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The effect of a chlorhexidine regimen on de novo plaque formation

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Abstract

Objective: To evaluate the effect of a pretreatment regimen that combined meticulous mechanical tooth cleaning with the daily use of chlorhexidine (rinse, gargle and tongue application) on de novo plaque formation and on the recolonization of various microbiological species in plaque and saliva during a 4-day period of no oral hygiene.

Material and Methods: Ten subjects aged 24–36 years with gingivitis were recruited. The study was designed as a double blind cross-over clinical trial including two phases. Each experimental phase comprised one *preparatory period* of 7 days and one *plaque accumulation period* of 4 days. During the *preparatory period*, the volunteers (i) performed meticulous mechanical tooth cleaning using toothbrush and dentifrice and (ii) were, in addition, given two sessions of professional tooth cleaning (PTC) The final PTC was delivered after bacterial sampling had been made on Day 0. In the Control group, no additional plaque control measures were included. In the Test group, the participants in addition to the mechanical measures (i) rinsed twice daily, for 60 s each time with a 0.2% chlorhexidine solution, (ii) gargled twice daily for 10 s with the chlorhexidine preparation, and finally (iii) brushed the dorsum of the tongue for 60 s, twice daily, with a 1.0% chlorhexidine gel. During the 4-day plaque accumulation period, the participants abstained from all mechanical and chemical plaque control measures. On Days 0, 1, 2 and 4 the quantity and quality of plaque formed was assessed by clinical means and by DNA probe techniques. The microbiota of the saliva was studied in samples obtained on Days 0 and 4.

Results: It was demonstrated that chlorhexidine used as a mouthrinse combined with gargling and tongue application during the preparatory period significantly retarded the amount of plaque that formed on tooth surfaces during the following 4 days of no oral hygiene. Further, the number of microorganisms present in the biofilm representing Days 0, 1 and 2 of the ''plaque accumulation period'' was apparently affected by the use of the antiseptic. Among the microorganisms influenced by the chlorhexidine regimen, a substantial number belonged to the genus *Actinomyces*. It was also observed that the adjunctive use of chlorhexidine reduced the number of bacteria present in saliva at the end of the preparatory period (i.e. on Day 0). After 4 days of no oral hygiene, the microbiota of the newly formed plaque in the Test and Control groups had many features in common.

Conclusion: Habitat is critical in controlling the bacterial composition of the dental biofilm. The microbiota will tend to go back to the one that is characteristic of a given subject, once chemical antimicrobial means are withdrawn.

Key words: checkerboard DNA–DNA hybridization technique; microorganisms; plaque formation

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A number of different antiseptic substances have been incorporated in mouthrinses and dentifrice preparations to improve the outcome of mechanical oral hygiene procedures. One of the most frequently used compounds, chlorhexidine, is a broad spectrum antiseptic with pronounced antimicrobial effects both on Gram-positive and Gram-negative bacteria as well as on fungi and some viruses. Chlorhexidine is a positively charged bisbiguanide, which can

adsorb to different negatively charged sites in the oral cavity including the mucous membranes and salivary proteins. Further, chlorhexidine binds to several components of the biofilm on the tooth surfaces such as bacteria, extracellular polysaccharides and glycoproteins (e.g. Rølla et al. 1970, Davies 1973).

Due to the high affinity of chlorhexidine to oral surfaces, elevated levels of the compound can be detected in saliva for several hours after a single dose. Thus, chlorhexidine in a mouthrinse (0.12% or 0.2% solution) can be administered at 12 h intervals and retain its ability to retard/prevent plaque formation (Bonesvoll et al. 1974, Lang et al. 1982).

At low concentrations, chlorhexidine causes damage to the cell membrane (Jones 1997) and low-molecular-weight molecules escape from the microorganisms. At higher concentrations, chlorhexidine causes precipitation and coagulation of the proteins in the cytoplasm of the exposed microbes.

