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A three-year prospective study of adult subjects with gingivitis II: microbiological parameters

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Abstract

Aim: To investigate whether the clinical benefits obtained with a periodontal prevention programme in subjects with periodontal health or minimal disease were accompanied by beneficial changes in the subgingival microbiota.

Material and Methods: One hundred and twenty-four subjects completed the study. Subjects were clinically and microbiologically monitored at baseline, 1, 2 and 3 years. Subgingival plaque samples were taken from the mesiobuccal aspect of every tooth and were analysed for the levels of 40 bacterial species using checkerboard DNA–DNA hybridization (total samples = 13,477). The mean counts of each of the 40 test species were calculated for each subject at each time point. Significance of differences over time was sought using the Friedman test. *p* values were adjusted for multiple comparisons.

Results: All clinical parameters, at the microbiologically sampled sites, improved over time. The clinical changes were accompanied by statistically significant decreases in the mean counts of 35 of the 40 test species. Major reductions occurred by year 2 for *Actinomyces*, *Capnocytophaga*, *Campylobacter*, *Fusobacterium* and *Prevotella* species. At year 3, there was a modest re-growth of the majority of the species.

Conclusions: The clinical improvements obtained through preventive measures were accompanied by a shift to a more host-compatible subgingival microbiota.

Key words: bacteria; biofilm; gingivitis; periodontal health; periodontal; subgingival plaque

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There have been a number of studies that have examined preventive procedures in periodontally healthy and maintenance subjects (Suomi et al. 1971, Soderholm & Egelberg 1982, Soderholm et al. 1982, Glavind et al. 1984, Albandar et al. 1994, Haffajee et al. 2001b, Heitz-Mayfield et al. 2003, Schatzle et al. 2003, Axelsson et al. 2004). However, there are a limited number of reports describing the microbial outcomes of these procedures (Loos et al. 1988, Murray et al. 1989, McNabb et al. 1992, al Yahfoufi et al. 1995, Hellstrom et al. 1996, Nogueira et al. 2000, Ximenez-Fyvie et al. 2000b, Haffajee et al. 2001a, Goodson et al. 2004). Most microbiological studies on periodontally healthy populations emphasized differentiation from gingivitis and/or periodontitis (Tanner et al. 1996, 1998, 2000, Haffajee et al. 1998, 2005, Maiden et al. 1998, Ximenez-Fyvie et al. 2000a, Yang et al. 1994). These studies implicated species such as *Tannerella forsythia* (Haffajee et al. 1998, Maiden et al. 1998, Tanner et al. 1996, Ximenez-Fyvie et al. 2000a, Yang et al. 1994), *Porphyromonas gingivalis* (Haffajee et al. 1998, Ximenez-Fyvie et al. 2000a, Yang et al. 1994), *Treponema denticola* (Haffajee et al. 1998, Ximenez-Fyvie et al. 1994a) and *Selenomonas noxia* (Tanner et al. 1996, 1998, Haffajee et al. 1998) as aetiological agents of chronic periodontitis. Longitudinal monitoring of subjects with periodontal health or minimal disease may be of use when examining the ecologic shifts that occur during the transition from health to periodontal disease. Tanner et al. (1996) monitored a group of periodontally healthy subjects for 12 months in an attempt to characterize the microbiological profile associated with early periodontal lesions. They described *Actinomyces naeslundii*, *Veillonella parvula*, *S. noxia* and *Prevotella nigrescens* as being associated with periodontal sites that increased in probing pocket depth (PPD) >1.5 mm. The same research group studied 56 systemically healthy adult subjects with minimal attachment loss and found that *T. forsythia*, *Campylobacter rectus* and *S. noxia* were associated with an increase in interproximal attachment loss $\geqslant 1.5$ mm (Tanner et al. 1998).

Several studies examined the effect of supragingival plaque removal on the composition of the subgingival micro-

biota (Loos et al. 1988, McNabb et al. 1992, al Yahfoufi et al. 1995, Hellstrom et al. 1996, Westfelt et al. 1998, Nogueira et al. 2000, Ximenez-Fyvie et al. 2000b, Haffajee et al. 2001a, Petersilka et al. 2002). It was found that repeated professional removal of supragingival plaque had a marked effect on the composition of the subgingival microbiota, reducing total counts as well as counts of individual species (al Yahfoufi et al. 1995, Hellstrom et al. 1996). This effect was more pronounced at sites with shallow pocket depths compared with deeper sites (McNabb et al. 1992, Westfelt et al. 1998). Self-performed plaque control using either powered or manual brushes also decreased counts of species in the subgingival plaque, although the effects were less marked (Haffajee et al. 2001a).

Studies that examine preventive procedures may have the immediate benefit of optimizing preventive strategies and providing clues to desired microbiological endpoints for new methods to prevent periodontal disease progression. The present paper examines the microbial features of adult subjects who are periodontally healthy or who exhibit minimal disease and their response to preventive regimens for 3 years. The purpose of the present investigation was to test the hypothesis that the clinical benefits attained through periodontal prevention programmes based on meticulous supragingival plaque control in these subjects (Bogren et al. 2006) would be accompanied by a shift to a more host-compatible subgingival microbiota.

Material and Methods

Subject population, study protocol and clinical monitoring

The study population, the study protocol and details regarding the clinical monitoring of patients were previously described (Bogren et al, 2006). In brief, a total of 160 periodontally healthy or minimally diseased subjects were selected and enrolled in the study. 124 of the 160 subjects had complete microbiological data for the four visits. The subjects were recruited in two geographic locations: Landskrona, Sweden ($N = 80$), and Boston, USA ($N = 80$). The Ethics Committee at The University of Goteborg and the Institutional Review Board at The Forsyth

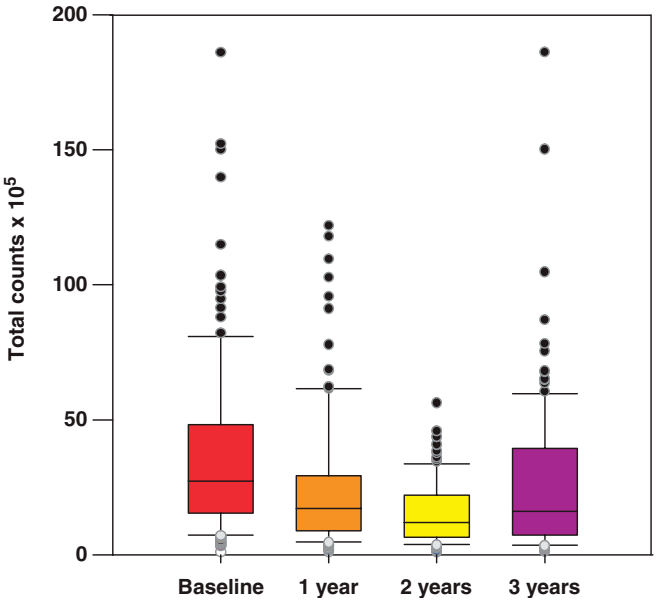

Fig. 1. Tukey box plots of the mean total DNA probe counts of bacterial species at baseline, 1, 2 and 3 years for the 124 subjects. The total DNA probe count was computed for each subject at each time point separately. In the figure, the boxes describe the lower and upper quartiles and the horizontal line within each box represents the median value. The whiskers represent the 10% and 90% values, while the circle outside the whiskers represents individual subjects outside this range.

