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# Bacteriology of Experimental Gingivitis in Young Adult Humans

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From replicate trials of experimental gingivitis in four periodontally healthy subjects, 166 bacterial species and subspecies were detected among 3,034 randomly selected isolates from 96 samples. Of these bacteria, *Actinomyces naeslundii* (serotype III and phenotypically similar strains that were unreactive with available antisera), *Actinomyces odontolyticus* (serotype I and phenotypically similar strains that were unreactive with available antisera), *Fusobacterium nucleatum*, *Lactobacillus* species D-2, *Streptococcus anginosus*, *Veillonella parvula*, and *Treponema* species A appeared to be the most likely etiological agents of gingivitis. Statistical interpretations indicated that the greatest source of microbiological variation of the total flora observed was person-to-person differences in the floras. The next greatest source of variation was the inflammatory status of the sample sites. Person-to-person differences were smallest at experimental day 4. The floras became more diverse with time and as gingivitis developed and progressed. Analyses indicated that sequential colonization by certain species was repeatable and therefore probably predictable. Variation was relatively small between replicate trials, between two sites on the same teeth sampled on the same day, and between the same sites sampled at the same relative time in a replicate trial.

Experimental gingivitis in humans (13) is a well-established model for studying the development of gingival inflammation (9, 11, 14, 17, 24, 25, 27). It offers an important basis for indicating the bacterial etiology of gingivitis. Causative relationships between particular species and gingivitis have not been established, however, partly because of the complexity of the flora and partly because of the incomplete information concerning the bacterial species present. Early studies, limited to morphological assessments of stained smears, associated gingivitis with a change from a gram-positive flora to a more complex flora including gram-negative and spiral forms (25). One previous cultural study (14, 24) of the interproximal plaque flora of samples collected from 25 subjects at days 0, 7, 14, and 21 partially characterized the isolates and placed them in 29 groups or species. They reported a shift from a *Streptococcus*-dominated plaque to an *Actinomyces*-dominated plaque with increasing time of supragingival plaque accumulation. Developing gingivitis was associated with increased numbers of *Actinomyces israelii*, and gingivitis with bleeding was associated with the presence of *Actinomyces viscosus* and non-saccharoclastic strains of pigmenting bacteroides that probably now would be identified as *Bacteroides gingivalis*.

The purpose of the present study was to enumerate the bacterial flora of the gingival sulcus in experimental gingivitis in adults, as completely as is now possible; to evaluate the predictability of flora changes in a given site; to determine the significance of sample time, tooth site, and person variation; and to attempt to correlate the observed changes in the flora with deterioration of gingival health.

## MATERIALS AND METHODS

**Subjects.** Four Caucasian males, ages 22, 22, 23, and 31 years, participated as subjects in this study. They were medically healthy by history and received no medications during the course of the study nor any antibiotics for 2 months preceding the study. There were no instances of probeable gingival sulcus depth greater than 3 mm, nor had any subject received periodontal surgery.

**Experimental gingivitis.** Before the study, all subjects were given a dental prophylaxis (scaling and polishing of the teeth) and were instructed in oral hygiene procedures. On day 0, all teeth were again polished, and the established model (13) was instituted. Each subject refrained from all efforts at tooth cleaning for 26 days. No type of mouthwash, mechani-

TABLE 1. Sample site[a] schedule for both replicates of experimental gingivitis in adults

| Day | Person 1 | Person 2 | Person 3 | Person 4 |
|---|---|---|---|---|
| 4 | 6F, D | 4F, M | 3F, M | 6F, D |
| | 27F, D | 30F, D | 27F, D | 29F, D |
| 11 | 3F, M | 3F, M | 6F, D | 4F, D |
| | 30F, M | 27F, D | 29F, M | 30F, M |
| 26 | 2F, M | 6F, D | 4F, M | 3F, M |
| | 31F, M | 29F, M | 19F, M | 27F, D |

[a] Military tooth numbering system 1-32, D = distal, F = facial, M = mesial.

cal cleaning, or systemic medication was permitted. Diet was not monitored. Samples for bacteriological study were obtained on days 4, 11, and 26. Whole-mouth gingival index (GI) (12) and plaque index (19) scores were determined on each sample day. All scores were determined by the same examiner. Exact replicability of GI scores by this examiner in a separate standardization trial was 82%, with a zero incidence of replicate scores differing by more than 1. After the day 26 sample, the teeth were scaled and polished, oral hygiene instructions were repeated, and oral hygiene procedures were reinstituted. After at least 1 month, the entire experiment was repeated for each subject.

**Sample sites.** On each of the three sample days in each replicate experiment in each subject, two samples were obtained (one facial, one proximal) from each of two teeth (one mandibular, one maxillary). Sampling of canine, premolar, and molar sites was distributed among subjects so that no sample time (day) was restricted to a single tooth type. Surfaces sampled had no carious lesions or restorations. To ensure that no interference with plaque development occurred, a different tooth surface was sampled each time within each experimental trial (replicate), and no proximal sample site was immediately adjacent to any other sample site. The sample sites are identified in Table 1. The same sites were sampled in each replicate trial. There were 24 facial and 24 proximal (mesial or distal) sites sampled in each trial.