Gram-positive microorganisms are generally more sensitive to chlorhexidine than Gram-negative species. There are no reports of acquired resistance even after long-term use of chlorhexidine in man (Briner et al. 1986a, b, 1989). Briner et al. (1989) studied the effect of a 2-year use of 0.12% chlorhexidine mouthrinse on plaque bacteria. The authors concluded that the regimen (i) reduced several species associated with gingivitis and periodontitis, such as members of the genera *Actinomyces*, *Veillonella* and *Fusobacterium* but (ii) caused no change in the resistance of oral bacteria to chlorhexidine.

The aim of the present study was to evaluate the effect of a regimen that combined a chlorhexidine rinse and chlorhexidine gel administration on the recolonization of various microbiological species in plaque and saliva during a 4-day period of de novo plaque formation.

Material and Methods

Ten subjects aged 24–36 years, with good general heath and no signs of destructive periodontal disease were recruited. They had a minimum of 24 teeth and had not used systemic antibiotics during the last 6 months. All subjects were informed about the study protocol and signed written informed consent forms. The study was approved by the local human investigational review board.

Screening examination

A screening examination disclosed that all 10 volunteers exhibited signs of gingivitis at different locations in the dentition. They were each given a case presentation, and instructed in proper self-performed plaque control procedures. In addition, they were given a series of supragingival debridement procedures including professional mechanical tooth cleaning (PTC, Axelsson & Lindhe 1976). The PTC was repeated once every 3 days during a 2-week period to establish and maintain healthy gingival conditions until the start of the preparatory period (see below).

Study design

The study was designed as a double-blind cross-over clinical trial including two phases.

Each experimental phase comprised one *preparatory period* of 7 days and one *plaque accumulation period* of 4 days.

Preparatory period

During the *preparatory period*, the volunteers (i) performed meticulous mechanical tooth cleaning using toothbrush (Colgate Precision®; Colgate Palmolive, Piscataway, NJ, USA) and dentifrice (Colgate Protection Caries®) and (ii) were, in addition, given two sessions of PTC. The final PTC was delivered after bacterial sampling had been made on Day 0 (see below).

Control group. No additional plaque control measures were included.

Test group. In addition to the mechanical regimen (see above), the participants (i) rinsed twice daily, for 60 s each time with the 0.2% chlorhexidine solution (Hexident®, Ipex, Sweden), (ii) gargled twice daily for 10 s with the same chlorhexidine preparation, and finally (iii) brushed the dorsum of the tongue for 60 s, twice daily, with a 1.0% chlorhexidine gel (Corsodyl Gel®, GlaxoSmithKline, Sweden).

Plaque accumulation period

During this 4-day interval, the participants abstained from all mechanical and chemical plaque control measures.

Wash-out period

Following the end of a plaque accumulation period, the participants received a new PTC and were instructed to reinstitute mechanical self-performed plaque control. Further, once every third day during the subsequent 10 days PTC was delivered to the participants by a dental hygienist.

Clinical measurements

Dental plaque

The dentition was disclosed with erythrosin (Diaplac®, Nordenta AB, Sweden) and plaque was scored at the disto-, mid-, mesio-buccal and disto-, mid-, mesio-lingual surfaces of each tooth according to the criteria of the Turesky modification of the Quigley and Hein Plaque Index (Quigley & Hein, 1962, Turesky et al., 1970).

Microbiological sampling and analysis

Plaque samples

The supragingival plaque samples were taken at Day 0 (immediately prior to the last PTC session) and after 1, 2 and 4 days of no oral hygiene. The samples were obtained from the following tooth surfaces:

Day 0: mesio-buccal surfaces of 14, 24, 34 and 44;
Day 1: disto-buccal surfaces of 14, 24, 34 and 44;
Day 2: mesio-lingual surfaces of 14, 24, 34 and 44;
Day 4: disto-lingual surfaces of 14, 24, 34 and 44.

Each sample was harvested with a sterile curette and the pooled samples from each examination interval were placed into a separate Eppendorf tube (Sarstedt, Germany) containing 150 μl of Tris EDTA buffer (TE: 10 mM Tris-HCL, 1 mM EDTA, pH 7.6). To each sample, 100 μl of 0.5 M NaOH was added and the suspensions boiled in water for 5 min. The samples were then neutralized with 800 μl of 5 M ammonium acetate. The plaque samples were evaluated using the checkerboard DNA–DNA hybridization technique (Socransky et al. 1994) as described by Sekino et al. (2003).