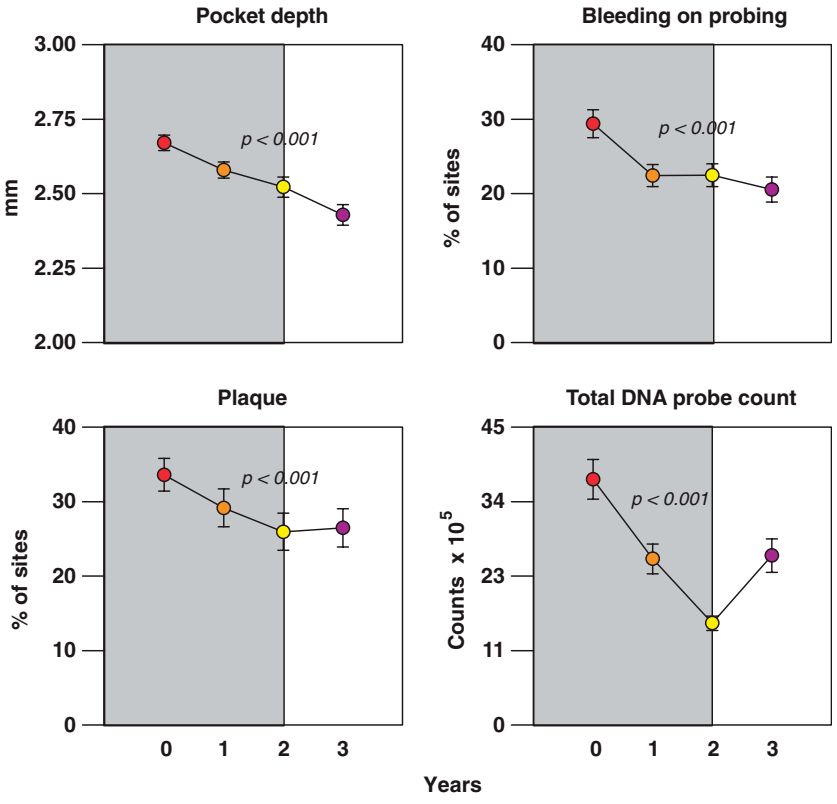

Fig. 2. Plots of the full-mouth mean values (± SEM) for PPD, % of sites with BoP, plaque and total DNA probe counts ($\times 10^5$) at baseline, 1, 2 and 3 years in all subjects. The circles represent the mean values and the whiskers represent the standard error of the mean. Values for each parameter were measured at up to 28 mesiobuccal sites in each subject, averaged within a subject and then averaged across subjects for each time point. Significance of differences over time was tested using the Friedman test.

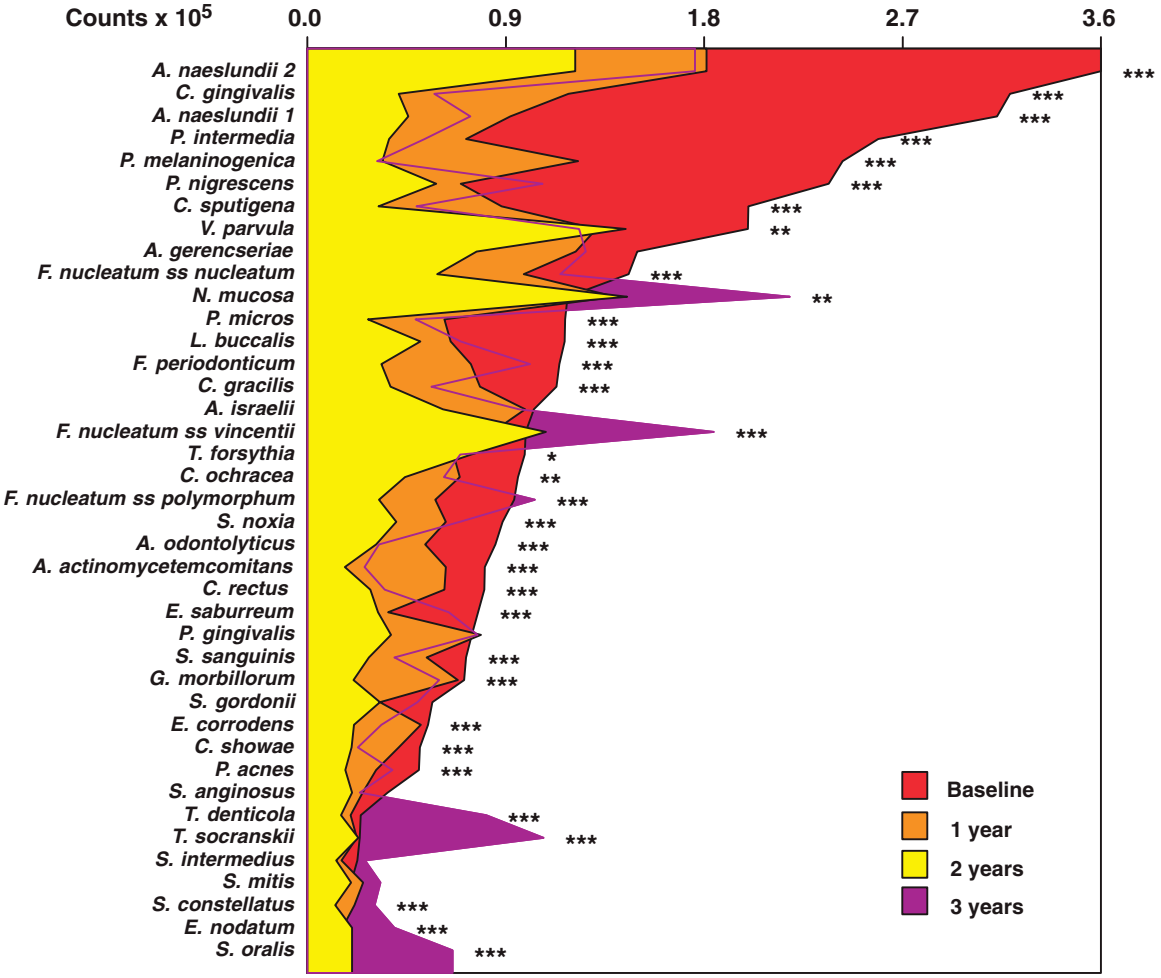

Fig. 3. Mean microbial profiles of 40 test species in 124 periodontally healthy or minimally diseased subjects from Sweden and USA at baseline, 1, 2 and 3 years. The mean counts for each species were computed for samples from up to 28 sites in each subject and then averaged across subjects at each visit. Significance of differences in mean counts among visits was determined using the Friedman test and adjusted for multiple comparisons (Socransky et al. 1991); $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. The species were ordered according to baseline values.