**Sampling.** The teeth were isolated with cotton rolls, and the sample sites were gently dried with sterile cotton swabs. The accumulated supragingival plaque at the sites to be sampled was removed as completely as possible with sterile toothpicks. The sample to be studied was then obtained from the plaque approximating the gingival margin and in the sulcus by making a single pass with a nickel-plated Morse 00 scaler in light contact with the tooth surface from the depth of the sulcus to just coronal to the gingival margin. Within 4 s, the scaler tip was transferred aseptically into a tube (12 by 75 mm) containing 1.5 ml of prereduced anaerobically sterilized diluent (two parts chopped meat broth, three parts salts solution [7]) and ca. 0.05 g of 110- to 150-μm-diameter glass beads. Each tube was flushed with oxygen-free $CO_2$ from a sterile cannula while it was open. The restoppered tubes were immediately taken to the laboratory for processing.

**Initial cultures.** Culture work was initiated within 5 to 20 min of sampling. The specimen on the scaler tip was dispersed in the dilution broth by vigorous shaking with the glass beads on a Vortex mixer for 5 to 10 s. Serial 10-fold dilutions were made in salts solution-gelatin diluent (7) under anaerobic gas containing 3% $H_2$, 12% $CO_2$, and 85% $N_2$. Duplicate plates and roll tubes were inoculated with four consecutive dilutions. The dilutions to be cultured were determined by rough estimates of the microscopic count in the original dispersed suspension. Separate tubes and plates were inoculated for isolation of spirochetes, mycoplasma, and *Campylobacter sputorum*.

The plates in anaerobe jars and the roll tubes were incubated under the same gas mixture for 5 days before the colonies were counted and picked. Spiral lines were drawn on the bottoms of the plates and around the roll tubes to aid in counting under ×12 magnification. Total colony counts were determined from plates and tubes that contained between 30 and 300 colonies. Colonies that touched each other and had the same morphology were counted as one.

To obtain a representative cross-section of the population, 15 well-isolated colonies were picked in sequence, regardless of size or morphology, from a suitable plate, and 15 were picked from a roll tube. Only colonies that were so close together as to probably yield mixed cultures were intentionally omitted. If there was a 10-fold or greater difference in the plate or roll tube count, 30 colonies were picked from the one giving superior counts. Cells from colonies were inoculated into isolation broth medium under oxygen-free $CO_2$ and incubated until growth was apparent.

If no visible growth was obtained in 48 h, Gram stains were made and portions of the broth were transferred to several media under different gas phases. Depending on cellular morphology, enrichment media contained fumarate and formate, pyruvate, IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.), gelatin, rumen fluid, pectin, nitrate, serum, or thiamine pyrophosphate, and cultures were incubated in air or air enriched to 5% with $CO_2$, 10% $CO_2$ in $N_2$, or $N_2$.

When growth was apparent, each original broth culture was gram stained and restreaked to a suitable medium, and all distinct colony types were picked for analysis. If two or more bacterial species were present in any original broth culture, all were isolated and identified.

**Identification.** Each pure culture was Gram stained and subcultured to brain heart infusion broth supplemented with yeast extract, heme, vitamin K, and $CaCO_3$ (7, 15) and any other additives known to be required by the isolate. Soluble proteins of all isolates were anlayzed electrophoretically (15). Isolates with similar morphology were placed on the same gels. Representative strains of each electrophoretic pattern group that was not clearly identical were tested for all characteristics listed previously (7). Routine tests for all strains characterized included chromatographic analysis of fermentation products and hydrogen, growth and pH in 30 media, and tests for indole, esculin hydrolysis, catalase, protein digestion, gas production, motility, aerotolerance, ammonia production, and nitrate reduction. These tests were supplemented with tests for flagellar arrangement, pigmenta-

tion on blood agar medium, inhibition by bile, metabolism of threonine, $NH_3$ from arginine, lactate or pyruvate metabolism, bile-esculin reaction, coagulase, inulin fermentation, salt tolerance, hemolysis, antibiotic susceptibility, hippurate hydrolysis, heat resistance, or growth in differential media as required for differentiation of species in several genera. All results that differed from those of previously defined species or were questionable were verified with repeat tests.

Strains of actinomyces were tested for fluorescent-antibody staining with monovalent conjugates prepared for the following serotypes: *A. israelii* I and II; *Actinomyces naeslundii* I, II, and III; *Actinomyces* strain W. Va. 963; *Actinomyces bovis* I and II; *A. viscosus* II; *Actinomyces meyeri*; and *Actinomyces odontolyticus* I and II.