Saliva samples

Prior to the clinical registrations at Days 0 and 4, a volume of 0.2–0.5 ml of saliva was collected in a sterile Eppendorf microcentrifuge tube. To a new Eppendorf tube containing 150 μl of TE, 200 μl of each saliva sample were transferred and mixed for 10 s. Of the resulting suspension, 200 μl were transferred to a fresh Eppendorf tube and 100 μl of 0.5 M NaOH was added and mixed for 10 s. The samples were then

boiled in a water bath for 5 min and neutralized with 800 μl of 5 M ammonium acetate. The samples were then analysed by checkerboard DNA–DNA hybridization as described by Sekino et al. (2003).

Data analysis

Subject mean values were calculated for all clinical and microbiological parameters. Analysis of variance (ANOVA) and the Student–Newman–Keuls (SNK) test were applied to evaluate if there were significant difference between treatments for clinical parameters.

The counts of 40 test species in samples taken from the test teeth and saliva were available for each subject at Days 0, 1, 2 and 4 (of no oral hygiene) for plaque samples and Days 0 and 4 for saliva samples. Significance of differences of species between groups at each time point was sought using the Mann–Whitney test.

Results

Clinical observations

Mean QHI plaque scores at sampled sites

The mean individual QHI scores for the different treatment regimens are presented in Fig. 1. In the Control group, the mean QHI at the sampling sites increased from 1.0 (Day 1) to 1.1 (Day 2) and 1.6 (Day 4) during the 4 days of no oral hygiene. The corresponding QHI scores for the Test group were 0.3, 0.6 and 0.7. At all re-examination intervals, more plaque had formed in the Control than in the Test group ($p < 0.01$).

Microbiological observations using DNA probe technique

Changes in total DNA probe count

Figure 2 presents the total DNA counts in plaque and saliva samples obtained at the different examination intervals. The median DNA counts from *plaque samples* obtained at Days 0, 1 and 2 in the Control group were significantly higher than in samples from the corresponding intervals in the Test group. The median DNA counts per millilitre of saliva were higher in the Control than in the Test group at both the Day 0 and the Day 4 examination intervals. However, the differences were not statistically significant.

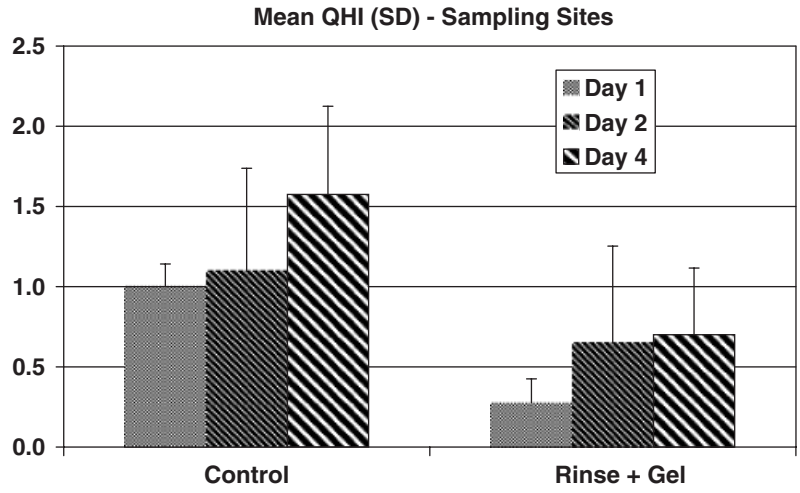

Fig. 1. Bar chart of the mean QHI (SD) of the sampled sites on Days 1, 2 and 4.