Institute approved the study protocol including the taking of clinical measurements and subgingival plaque samples. The subjects signed informed consent before entering the study. To be included in the study, subjects had to be $\geqslant$20 years of age, have at least 24 natural teeth, $\leqslant$2 sites with PPD $>$4 mm and no proximal sites with clinical attachment loss (CAL). Subjects were excluded if they had any systemic condition, which would influence the course of periodontal disease or treatment, and medical conditions, which would require antibiotic prophylaxis for routine dental procedures. Individuals who had taken antibiotics in the previous 3 months or were either pregnant or nursing were also excluded.

Subjects were clinically and microbiologically monitored at baseline, 1, 2 and 3 years. The clinical measurements were taken at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual) as described by Bogren et al. (2006). All subjects received dental prophylaxis performed by a dental hygienist as well as instructions in and reinforcement of proper home care procedures at the beginning of the study and every 6 months for 3 years.

Microbiological assessment

Subgingival plaque samples were taken at baseline, 1, 2 and 3 years from the mesiobuccal aspect of all teeth, excluding third molars. The subgingival plaque samples were collected using sterile Gracey curettes (LM-instruments Oy, Parainen, Finland/Hu-Friedy®, Chicago, IL, USA) after removal of supragingival plaque (if present). The samples were placed in separate microcentrifuge tubes containing 0.15 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and 0.15 ml of 0.5 M NaOH was added. All samples were processed in the same laboratory at The Forsyth Institute. Samples were individually analysed for their content of 40 bacterial species using the checkerboard DNA–DNA hybridization technique (Socransky et al. 1994, 2004). In brief, the samples were lysed and the DNA placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA, USA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics), with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-

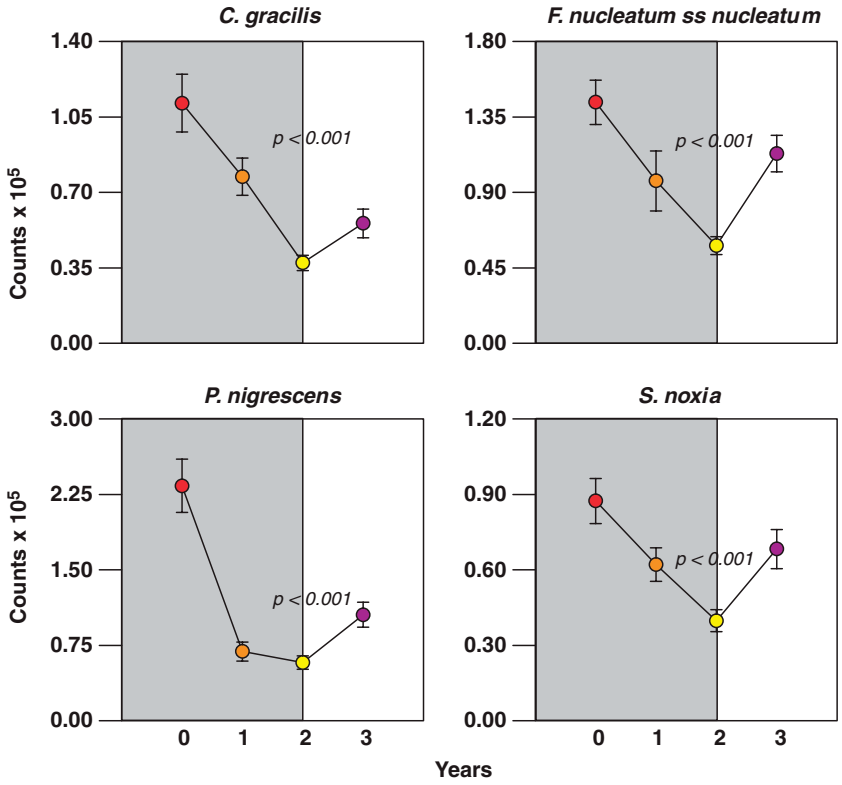

Fig. 4. Mean counts (x10^5 ± SEM) of *Campylobacter gracilis*, *Fusobacterium nucleatum ss nucleatum*, *Prevotella nigrescens* and *Selenomonas noxia* in subgingival plaque samples taken at baseline, 1, 2 and 3 years. The mean counts for each species were computed for a subject for each visit, and then values were averaged across subjects at each time point. The whiskers indicate the SEM. Significance of differences over time was determined using the Friedman test and adjusted for multiple comparisons (Socransky et al. 1991).

labelled whole genomic DNA probes to 40 subgingival species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using antibody to digoxigenin, conjugated with alkaline phosphatase and chemi-fluorescence detection. Signals were detected using the AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL, USA) and were read using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA), a computer-linked instrument that read the intensity of the fluorescence signals resulting from the probe-target hybridization. Two lanes in each run contained standards at the concentration of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the Storm Fluorimager and converted to absolute counts by comparison with standards on the same membrane. Failure to detect a signal was recorded as zero. A total of 13,477 subgingival samples were evaluated for the 124 subjects (27 samples/visit/subject).

Preventive regimens

Subjects received dental prophylaxis including supragingival scaling and polishing, performed by a dental hygienist, as well as instructions in and reinforcement of proper home care procedures at the beginning of the study and subsequently every 6 months for 3 years. A self-performed plaque removal programme was introduced that included 2 × a day brushing using a manual or powered toothbrush and fluoride-containing dentifrice (Protection Caries® or Total®, Colgate, Piscataway, NJ, USA). Inter-dental cleaning with dental floss and/or toothpicks was also performed. These procedures were carried out for the 3 years of the study. Fresh supplies for the self-performed cleaning procedures were provided at each 6-month recall.