The recorded reactions of each species were compared with those of the type or reference strains of the species, and the electrophoretic patterns of soluble proteins were compared by matching cut photographs of the patterns with those of the type strains and reference strains.

**Media.** Most media contained (per 100 ml) 0.5 mg of hemin, 0.1 mg of vitamin $K_1$, 0.05 g of cysteine-hydrochloride, and 0.25 mg of resazurin (7). Most isolation media also contained yeast autolysate, sterile serum, and thiamine pyrophosphate. Yeast autolysate was prepared by incubating 1 ounce of Fleishman's yeast powder in 100 ml of water at 56°C for 72 h. The mixture was clarified by filtering through cheesecloth and filters of decreasing pore size until it would pass through a 0.2-μm pore size filter (Sartorius Inc., San Francisco, Calif.) for sterilization. Nonhemolytic serum from mature rabbits was purchased from Pelfreeze, Little Rock, Ark., sterilized by passage through a 0.2-μm pore size filter (Sartorius), and inactivated at 56°C for 30 min. The stock thiamine pyrophosphate (TPP) solution contained 0.5 g of TPP in 100 ml of distilled water and was filter sterilized. Fifty milliliters of sterile serum and 50 ml of yeast autolysate were mixed, and TPP was added (4 ml of TPP solution for treponemes and 0.5 ml of TPP solution for other isolation media). One milliliter of the serum-yeast autolysate-TPP mixture was added per 10 ml of basal medium unless specified otherwise.

**Roll tube isolation medium.** Nonselective D-4 isolation medium was prereduced and anaerobically sterilized and contained 3.7 g of brain heart infusion broth base (BBL Microbiology Systems), 0.5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.5 ml of 6% (wt/vol) ammonium formate solution, resazurin, and 100 ml of distilled water. After the medium was boiled and cooled under oxygen-free $CO_2$, cysteine, vitamin $K_1$, and hemin were added. The pH was adjusted to 7.0, and the broth was dispensed under oxygen-free $N_2$ (12 ml per tube [25 by 147 mm] containing 0.3 g of agar). The stoppered tubes in racks were autoclaved in presses at 121°C for 10 min with fast autoclave exhaust. Before sample collection, the agar medium in roll tubes was melted and cooled to 54°C, and 1.2 ml of the serum-yeast autolysate-TPP mixture was added to each tube under $N_2$–$CO_2$–$H_2$ (85:12:3) gas. The tubes of molten agar were inoculated under the anaerobic gas, restoppered, spun and chilled until the agar solidified, and incubated at 37°C.

**Nonselective blood agar plates for isolation.** Medium for plates had a composition similar to D-4 roll tube media, except 5.2 g of brain heart infusion agar (BBL Microbiology Systems) replaced the brain heart infusion broth base and agar, 4.0 ml of defibrinated rabbit blood replaced the serum, and resazurin was omitted. The basal medium was autoclaved at 121°C for 15 min, cooled to 56°C, and blood, yeast autolysate, TPP, vitamin K, and hemin were added. Plates were poured in an aerobic atmosphere and stored at room temperature in anaerobe jars with a gas mixture containing 10% $CO_2$ and 90% $H_2$.

**Subculture broth.** Colonies were picked to prereduced anaerobically sterilized D-5 broth which contained 3.7 g of brain heart infusion broth base (BBL Microbiology Systems), 0.5 g of yeast extract (Difco), 0.05 ml of pyruvic acid, 0.5 ml of ammonium formate (6% [wt/vol] in water) solution, 0.2 g of pectin, 2 ml of IsoVitaleX (BBL Microbiology Systems), 1.0 g of agar, 0.1 g of $KNO_3$, resazurin, and 100 ml of distilled water. After the basal medium was boiled and then cooled under $CO_2$, cysteine, vitamin $K_1$, and hemin were added. The pH was adjusted to 7.0 with 1 N NaOH. The medium was dispensed under $O_2$-free $N_2$ (3 ml per tube) and sterilized. Before use, 0.3 ml of sterile serum-yeast autolysate-TPP solution was added aseptically to each tube.

***Treponema* isolation medium.** Oral treponeme isolation (OTI) medium contained 5.0 g of peptone (Difco), 5.0 g of heart infusion broth base (BBL Microbiology Systems), 5.0 g of yeast extract (Difco), 0.8 g of ribose, 0.8 g of pectin, 0.8 g of glucose, 0.4 g of fructose, 0.8 g of starch, 0.4 g of sucrose, 0.8 g of maltose, 0.8 g of sodium pyruvate, 2.0 g of $K_2HPO_4$, 5.0 g of NaCl, 2.0 g of $(NH_4)_2SO_4$, cysteine-hydrochloride, hemin, vitamin $K_1$, resazurin, 500 ml of distilled water, 500 ml of clarified rumen fluid, and 100 ml of serum-yeast autolysate-TPP.