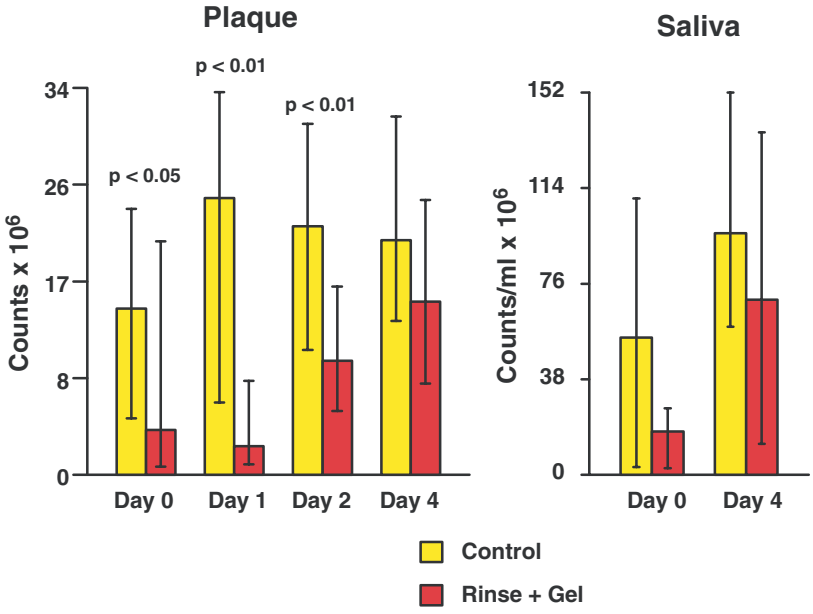

Fig. 2. Bar chart of the median total DNA probe counts in plaque and saliva samples taken at different time points after the test and control periods. The left panel presents the median total DNA probe counts ($\times 10^6$, $\pm$ 95%CI) in plaque samples taken from the 10 subjects at Days 0, 1, 2 and 4 after the two preparatory periods. The right panel is a bar chart of the median total DNA probe counts per milliliter of saliva ($\times 10^6$, $\pm$ 95%CI). The bars represent the median counts and the whiskers indicate the 95% confidence intervals. Significance of differences between groups at each time period was determined using the Mann–Whitney test.

Microbiologic changes in plaque samples

The median counts of the 40 test taxa in the plaque samples at each visit after the preparatory period are presented in Fig. 3. The species are ordered according to the complexes described by Socransky et al. (1998). The numerically most dominant species detected on Day 0 (the last day of the preparatory phase) in the Control group were members of the genus *Actinomyces*. In the Day 0 samples, these species were significantly higher in the Control group than in the Test group. In the Control group, the higher counts of the *Actinomyces* persisted at Days 1 and 2 of no plaque control. By Day 4, the differences in median counts of the *Actinomyces* species between the Control and Test groups had diminished due to a reduction in the counts in the Control group and an increase in the Test group.