Data analysis

Microbiological data available for each subject were the total DNA probe counts and the counts of each of the 40 test species for up to 28 subgingival plaque samples per subject at baseline, 1, 2 and 3 years. The data for each species were expressed as counts × 10^5 at each site, averaged within each subject and then averaged across subjects at each time point separately. Significance of differences in mean counts for each species and total DNA probe counts over time was sought using the Friedman test. In a similar manner, the % of sites with counts of each species $>10^5$ was computed for each subject at each visit. These data were averaged within each subject and then averaged across subjects at each time point separately and the significance of difference over time determined using the Friedman test. For certain analyses, the data were subset according to baseline pocket depth categories of $\leqslant 3$ mm and >3 mm, averaged within a subject within each pocket depth category and then averaged across subjects for each time point. Significance of differences of bacterial species between the two pocket depth categories at each time point was determined using the Mann–Whitney test. Significance of differences in counts of bacterial species over time was determined using the Friedman test. All microbiological analyses were adjusted for multiple comparisons (Socransky et al. 1991).

The relationship between mean pocket depth change from baseline to 3 years in each subject and mean baseline pocket depth, age, smoking status and the baseline mean counts of the 40 test species or change in mean counts from baseline to 3 years of the test species was examined using stepwise forward multiple linear regression. The analyses were terminated when a variable entering the model had a p value >0.10.

Results

One hundred and twenty-four of the original 160 subjects had microbiological data for all four visits and completed the study. There was considerable variability in the total counts of bacteria among subjects at the different time points (Fig. 1). At baseline, the counts ranged from 0.94 to 186.1 (× 10^5) with a median value of 27.31 (× 10^5). These counts diminished over time with median values of 17.14, 11.98 and 16.12

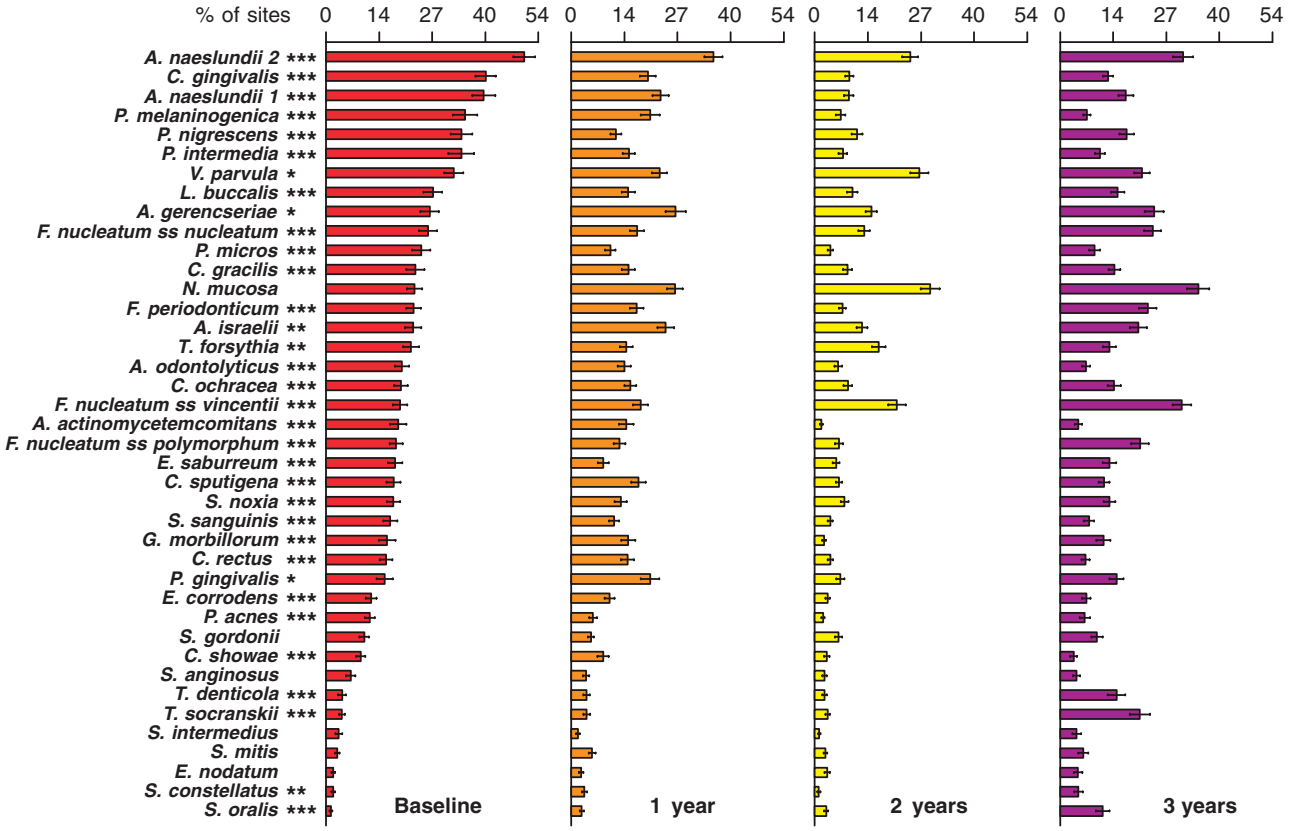

Fig. 5. Bar charts of the % of sites colonized at levels $>10^5$ for the 40 test species at baseline, 1, 2 and 3 years. The % of sites colonized by each species at levels $>10^5$ was determined in each subject and averaged across subjects at each time point separately. The bars represent the mean values of each species and the whiskers represent the SEM. The species were ordered according to the baseline % of sites colonized. Significance of difference over time was determined using the Friedman test, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and adjusted for multiple comparisons (Socransky et al. 1991).

($\times 10^5$) at 1, 2 and 3 years, respectively. It is interesting that 80% of the counts at baseline ranged from 7.34 to 80.91 ($\times 10^5$) and at 2 years 80% of counts ranged from 3.83 to 33.75 ($\times 10^5$).

Changes in clinical and microbiological parameters

Figure 2 presents the changes in periodontal parameters over the 3 years of observation for the 124 subjects who completed the 3 years of the study. Only data from mesiobuccal sites (i.e. microbiologically sampled sites) are presented. The mean PPD decreased from 2.7 to 2.4 mm ($p<0.001$). There was a statistically significant decrease in the percentage of sites with bleeding on probing (BoP) from 29% to 21% ($p<0.01$) and sites with visible plaque (34–26%, $p<0.01$) over time. The improvement in clinical parameters over time was accompanied by a statistically significant ($p<0.001$) decrease in the mean total DNA-probe counts of subgingival species from 39.6×10^5 at baseline to 15.9×10^5 at 2 years, rebounding to 26.6×10^5 at 3 years.