Agar (1.58 g/100 ml) was added for solid OTI medium. After the OTI agar hardened in petri dishes, a sterile 50-mm-diameter membrane filter with an average pore size of 0.15 μm (Sartorius Inc.) was placed on the surface of the agar. A 1-inch (2.54-cm)-diameter sterile O ring was placed on the filter and sealed to it with sterile 3% agar (wt/vol in distilled water). One-tenth milliliter of inoculum was placed on the membrane filter, and the plates were incubated at 37°C for 2 weeks in an atmosphere of 90% $H_2$–10% $CO_2$. Spirochetes and some other motile bacteria migrated through the filter, and the resultant mixed cultures that were enriched with spirochetes were subsequently purified by streaking of agar.

RIP broth (prereduced OTI broth containing 2 μg of rifampin per ml and 800 U of polymyxin per ml) was also used as a selective isolation medium for spirochetes. Ten milliliters of broth was inoculated under $N_2$–$CO_2$–$H_2$ (85:12:3) with 0.1 ml of the dilution to be cultured and incubated at 37°C for 1 week.

Peptone-yeast extract-serum agar used for selective isolation of serum-requiring treponemes contained 0.5 g of heart infusion broth base (BBL Microbiology Systems), 2.0 g of Trypticase (BBL Microbiology Systems), 1.0 g of yeast extract (BBL Microbiology Systems), 0.2 g of $K_2HPO_4$, 1.3 g of agar, cysteine-hydrochloride, and 90 ml of distilled water. After the medium was autoclaved and cooled to 45°C, 10 ml of

TABLE 2. Distribution of GI scores among sites sampled

| Trial | Day | GI scores[a] | | | | | | | | | | | | Mean GI Score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Person 1 | | | | Person 2 | | | | Person 3 | | | | Person 4 | | | | |
| | | Site[b] | | | | Site | | | | Site | | | | Site | | | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | |
| I | 4 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0.56 ± 0.13 |
| | 11 | 2 | 2 | 2 | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1.25 ± 0.17 |
| | 26 | 1 | 0 | 1 | 2 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 1.44 ± 0.16 |
| II | 4 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0.56 ± 0.13 |
| | 11 | 2 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 2 | 1 | 1.44 ± 0.16 |
| | 26 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 2 | 2 | 2 | 2 | 1.88 ± 0.09 |

[a] The criteria of Loe and Silness (11) were used to determine GI scores; essentially, 0 = healthy, 1 = mild inflammation, 2 = moderate inflammation, bleeding when gently probed.

[b] Sites sampled are given in Table 1, Methods. Sites 1 and 2 were facial and proximal, respectively, of the first tooth listed; sites 3 and 4 were the facial and proximal, respectively, of the second tooth listed.

sterile inactivated rabbit serum containing 0.4 ml of TPP solution was added, and the medium was poured into petri dishes. Membrane filters and O rings were placed on the surface of the hardened agar. Inocula were placed on the filters, and the plates were incubated for 2 weeks at 37°C in anaerobic jars in an atmosphere of 90% $H_2$–10% $CO_2$.

**Mycoplasma medium.** Mycoplasma isolation medium contained 34 g of mycoplasma agar (BBL Microbiology Systems), 10 g of arginine-hydrochloride, and 700 ml of distilled water. After the medium was autoclaved, 200 ml of inactivated sterile rabbit serum, 100 ml of yeast autolysate, thallium acetate (10 ml of a 1:20 solution), and penicillin (10 ml of a 100,000-U/ml solution) were added. Mycoplasma isolation plates were streaked with 0.1 ml of the original dilution of gingival sample, and the plates were incubated at 37°C in anaerobic jars, using GasPak envelopes (BBL Microbiology Systems).

**Campylobacter medium.** *Campylobacter sputorum*-selective medium contained brucella agar (BBL Microbiology Systems) reconstituted as directed with (per 250 ml) 0.25 ml of bacitracin solution (50,000 U in 25 ml of distilled water) and 0.1 ml of novobiocin solution (500 mg in 100 ml of distilled water). Duplicate plates were streaked with the original dilution of the sample. One plate was incubated in an atmosphere containing 5% $O_2$, and the other was incubated under 10% $O_2$.

**Subculture and identification of treponemes.** After incubation of OTI and peptone-yeast extract-serum plates, membrane filters were removed, and the agar was observed for a white haze in the medium. A piece of agar from a white hazy area was examined for treponemes by dark-field microscopy, and a plug of the hazy area was inoculated into OTI broth containing 0.16% agar. To obtain isolated colonies, about 0.1 ml of a broth culture was inoculated into 20 ml of melted prereduced OTI agar in 6- or 8-ounce (180- or 240-ml) rubber stoppered prescription bottles. The agar and inoculum were mixed, and the agar was allowed to harden on the flat side of the bottle. The bottle-plates were incubated at 37°C and observed daily for treponemal colonies. Single well-isolated colonies were picked and subcultured in OTI or peptone-yeast extract-serum broth.