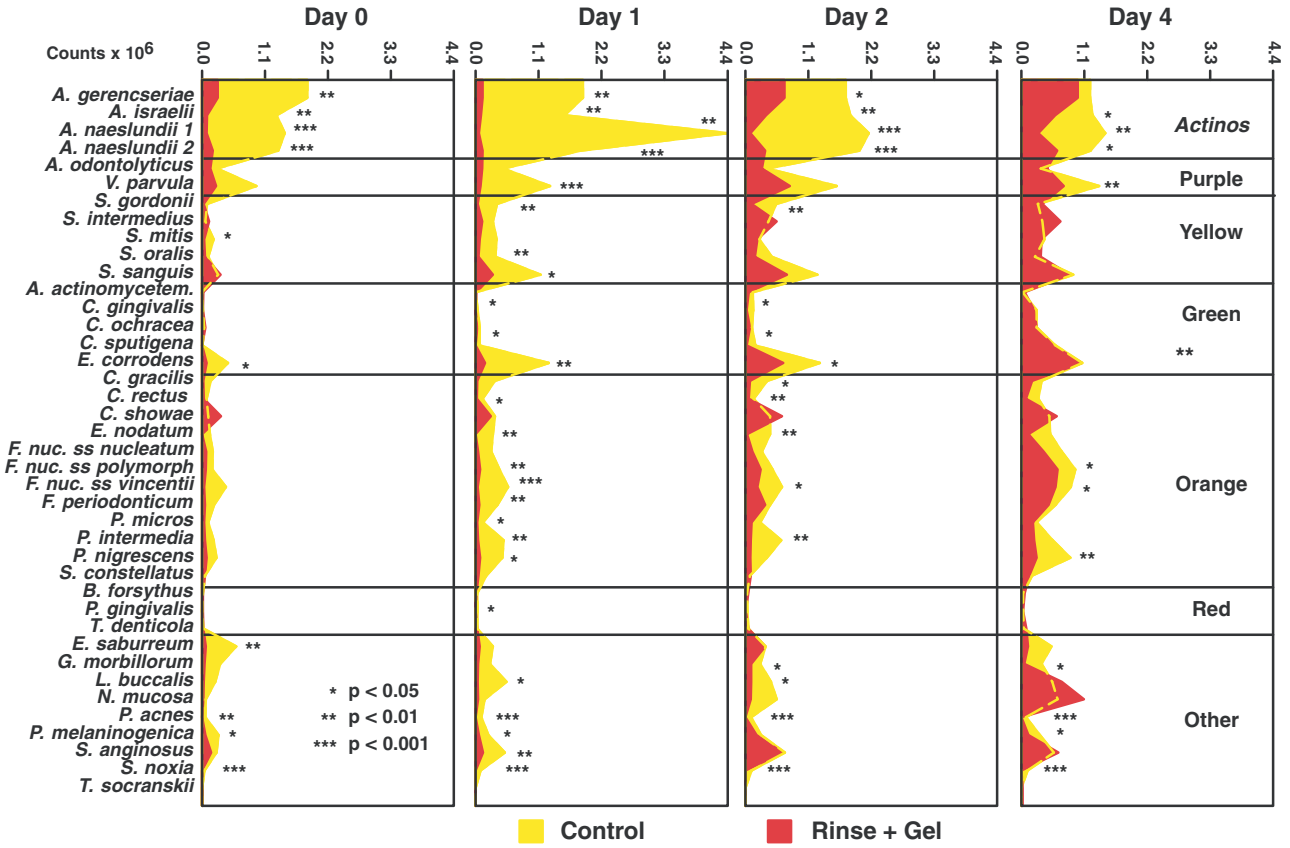

Fig. 3. Microbial profiles of the median counts $\times 10^6$ of the 40 test species in plaque samples taken from the 10 subjects at Days 0, 1, 2 and 4 after the three preparatory periods. Significance of differences between groups at each time period was sought using the Mann–Whitney test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The species have been ordered according to the complexes described by Socransky et al. (1998).

Many other species were lower in median counts in the Test than in the Control group. This was particularly noticeable in samples from Days 1 and 2 (Fig. 3). After 4 days of no oral hygiene, the differences in microbial profiles in the two treatment groups had noticeably diminished although a few statistically significant differences in some of the complexes could still be detected.

Microbiologic changes in saliva samples

The median counts of the 40 test taxa in saliva samples obtained on Day 0 and after 4 days of no oral hygiene in the two treatment groups are presented in Fig. 4. The Day 0 samples exhibited higher levels of *Streptococcus oralis*, *Eubacterium nodatum* and *Fusobacterium nucleatum* ss *nucleatum* in the Control than in the Test group. By 4 days of no oral hygiene, the levels of most taxa had increased in both groups. Significant differences at this time point were observed primarily for species in the ''orange complex'' and ''other'' categories.

Discussion

The results of the present investigation demonstrated that chlorhexidine used as a mouthrinse combined with gargling and tongue application during the preparatory period significantly retarded the amount of plaque that formed on tooth surfaces during the following 4 days of no oral hygiene. Further, the number of microorganisms present in the biofilm representing Days 0, 1 and 2 of the ''plaque accumulation period'' was apparently affected by the use of the antiseptic. Among the microorganisms influenced by the chlorhexidine regimen, a substantial number belonged to the genus *Actinomyces*.

In the current study, it was also observed that the adjunctive use of chlorhexidine reduced the number of bacteria present in saliva at the end of the preparatory period (i.e. on Day 0).