The mean counts of the 40 test species in subgingival plaque samples from all subjects at baseline, 1, 2 and 3 years are presented in Fig. 3. The species are ordered according to baseline values. The mean levels of the 40 test species were quite low at baseline, but significant changes for 35 of the 40 test species occurred during the 3-year study. Major reductions occurred by year 2 for members of the genera *Actinomyces*, *Capnocytophaga*, *Campylobacter*, *Fusobacterium* and *Prevotella*. At year 3, there was a modest increase in the mean counts of the majority of the species, although only 10 of the 40 species achieved levels greater than the baseline values. These species included members of the genera *Streptococcus*, *Fusobacterium* and *Treponema*.

Figure 4 presents the mean counts ($\times 10^5$, $\pm$ SEM) of three orange complex species and *S. noxia* at baseline, 1, 2 and 3 years. All species demonstrated the same trend displayed by the total DNA probe count data, decreasing to 2 years, followed by an increase at year 3.

Figure 5 presents the % of sites colonized by the 40 bacterial species at levels $>10^5$ at each monitoring time point. In accord with the data from Fig. 3, there was a significant reduction in the percentage of sites colonized at $>10^5$ at 2 years for 34 of the 40 test species. The exceptions were four *Streptococcus* species, *Neisseria mucosa* and *Eubacterium nodatum*.

Figure 6 presents percentile plots of mean PPD, at sampled sites only, and the mean counts of *Fusobacterium nucleatum ss nucleatum*, *F. nucleatum ss polymorphum* and *S. noxia* for each individual at baseline, 2 and 3 years. These plots illustrate the mean counts for each subject and thus, display the variability among subjects. The PPD plot demonstrated that the majority of subjects exhibited an improvement in mean PPD from baseline (red circles) to 2 years (yellow circles) and 3 years (purple circles). The distributions of the

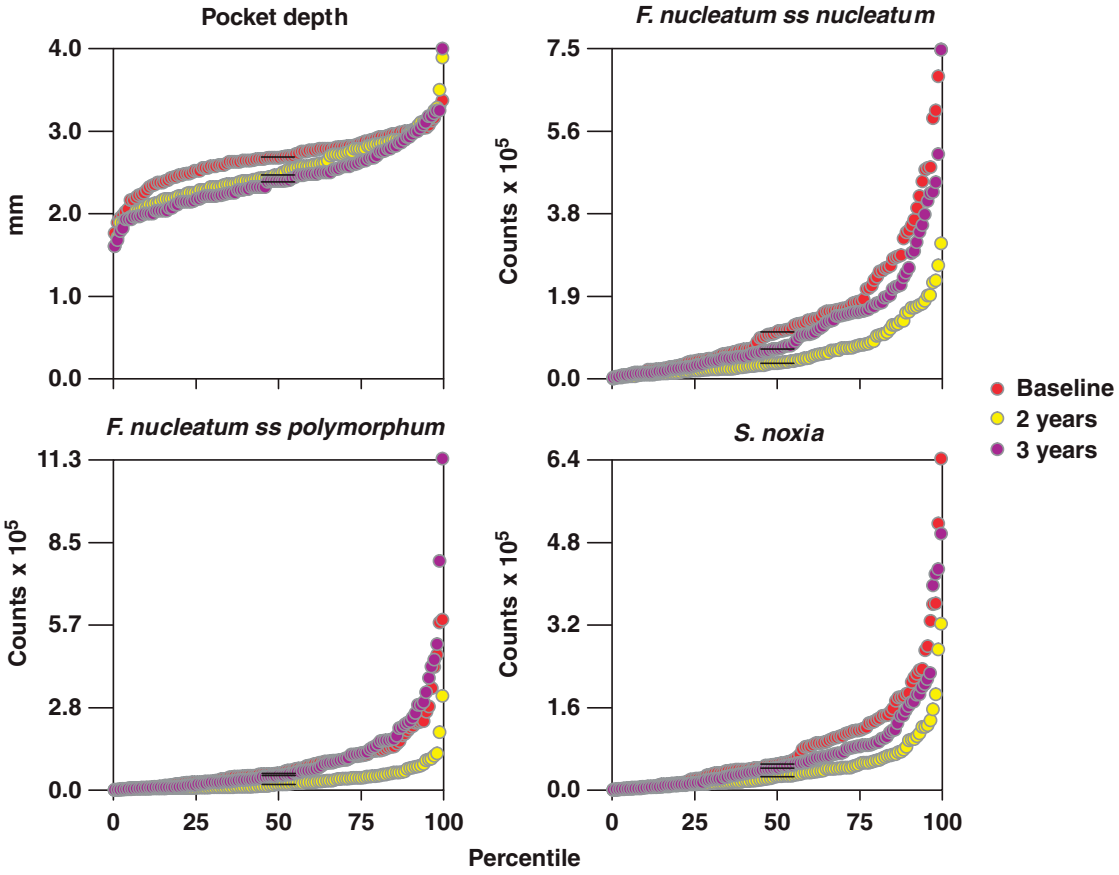

Fig. 6. Percentile plots of mean PPD and of mean counts of *Fusobacterium nucleatum ss nucleatum*, *F. nucleatum ss polymorphum* and *Selenomonas noxia* at baseline (red circles), 2 years (yellow circles) and 3 years (purple circles). For the first plot, the y-axis represents the mean PPD in mm, while for the other 3 plots, it represents the mean counts ($\times 10^5$). The *x*-axis of all plots represents the percentiles. Each circle depicts the mean value for an individual subject and the horizontal lines in each panel indicate the median values (50th percentile) for baseline, 2- and 3-year values.

mean counts for the 3 selected bacterial species demonstrated the decreasing levels of these species from baseline to 2 years and the increase approaching baseline values by year 3. Indeed, the distribution of the baseline and 3-year values for *F. nucleatum ss polymorphum* are almost identical.

Changes in microbiological parameters at sites with different baseline pocket depths

The microbiological data at sites subset into two baseline pocket depth categories ($\leqslant 3$ mm and >3 mm) are presented in Figs 7 and 8. Figure 7 presents the mean total DNA probe counts at baseline, 1, 2 and 3 years for the sites that were $\leqslant 3$ mm or >3 mm at baseline. The data indicate that there was a significant reduction in mean total DNA counts ($\times 10^5$) for both baseline pocket depth categories over time up to 2 years, with some rebound by year 3. In addition, there were clearly higher mean counts at sites with greater baseline pocket depths at each time point, although differences between groups were significant at baseline only.