Treponemal isolates were characterized with methods and media described previously (7). All media were supplemented with (per 100 ml) serum (5 ml), thiamine pyrophosphate (0.75 mg), rumen fluid (2 ml), volatile fatty acid solution (0.025 ml), yeast autolysate (4 ml), and hemin. The volatile fatty acid solution contained 5 ml of glacial acetic acid, 4 ml of *n*-butyric acid, 1.0 ml of *n*-valeric acid, 1.0 ml of isovaleric acid, 1.0 ml of isobutyric acid, and 100 ml of distilled water. The pH was adjusted to 7.0, and the solution was filter sterilized.

All isolates were preserved in liquid nitrogen in OTI broth.

## RESULTS

**Clinical measurements.** Of the 96 samples, 18 were from sites with a GI of 0, 42 were from sites with a GI score of 1, and 36 were from sites with a GI score of 2. The distribution and means of GI scores observed at the sites sampled are shown in Table 2.

The mean ± standard error whole-mouth GI scores for trials I and II, respectively, were 0.62 ± 0.02 and 0.70 ± 0.02 for day 4, 0.91 ± 0.03 and 0.98 ± 0.02 for day 11, and 1.35 ± 0.01 and 1.47 ± 0.01 for day 26. In general, these results were typical of the subjects in group III of the original description of the experimental gingivitis model (13).

The standard errors here are somewhat misleading, because the observations on any day

TABLE 3. Percent coverage for trials, days, persons, and GI scores

| Sample | No. of bacterial taxa seen once | No. of bacterial isolates | Percent coverage ± SE[a] | No. of bacterial taxa |
|---|---|---|---|---|
| Total | 50 | 3034 | 98.4 ± 0.2 | 153 |
| Trial I | 35 | 1525 | 97.7 ± 0.3 | 114 |
| Trial II | 33 | 1509 | 97.8 ± 0.3 | 113 |
| Day 4 | 20 | 1004 | 98.0 ± 0.4 | 69 |
| Day 11 | 38 | 1027 | 96.3 ± 0.6 | 101 |
| Day 26 | 35 | 1003 | 96.5 ± 0.5 | 106 |
| GI score 0 | 22 | 562 | 96.1 ± 0.8 | 64 |
| GI score 1 | 27 | 1331 | 98.0 ± 0.4 | 98 |
| GI score 2 | 33 | 1141 | 97.1 ± 0.5 | 113 |
| Person 1 | 22 | 769 | 97.1 ± 0.5 | 75 |
| Person 2 | 25 | 757 | 96.7 ± 0.6 | 67 |
| Person 3 | 21 | 764 | 97.3 ± 0.5 | 72 |
| Person 4 | 33 | 744 | 95.6 ± 0.7 | 101 |

[a] The formula used for the appropriate standard error of the estimate of the percent coverage was $(90 \cdot n_1^{1/2})/N$, which is based on formulae 27 and 65 of Good (5) where $n_1$ = the number of taxa occurring once, and $N$ = total number of bacterial isolates.

presumably are strongly correlated. In particular, the discrepancy between the two trials on day 26 is not as significant as it looks. In fact, if the data on day 26 are expressed as a 2 × 3 contingency table with numbers 1, 7, and 8 (trial I: 1 [GI = 0], 7 [GI = 1], 8 [GI = 2]) and 0, 2, and 14 (trial II), the value of $\chi^2$ is 5.4 with two degrees of freedom, corresponding to a tail-area probability of 1/15 as compared with 1/70 if the comparison is done by a $t$ test.

The mean ± standard error plaque index scores of the 32 sample sites for each day (both trials) were 1.28 ± 0.08 for day 4, 1.56 ± 0.12 for day 11, and 2.09 ± 0.07 for day 26.

**Bacteriological data.** From the 96 samples of two replicates from the four persons, 153 nonspirochetal species, subspecies, and serovars and 13 species of spirochetes were isolated. These bacteriological data were compared by trial, person, day, GI score, tooth, and site by coverage analyses and by similarity comparisons. Comparisons of days (with equal numbers of isolates) were used to verify the accuracy of the experimental procedures. Comparisons of floras of sites with different GI scores were used to interpret the significance of different species in the etiology of gingivitis.

**Coverage analysis.** The crude coverage value [1 − (number of kinds seen once/total observations) ×100] (5) is an estimate of the complexity of the flora in which, for samples of a given size, the coverage value becomes lower as the flora becomes more complex. The relative homogeneity of floras can be determined by considering both the number of species or taxa observed and the percent coverage. The observed 153 nonspirochetal taxa accounted for 98.4% of the cultivable cells in the total samples from this population (Table 3). The estimate does not tell how many additional different bacterial species might occur in the 1.6% cultivable bacterial flora that was not included.