Plaque samples representing different intervals of no oral hygiene were analysed with respect to the presence of a variety of bacterial species that normally occur in the dental biofilm and may be associated with different forms of caries and gingivitis/periodontitis. It was observed that species of *Actinomyces*, early colonizers of plaque (Socransky et al. 1977, Syed et al. 1978, Moore & Moore 1994) occurred in much lower numbers in plaque samples from the Test group on Days 0, 1 and 2 than in samples from the corresponding intervals from the Control group. These results are in agreement with findings reported by Briner et al. (1986). They studied the effect of chlorhexidine (0.12%) rinsing, twice daily during a 6-month period, on certain plaque bacteria. The authors reported that the number of *Actinomyces* recorded from samples obtained at the baseline examination was reduced between 81% and 93% during the 6 months of monitoring. However, in samples obtained 3 months after the termination of the rinsing

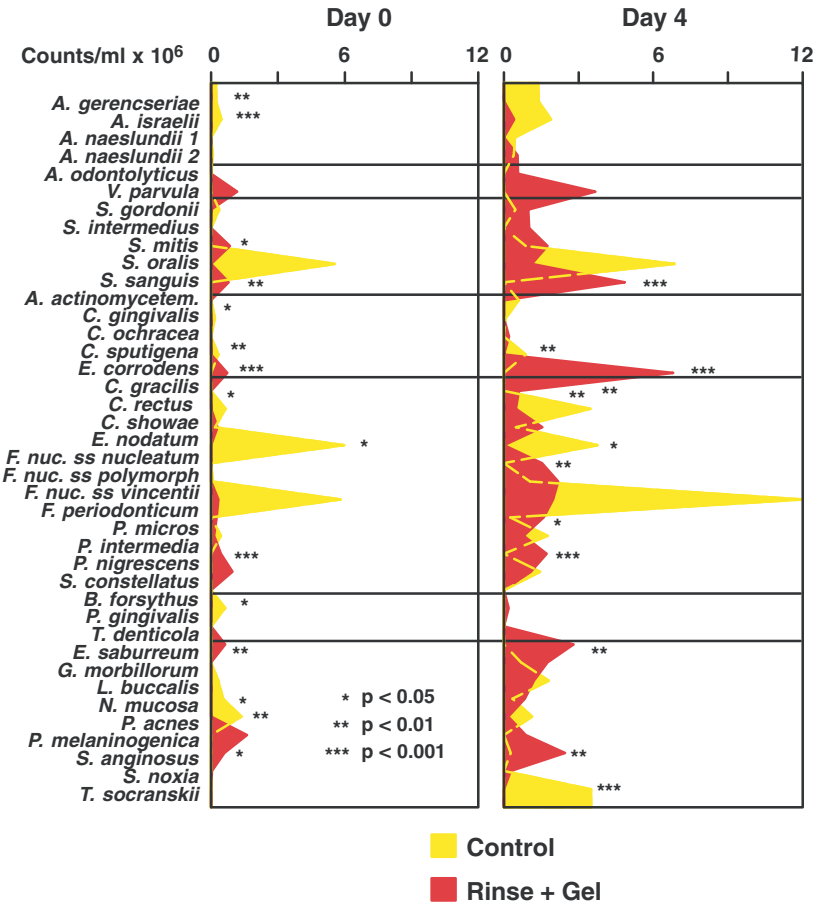

Fig. 4. Microbial profiles of the median counts $\times 10^6$ of the 40 test species in saliva samples taken from the 10 subjects at Days 0 and 4 after the two preparatory periods. Significance of differences among groups at each time period was sought.

regimen, no differences between the placebo and the chlorhexidine groups were found with respect to the number of *Actinomyces* species. The current results are also in line with findings presented by Brownstein et al. (1990) who reported that in subjects who rinsed daily with 0.12% chlorhexidine during a 2-month period there was a significant reduction of the number of *Actinomyces* in plaque samples as compared with subjects who rinsed with a placebo solution.

Findings from the present Control group revealed that (i) the total number of bacteria as well as (ii) the number of *Actinomyces* species present in plaque at all examination intervals during the 4 days of no oral hygiene and in saliva samples representing Days 0 and 4 did not markedly vary over time.

This observation is not in agreement with data presented by Scheaken et al. (1987). They studied the presence of bacteria in plaque and saliva in human volunteers who abstained from oral hygiene procedures. The authors reported that after 7 days of no oral hygiene, the total number of *Actinomyces* species in both plaque and saliva had increased by approximately 2 log units. In this context, it must be realized that the period of no oral hygiene was almost twice as long in the study by Schaeken et al. (1987), compared with the current trial.