The mean counts of individual species at sites with initial PPD of $\leqslant 3$ and >3 mm at baseline, 1, 2 and 3 years are presented in Fig. 8. At baseline, there were differences in the mean counts of many species in the two pocket depth categories, although the differences were not statistically significant after adjusting for multiple comparisons. The *Actinomyces* and *Capnocytophaga* species, as well as many of the orange complex species were at higher levels in the samples from the sites with baseline PPD >3 mm. By 1 year, there was a clear reduction in counts of many species, particularly at the sites with initial PPD of >3 mm. This was most marked for the *Actinomyces*, *Fusobacterium* and *Prevotella* species (Fig. 8, second panel). At the 2-year monitoring visit, the mean counts of most species were reduced even further. At the end of the study (year 3), most species exhibited some regrowth in both pocket depth categories, although levels were lower than baseline values for the majority of species.

Relation between change in mean pocket depth and clinical and microbiological parameters

The relationship of the change in mean pocket depth in a subject and baseline counts of the 40 test species as well as baseline pocket depth, age and smoking status was examined using stepwise forward multiple linear regression (Table 1). Not surprisingly, the data indicated that the most significant predictor of change in mean pocket depth was the mean baseline pocket depth of the subject. The greater the baseline pocket depth, the greater the reduction in pocket depth over time. High mean counts of *Treponema socranskii*,

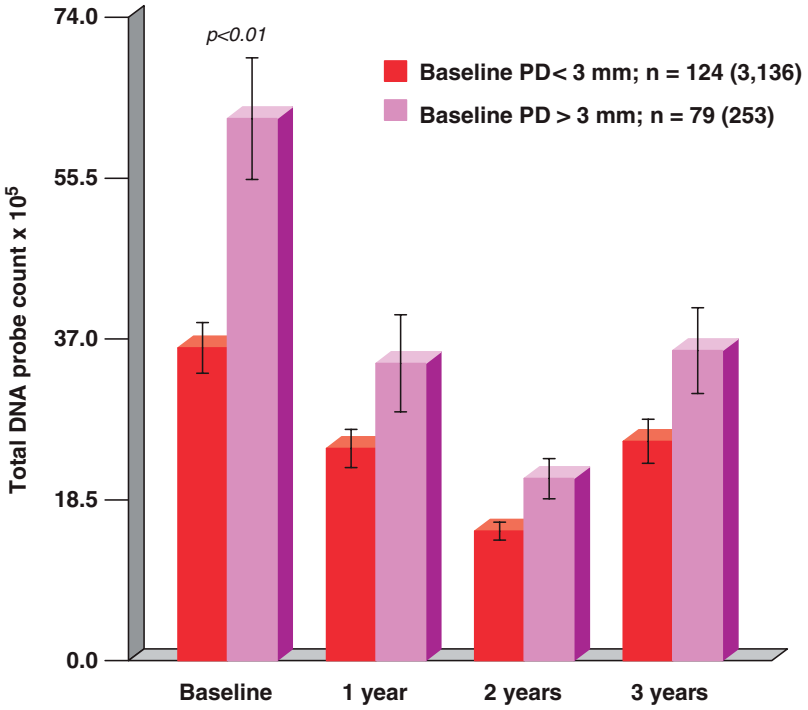

Fig. 7. Mean total DNA probe counts ($\times 10^5 \pm$ SEM) for samples from sites with baseline pocket depths $\leqslant 3$ and > 3 mm for all subjects combined. The data for the baseline, 1-, 2- and 3-year visits were subset according to their baseline pocket depth, not their pocket depth at years 1, 2 and 3. The bars represent the mean values and the whiskers the SEM. At each time point separately, the total counts for the 40 subgingival species were determined at each site type separately in each subject, averaged within a subject and then across subjects. Differences over time for both pocket depth categories were significant using the Friedman test. The significance of differences between the two pocket depth categories at each time point was determined using the Mann–Whitney test.

C. rectus and *Streptococcus oralis* at baseline were associated with mean reduction in pocket depth while high counts of *Actinomyces gerencseriae* and *S. mitis* as well as increased age were associated with a mean increase in pocket depth at 3 years. Table 2 presents similar data examining the relationship between mean pocket depth change from baseline to 3 years and change in mean counts of the 40 test species after adjusting for baseline pocket depth. A mean pocket reduction was associated with an increase in mean counts of *A. odontolyticus* and *S. constellatus* and a decrease in means counts of *F. periodonticum*, *S. oralis* and *C. rectus*.

Discussion

The goal of the present investigation was to determine whether, the clinical improvements observed in a population of healthy or minimally diseased subjects (Bogren et al. 2006) enrolled in a prevention programme, were accompanied by beneficial shifts in the subgingival microbiota. The data demonstrated a significant reduction in the mean total DNA probe count and in the mean counts of 35 of the 40 tested species over the 3 years of the study. Major reductions occurred by year 2 for members of the genera *Actinomyces*, *Capnocytophaga*, *Campylobacter*, *Fusobacterium* and *Prevotella*. These changes were reflected in the percentage of sites colonized at $> 10^5$ at the various time points (Fig. 5). Our findings confirm the 6-month microbiological effects reported in periodontal maintenance subjects using manual or powered toothbrushes (Haffajee et al. 2001b). In that study, significant reductions were observed for *Actinomyces* species, *V. parvula*, *Peptostreptococcus micros* and *P. intermedia/nigrescens,* indicating a dramatic effect of supragingival plaque control on the subgingival microbiota. These species were also reduced significantly in the 3 years of the present investigation. Furthermore, *T. forsythia* as well as counts of all the orange complex species were significantly decreased. Socransky et al. (1998) described a possible microbial succession of the subgingival microbial communities, and highlighted the rarity of finding red complex species in the absence of orange complex species. As levels of red complex species in healthy or minimally diseased subjects are low (Haffajee et al. 1998, van Winkelhoff et al. 2002, Yang et al. 1994), and orange complex species may play a role in the colonization of these species, targeting the orange complex in this population would appear to be a reasonable goal. The results of microbiological studies are quite encouraging and stress the well-documented clinical benefits of maintaining a high level of oral hygiene (Suomi et al. 1971, Soderholm et al. 1982, Albandar et al. 1994, Haffajee et al. 2001b, Axelsson et al. 2004).