The percent coverage values for replicate trials were very similar for the estimated coverage, suggesting, but certainly not proving, that 1,500 isolates provided good reproducibility of the repeated experiment in the four people. The data demonstrated a rapid increase in the complexity of the flora; half again more species were detected at days 11 and 26 than at day 4, but this larger number of species accounted for a slightly smaller percentage of the flora.

There was a measurable difference in the complexity of the floras of sites with different gingival indices and of different people in the experiment. There were fewer species in the total sites with a GI score of zero than in total sites with a GI score of 1 or 2. Person 4 had a flora estimated to contain 36% more bacterial species than those of the other subjects. This led us to question whether the bacterial flora of person 4 was more complex at the start of the experiments or whether he had a greater response than the other subjects in terms of increased complexity of the flora. This informa-

TABLE 4. Person-day floras as evaluated by number of taxa and percent coverage among approximately 250 isolates (from 2 sites on 2 teeth in both replicate trials)[a]

| Person | Day 4 | | Day 11 | | Day 26 | |
|---|---|---|---|---|---|---|
| | No. of taxa | Coverage ± SE | No. of taxa | Coverage ± SE | No. of taxa | Coverage ± SE |
| 1 | 36 | 95.3 ± 1.2 | 44 | 94.7 ± 1.3 | 46 | 91.6 ± 1.6 |
| 2 | 32 | 96.4 ± 1.1 | 42 | 93.7 ± 1.4 | 41 | 92.0 ± 1.6 |
| 3 | 34 | 95.2 ± 1.2 | 52 | 93.1 ± 1.5 | 48 | 94.1 ± 1.4 |
| 4 | 42 | 92.3 ± 1.6 | 54 | 90.3 ± 1.8 | 68 | 90.8 ± 1.7 |

[a] The mean coverage of the 12 person-day floras = 93.3 ± 0.6.

TABLE 5. Person-day-trial floras as evaluated by number of taxa and percent coverage among approximately 125 isolates (from 2 sites on 2 teeth) in each trial[a]

| Person | Trial | Day 4 | | Day 11 | | Day 26 | |
|---|---|---|---|---|---|---|---|
| | | No. of taxa | Coverage ± SE | No. of taxa | Coverage ± SE | No. of taxa | Coverage ± SE |
| 1 | I | 26 | 91.5 ± 2.3 | 29 | 95.5 ± 1.7 | 32 | 90.5 ± 2.5 |
| | II | 20 | 95.2 ± 1.8 | 28 | 90.8 ± 2.4 | 30 | 88.7 ± 2.7 |
| 2 | I | 25 | 92.9 ± 2.1 | 25 | 91.2 ± 2.4 | 31 | 86.8 ± 2.9 |
| | II | 23 | 94.4 ± 1.9 | 32 | 87.7 ± 2.8 | 25 | 91.0 ± 2.4 |
| 3 | I | 26 | 93.6 ± 2.1 | 38 | 85.3 ± 3.0 | 32 | 92.2 ± 2.2 |
| | II | 23 | 91.9 ± 2.3 | 35 | 90.1 ± 2.5 | 34 | 91.9 ± 2.3 |
| 4 | I | 23 | 90.0 ± 2.6 | 35 | 88.0 ± 2.7 | 43 | 86.4 ± 3.0 |
| | II | 31 | 89.8 ± 2.6 | 28 | 89.4 ± 2.6 | 46 | 83.1 ± 3.3 |

[a] Mean coverage of the 24 person-day-trial floras = 90.3 ± 0.62.

tion was obtained from comparisons of each person on each day (Table 4).

It is evident (Table 4) that the complexity of the bacterial flora increased between days 4 and 11 or 26 in each of the subjects and that person 4 had a more diverse flora at all sample times. This observation raised the question of whether the flora in each person responded similarly in both trials or whether there was any detectable carry-over of flora from trial I to trial II, especially in subject 4. Each subject responded in a similar manner in each of the two trials (Table 5). The fairly high number of species in person 4, day 4, trial II suggests that there may have been some carry-over effect in this person from trial I, but the effect is not statistically significant ($P$ = 0.11). However, by day 26 in both trials, person 4 developed the most complex flora seen in these subjects.

The coverage analyses in Tables 3 to 5 clearly demonstrate an increasingly complex subgingival flora during the absence of oral hygiene. Colonization by additional species, rather than replacement of existing species, is typical of other developing mixed floras in nature (e.g., trickling filters in sewage plants, development of intestinal floras in germfree animals exposed to fecal inocula, or the colonization of sterilized soils containing organic matter). If we predict similar changes in the periodontal flora, concern should be focused on changes in the relative concentrations of different bacterial species during each trial.

**Percent similarity analyses.** One simple method of comparing complex floras, reported by Socransky et al. (S. S. Socransky, A. C. R. Tanner, and J. M. Goodson, J. Dent. Res. **60A**:486, 1981) measures the similarity of the composition of floras as the sum of the percentages of each species in the two floras that is actually shared. We adopted this measure of similarity in this report because of its simplicity, although the use of the "min" function is liable to lead to intractable mathematical complexities in some contexts. On the other hand, an ordinary product moment correlation coefficient between two samples would not be appropriate because the frequencies have nowhere near normal distributions. (It might be possible to overcome this objection to the use of standard statistical tests by first transforming the frequencies $n$ by a formula of the form $\sqrt{n + k}$ while ignoring rare species. We have not yet tried this approach.)