Of interest in the present study was the observation that the levels of ''red complex'' species (Socransky et al. 1998), i.e. *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Treponema denticola*, in plaque samples from Days 0 through 4 of no oral hygiene were only minimally altered by the repeated chlorhexidine administration during the preparatory period. This is probably explained by the low levels of these species that occurred in this subject group that exhibited overt gingivitis, but without signs of destructive periodontitis.

The data in the present investigation are in accord with earlier studies that indicated that the use of chlorhexidine as an adjunct to mechanical forms of tooth cleaning led to a lowered accumulation of microorganisms on the tooth surfaces. Examination of the kinetics of bacterial re-population after the withdrawal of the home-care procedures suggested that there was a delay in plaque accumulation after the subjects had received adjunctive chlorhexidine during the preparatory period (Fig. 3). While the total DNA probe counts (Fig. 2) and the counts of specific species (Fig. 3) increased between Days 0 and 1 in the plaque samples from the Control group, there was little change in counts over the corresponding periods in the Test group. However, in the Test group the number of bacteria in plaque increased from Day 1 to Days 2 and 4. Indeed, by Day 4 the total number of bacteria and counts of specific taxa were more similar in the Test and Control groups than at earlier examination intervals. These findings suggest that the marked decrease in plaque bacteria in the Test group towards the end of the preparatory period may have led to a sufficient decrease in salivary bacteria to maintain reduced levels for at least 1 day. Alternatively, the substantivity of chlorhexidine for oral surfaces, particularly tooth surfaces, may have been a major contributor in delaying plaque regrowth. In either event, the data suggest that the study of alternative dosing schedules for chlorhexidine administration to control dental plaque may be a worthy avenue of investigation.

As indicated above the differences in levels of plaque bacteria observed on Day 0 were mirrored by lower counts of salivary bacteria (Figs. 2 and 4). However, the differences in total salivary counts between Test and Control groups were not statistically significant, nor were they as compelling as the differences in the plaque counts. This finding is not fully in accord with data previously published by Dahlén et al. (1984) who in a cross-over study monitored 18 healthy human volunteers who rinsed, 2–4 times daily for 7 days, with either a 0.2% chlorhexidine mouthwash, Ascoxal® (AstraZeneca, Sweden) or with saline. They reported that the total viable count of salivary bacteria detected in samples obtained after 7 days of chlorhexidine rinsing had been reduced by more than 90%.

The observation that at the end of the preparatory period there were somewhat lower counts of bacteria in saliva

in the Test than in the Control group may be related to an observed reduction in the number of organisms in plaque on the mucous membranes or both. The comparatively low number of bacteria in saliva may in turn have played a role in retarding de novo plaque formation. However, the magnitude of the contribution of salivary bacteria to the plaque build-up, could not be evaluated in this clinical trial.

The observations made in the present study indicate that it is possible to decrease the counts of bacteria in both biofilms and in saliva (and by extension, on soft tissues) by the use of antimicrobial agents. The data also indicate that when oral hygiene procedures, whether mechanical or mechanical plus chemical are withdrawn, the microbiota rapidly returns to its ''climax community'' state. In other words, the numbers of the organisms and the complexity of the microbiota increase to a point that may be sustained by the nutrients available in saliva, from the diet and from the gingival crevice fluid. The data also suggest that even though the microbiota is markedly suppressed and even ''distorted'' by administration of an agent such as chlorhexidine, the microbiota rapidly returns to one similar to that observed after suppression of the microbiota by mechanical means only. This reinforces the recognition that habitat is critical in controlling the bacterial composition of the dental biofilm and that the microbiota will tend to go back to the one that is characteristic of a given subject, once antimicrobial means are withdrawn. Apparently, making major shifts in the microbiota over short periods of time (such as the 7 days of preparation) is not adequate to make sustained changes in the dental ecosystem. This reinforces the need for more research on methods to alter the microbiota in a more sustainable fashion and the requirement for careful home care on the part of the patient until more easily sustained methods of microbial alterations can be devised.

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