The data of the present study extended the findings of earlier reports by following periodontally healthy and minimally diseased subjects for the longer time period of 3 years. The results indicated that the favourable microbiological changes found in other studies in time periods of up to 9 months following meticulous supragingival plaque control could be maintained for longer periods. Our findings are in accord with the hypothesis that the effect of supragingival plaque control on subgingival biofilms occurs as a result of a ''change in habitat'' (Ximenez-Fyvie et al. 2000a) with decreased inflammation and gingival crevicular fluid flow, resulting in lower levels of nutrients for subgingival organisms (Daly & Highfield 1996, Ramberg et al. 1996). This was substantiated by the continued decrease in % of sites with BoP over the entire study. Moreover, removal of supragingival biofilm could directly affect the contiguous subgingival plaque as the supragingival bacteria may provide an important source of nutrients for the subgingival plaque (Haffajee et al. 2001b). It was demonstrated in an earlier study that professional supragingival plaque control can have a dramatic effect on the subgingival plaque composition in periodontal patients under maintenance, which would last up to 9 months (Ximenez-Fyvie et al. 2000a). Murray et al. (1989) highlighted the importance of following the microbial changes resulting from supragingival plaque control for longer periods. They reported a reduction in *F. nucleatum* that took a full year to occur, suggesting that in order to affect

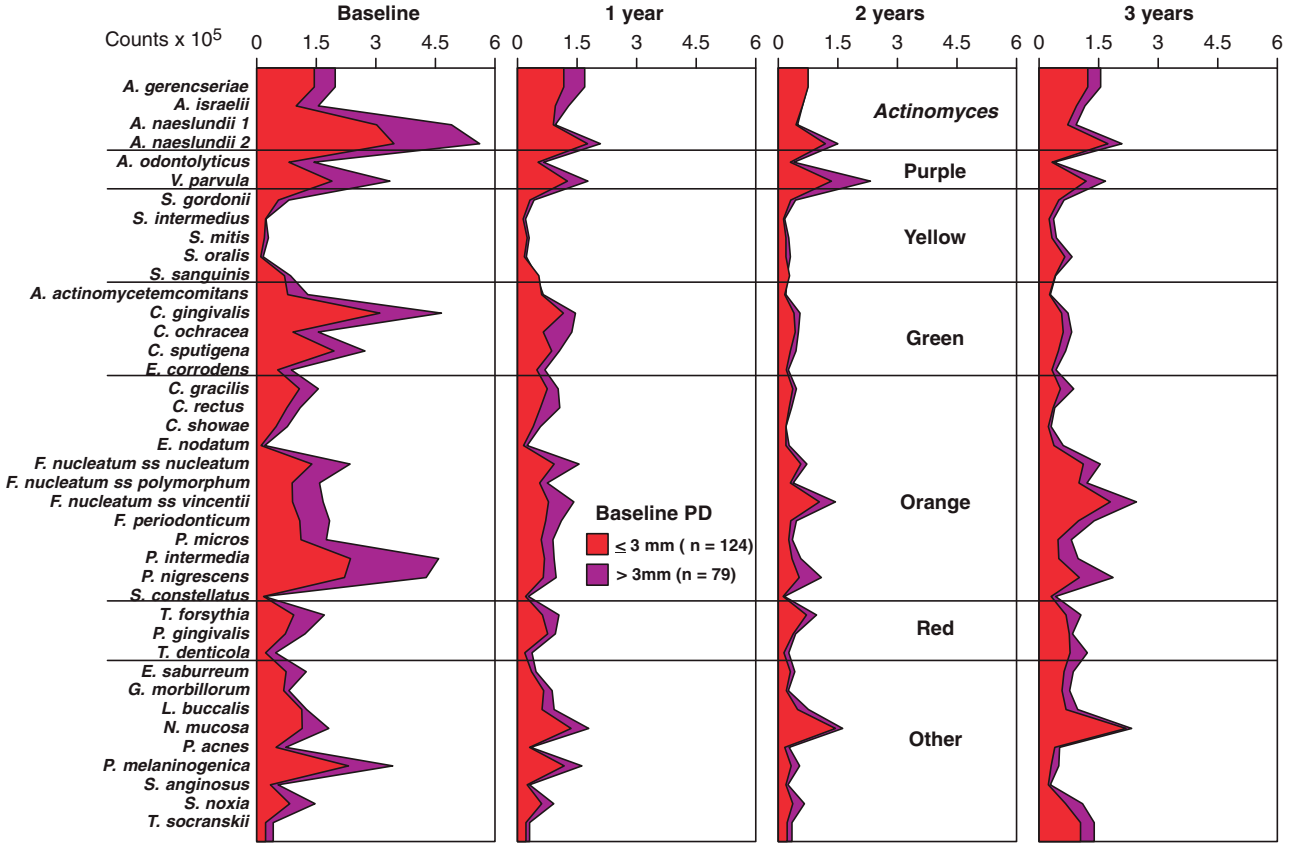

Fig. 8. Mean counts ($\times 10^5$) of 40 species in samples from sites with baseline pocket depths of $\leqslant 3$ and >3 mm for all subjects combined at baseline, 1, 2 and 3 years. The data for the baseline, 1-, 2- and 3-year visits were subset according to their baseline pocket depth, not their pocket depth at years 1, 2 and 3. The mean counts of each species were computed by averaging the data for each baseline pocket depth category separately in each subject, and then averaging across subjects at each time point separately. Significance of differences between pocket depth categories was determined using the Mann–Whitney test; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ after adjusting for multiple comparisons (Socransky et al. 1991). The species were ordered and grouped according to the complexes described by Socransky et al. (1998).

Table 1. Stepwise forward multiple linear regression relating the outcome variable, change in mean pocket depth from baseline to 3 years, to baseline mean counts of 40 bacterial species, mean baseline pocket depth, age and smoking status

	Coefficient	SE	*p*
Intercept	0.5513		
Baseline pocket depth	– 0.4164	0.1049	0.000135
Actinomyces gerencseriae	0.0421	0.0176	0.018565
Treponema socranskii	– 0.2165	0.1248	0.085834
Age	0.0074	0.0029	0.012154
Streptococcus mitis	0.7864	0.2228	0.000625
Campylobacter rectus	– 0.0913	0.0386	0.019863
S. oralis	– 0.5363	0.2885	0.065947

Analysis was terminated when the *p* value of an entering variable was >0.10.
Overall *p* value = 0.000003.

some members of the subgingival microbiota, the ecological alterations brought about by supragingival plaque control might have to persist for a long time. Based on these and other studies, the reduction in counts of most species in the subgingival microbiota was anticipated for the first year. However, we hypothesized that the levels of most, if not all, species would level off after the initial improvement. One factor that might have led to this sustained effect was the well-known Hawthorne effect; that is, when a subject is involved in a clinical study, the subject has a tendency to improve whether he or she receives the test or placebo therapies. In this study, this may have involved more intensive self-administered home care because the subject knew that he or she would be monitored for plaque levels and other parameters. A second factor may have been the minimal disease status of the subject population. The close proximity of the supra and subgingival habitats in the vast majority of sites with shallow sulci may have intensified the effect on the subgingival microbiota. However, supragingival professional cleaning can decrease the subgingival levels of periodontal pathogens even in moderately deep pockets of 4-5 mm (McNabb et al. 1992).