An abbreviated example calculation of a "similarity" is as follows. The percentages of flora of species A in samples X and Z are 12 and 1%, respectively; those of species B are 6 and 7%, respectively; and those of species C are 0 and 10%, respectively. Therefore, species A, B, and C have percentages of contribution to the similarity of 1, 6, and 0%, respectively. The variation between samples is emphasized by using only the lower percentage (in either sample) of each shared species and is accentuated further by the degree to which isolates were separated into different groups and subgroups. For example, if all serological groups of *A. naeslundii* were combined, the apparent similarity would be greater than when three serotypes are differentiated. We preferred to identify the isolates as thoroughly as possible because different subgroups or serotypes may have different significances in periodontal diseases.

The floras seen in combined samples were compared to determine the similarity of the floras of trials, anatomical sites, people, sites with different GI scores, and teeth (Table 6).

The similarity values of the combined samples (Table 6) are greatly affected by the number of

TABLE 6. Percent similarities of flora of combined samples

| Combined samples compared (a vs b) | Approx. (*N*) isolates/sample | Number of taxa[a] | | | % Similarity |
|---|---|---|---|---|---|
| | | Shared | In a only | In b only | |
| Trial I vs II | 1515 | 74 | 40 | 39 | 73.2 |
| Proximal vs facial | 1515 | 71 | 46 | 36 | 67.0 |
| Gingival Index | | | | | |
| 0 vs 1 | 562 vs 1331 | 47 | 17 | 51 | 64.8 |
| 0 vs 2 | 562 vs 1141 | 47 | 17 | 66 | 51.2 |
| 1 vs 2 | 1331 vs 1141 | 71 | 27 | 42 | 66.1 |
| Day | | | | | |
| 4 vs 11 | 1000 | 52 | 17 | 49 | 61.4 |
| 4 vs 26 | 1000 | 48 | 21 | 58 | 53.7 |
| 11 vs 26 | 1000 | 66 | 35 | 40 | 67.5 |
| Person | | | | | |
| 1 vs 2 | 750 | 39 | 36 | 28 | 59.7 |
| 1 vs 3 | 750 | 44 | 31 | 28 | 50.4 |
| 1 vs 4 | 750 | 51 | 24 | 50 | 51.7 |
| 2 vs 3 | 750 | 45 | 22 | 27 | 53.9 |
| 2 vs 4 | 750 | 46 | 21 | 53 | 45.3 |
| 3 vs 4 | 750 | 52 | 20 | 49 | 50.7 |
| Teeth (2 sites, 2 trials) (mean of 276 comparisons) | 125 | | | | 32.3 ± 0.6 |
| Each tooth vs other tooth sampled at the same time (mean of 24 comparisons) | 60 | | | | 36.2 ± 2.4 |
| Same tooth, trial 1 vs 2 (mean of 24 comparisons) | 60 | | | | 36.1 ± 2.6 |
| Teeth (2 sites, 1 trial) (mean of 1128 comparisons) | 60 | | | | 24.7 ± 0.3 |

[a] Isolates that did not survive through identification (3.5% of the total) were not included in the calculations.

isolates that are included in the samples being compared. However, the comparisons suggested several potentially important differences that were tested statistically by another analysis (see below). The similarity of the flora of all facial sites and that of all proximal sites was less than the similarity of the floras detected in the two trials (although the sample sizes, i.e., numbers of isolates, were identical). This suggests that there was a difference between the facial and proximal floras. The similarity values indicated (as did the coverage values) that the flora became more diverse as the GI scores increased and as the time without oral hygiene increased. Person 4 had the greatest number of species or taxa that were not shared with other people, which is further evidence that his flora was more complex than that of the others.

The floras of each tooth (both sites combined) versus the other tooth sampled on the same day in the same person and the flora of each tooth compared with itself in the two trials were far more similar ($t$ = 4.71 and 4.62, respectively) than the mean of all 1,128 possible tooth-to-tooth comparisons. Here, $t$ was calculated as [(mean similarity of 48 pairs of samples, e.g., both teeth sampled at the same time) − (mean similarity of 1,128 other possible tooth-to-tooth comparisons)] ÷ [(square root of the standard error of the 48-sample mean squared) + (standard error of the 1,128-sample mean squared)] (see Table 8, footnote *a*.) These results indicate that the flora was relatively uniform between different teeth within each person at each sample time and that the progressive change in composition of the flora may be repeatable and therefore, to some extent, predictable.

Although some species may have disappeared from the flora as gingivitis developed and progressed, the greater proportion of new species

TABLE 7. Cultural counts (times $10^5$) per sample (means of 8 samples)

| Person | Day 4 | | Day 11 | | Day 26 | |
|---|---|---|---|---|---|---|
| | RT[a] | BAP | RT | BAP | RT | BAP |
| 1 | 17.0 | 27.9 | 25.8 | 42.9 | 16.1 | 15.7 |
| 2 | 53.0 | 49.2 | 36.8 | 51.6 | 22.2 | 25.2 |
| 3 | 86.5 | 38.0 | 30.1 | 29.1 | 43.3 | 50.0 |
| 4 | 8.4 | 5.1 | 18.0 | 38.8 | 27.9 | 23.2 |

[a] RT = roll tube count, BAP = blood agar plate count.

decreased the chance of detecting all of the members of the original population that might remain. Species that maintained or increased their relative concentrations might assume greater significance in proportion to their relative numbers than species that were part of the initial flora but did not maintain their original proportions. This assumption would remove many bacterial species from consideration as primary agents of gingivitis.

Alternatively, it has been suggested that the original flora itself may be a primary agent of disease simply by proliferation to a greater density, therefore producing greater irritation. The mean ± standard error plaque index scores of the sample sites ranged from 1.28 ± 0.08 on day 4 to 2.09 ± 0.07 on day 26. Although there was an increase in supragingival plaque with time, there was no evidence in the present work that the total numbers of viable bacteria increased in the gingival sulci (Table 7), nor was there an increase with increasing GI scores. In fact, the mean counts ± standard error × $10^5$ for samples from sites with each GI score were as follows. GI 0: roll tubes, 39.8 ± 19.8 ($n$ = 18); plates, 21.0 ± 9.3 ($n$ = 15). GI 1: roll tubes, 25.2 ± 5.3 ($n$ = 39); plates, 32.1 ± 8.1 ($n$ = 37). GI 2: roll tubes, 31.3 ± 7.4 ($n$ = 36); plates, 35.7 ± 8.3 ($n$ = 36).

Although there is no evidence that either increased plaque index values (between 1 and 2) or GI scores (0 to 2) led to an increase in viable counts, both the coverage analysis and the percent similarity comparisons indicated that the flora became more diverse as gingivitis progressed.

The observations above were tested by comparing the individual samples within subsets (e.g., of each sample from sites with a given GI score compared with all other samples from sites with the same score) to determine whether such samples were more (or less) alike than were all of the other samples in the experiment. Among the 96 samples there were 96 × 95/2 = 4,560 possible comparisons that contained all of the percent similarity variation between samples for the entire experiment. The results of comparisons of individual sample subsets are shown in Table 8. These data provide evidence concerning the sources of variation in the entire experiment. As shown in Table 8, footnote *a*, and the Appendix, the $t$ values are analogous to Student's $t$ but are not directly comparable because they are not derived from independent observations.

The samples in trial I were more heterogeneous than those in trial II ($t$ = 4.78). This strongly indicates a microbial carry-over between the two trials, as suggested by the whole-mouth GI scores and the generalized tests described above. Analysis of the person-trial subsets (individual comparisons not shown) indicated that nearly all of the apparent carry-over effect was due to person 2. The mean similarity ± standard error of person 2-trial I samples was 27.27 ± 1.93%, and that of his trial II samples was 38.90 ± 1.33% ($t$ = 4.97). The differences between the two trials were by no means significant in the other three people. The difference in person 2 was almost certainly a "person" effect rather than a subtle "time" difference in technique, because the samples from persons 2 and 3 were taken concurrently. In trial II, person 2 developed a GI score of 2 in the sample sites by day 11 (and day 26), suggesting that the species that may have been carried over could be of special interest. *A. israelii* serotype II, *A. naeslundii* serotype I, *"A. naeslundii-viscosus"* (serotype cross), *A. odontolyticus* serotype I and serotype unreactive with available antisera, coccus SM1 (undescribed species), *Streptococcus mitis*, *Streptococcus* D-16, *Veillonella parvula*, and *Wolinella recta* were each in larger concentrations on day 4, trial II, than on day 4, trial I.

The floras of facial site samples and of proximal site samples were each more homogeneous than when compared with samples from the other anatomical area. Facial-versus-facial plus proximal-versus-proximal comparisons were more homogeneous than the other 2,304 possible comparisons of facial versus proximal sites, thus supporting the observation (Table 6) that there was a difference in the flora of the two anatomical sites. Of the facial flora, 7.2% (35 species including several facultative aerobes) was not seen in the proximal flora, and 11.2% (46 species) of the proximal flora was not detected in the facial flora. The difference between these two floras contributes a small but measurable source of experimental variation. The difference in bacterial composition is probably due to an environmental difference and might have contributed to the higher whole-mouth GI scores for proximal sites (mean ± standard error, 1.26 ±

TABLE 8. Analysis of the similarities within subsets of individual samples

| