément, dans la plaque sous gingivale, ont été saisis par informatique pour chaque sujet. La signification des différences entre les plaques supra et sous gingivales pour chaque espèce a été recherchée en utilisant le Wilcoxon signed rank test ajusté pour des comparaisons multiples. Toutes les espèces ont été détectées aussi bien dans la plaque supra que dans la plaque sous gingivale. L'espèce *Actinomyces* était le taxon le plus prévalent dans les deux habitats, 75 à 100% des sites supra gingivaux et 62 à 100% des sites sous gingivaux étant colonisés par au moins 1 des 5 espèces d'*Actinomyces*. Les échantillons de plaque supragingivale présentaient des comptages plus importants pour *Actinomyces naeslundii* *genospecies 1*, *Actinomyces israelii*, *Actinomyces odontolyticus*, *Neisseria mucosa*, *Streptococcus gordonii*, *Capnocytophaga ochracea* et *Capnocytophaga sputigena* par comparaison avec le comptage moyen des échantillons sousgingivaux obtenus sur la même surface dentaire. Les échantillons de plaque sous gingivales présentaient des comptages significativement

supérieurs de *Prevotella nigrescens*, *Prevotella intermedia*, *Bacteroides forsythus* et *Porphyromonas gingivalis*. Les échantillons sous gingivaux présentaient des proportions significativement supérieures d'espèces appartenant aux complexes rouges et oranges, alors que les échantillons supragingivaux présentaient des proportions supérieures d'espèces appartenant aux complexes vert et violet ainsi que de l'espèce *Actinomyces*. Des pathogènes parodontaux potentiels purent être détectés dans la plaque supragingivale dans des sites où les échantillons sous gingivaux se révélaient négatifs pour ces mêmes espèces. Ces données indiquent que la plaque supragingivale peut contenir des pathogènes parodontaux potentiels, suggérant un rôle possible de cet environnement comme réservoir pour de telles espèces, pour la dissémination ou la réinfection de sites sous gingivaux.

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