The levels of most species were quite low in the subgingival samples at baseline, reflecting the relative periodontal health of our population. No species exhibited mean counts for the entire population above 4×10^5 and 80% of all species had mean levels below 2×10^5. Hence, it was surprising that these numbers were consistently reduced in both populations, and in

Table 2. Stepwise forward multiple linear regression relating the outcome variable, change in mean pocket depth from baseline to 3 years, to change in mean counts of 40 bacterial species from baseline to 3 years, after adjusting for mean baseline pocket depth

	Coefficient	SE	*p*
Intercept	0.8887		
Baseline pocket depth	− 0.4260	0.1049	0.000098
Actinomyces odontolyticus	− 0.1045	0.0359	0.004500
Fusobacterium. periodonticum	0.0670	0.0222	0.003259
Streptococcus constellatus	− 0.4221	0.1211	0.000737
S. oralis	0.1018	0.0463	0.030468
Campylobacter rectus	0.0713	0.0328	0.032063

Analysis was terminated when the *p* value of an entering variable was >0.10.
Overall *p* value = 0.000004.

both pocket depth categories for up to 2 years. In fact, the 2-year data demonstrated that only 3 species had mean levels above 1×10^5. The mean total DNA probe count was reduced from 37.1×10^5 to a value of 15.4×10^5 by the second year of the study. This value might represent the lowest practically achievable level for subgingival bacteria that may be obtained with common preventive measures. Data from a study investigating early biofilm formation revealed that the amount of bacterial DNA that could be recovered from a polished tooth surface, immediately after the tooth was pumiced, was 4.6×10^5 (Li et al. 2004). Further, recent data from an ongoing study in our department were in accord with these findings. In that study, subgingival plaque samples were obtained from up to 28 teeth of 36 periodontally healthy subjects immediately after full-mouth prophylaxis. The mean total DNA probe count was 4.5×10^5. Thus, in the current investigation, patient-initiated preventive procedures with regular (6 month) professional maintenance were able to reduce plaque levels for prolonged periods of time that were only three to four times greater than those achieved immediately after meticulous supragingival plaque removal.

There was a tendency for plaque levels to increase in many subjects at the 3-year monitoring visit. This trend was true not only for the total DNA probe count, but for most of the test species also (Figs 1 and 3). Nevertheless, only 10 species had exceeded mean baseline levels at 3 years including *N. mucosa*, *F. nucleatum ss vincentii*, *F. nucleatum ss polymorphum*, *T. denticola*, *T. socranskii*, *S. intermedius*, *S. mitis*, *S. constellatus*, *E. nodatum* and *S. oralis*. Despite the increase in mean counts of several species between years 2 and 3, the clinical parameters continued to show improvement, with further reductions in mean PPD and mean % of BoP. The percentile plot of the mean PPD values presented in Fig. 6 demonstrated that this trend was consistent throughout the subject distribution. On the other hand, the percentile plots of three representative sample species in Fig. 6 illustrated the tendency for increased colonization by these species in at least 75% of the subjects. This tendency for re-colonization at 3 years was observed in samples from sites with initially ''shallower'' and ''deeper'' pockets (Fig. 8). It is tempting to speculate that, if followed for a longer period of time, the clinical benefits attained through the prevention programmes would also show signs of relapse. Our data are in accord with the hypothesis that increases in subgingival species lead to an inflammatory response, which in turn leads to the clinical deepening of the gingival sulcus. Indeed, there was a statistically significant increase in several members of the orange complex (primarily members of the genera *Fusobacterium*) and in *P. gingivalis* and *T. denticola* between years 2 and 3 (data not shown), which may eventually lead to ''active disease''. The parallel increase in the % of sites with visible plaque and the total DNA probe count in the last year of the study suggested that the re-colonization of subgingival species was likely the result of a decrease in the quality of home care by the participants. Less effective home care procedures would reverse the beneficial ecological alterations obtained through the prevention programmes, returning the subgingival microbiota to the original climax community.

The factors that might have affected mean pocket depth change from baseline to 3 years, in individual subjects, were examined using multiple linear regression. The most important factor, as described in this and a previous paper (Bogren et al. 2006), was baseline pocket depth, with the subjects exhibiting the greatest baseline mean pocket depth being more likely to exhibit pocket depth reduction. Conspicuously absent from the species that related to pocket depth change in these minimally diseased subjects were the members of the infamous red complex. Rather, reductions in the orange complex species, *F. periodonticum* and *C. rectus* as well as *S. oralis* were most strongly related to pocket depth reduction.

The results of the current investigation clearly demonstrated that the clinical improvements obtained through preventive measures aimed at supragingival plaque control were accompanied by shifts to a more host-compatible subgingival microbiota. This effect was anticipated in the 1-year data as our group and others have reported such changes. However, the extent and, more importantly, the duration of the shifts in the composition of the subgingival biofilm were surprising. Our findings also suggested that a return of the subgingival microbiota to baseline levels might precede clinical changes at the gingival margin. The subgingival biofilm was clearly returning to its original composition by 3 years, despite continued clinical improvements. A decrease in the efficiency of plaque control in the last year of the study was evidenced by an increase in % of sites with plaque and an increase in the mean total DNA probe count. This may initiate an inflammatory process in the gingiva, leading to environmental changes such as an increase in the gingival crevicular flow, which could foster further growth of pathogenic species. Nonetheless, the present study demonstrated that well-performed patient-initiated prevention procedures can lead to both clinical and microbiological improvements for prolonged periods of time in periodontally healthy subjects and those with minimal periodontal disease.

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Clinical Relevance

Scientific rationale for the study: Limited information exists regarding changes in clinical and microbiological parameters that may occur over time in subjects who are periodontal healthy or exhibit minor signs of gingivitis and are exposed to preventive measures.

Principal findings: Clinical improvements obtained by a 3-year prevention programme were followed by shifts in the subgingival microbiota.

Practical implications: Adult individuals with gingivitis may benefit in terms of improved periodontal health and reduced bacterial load from regularly performed prophylaxis.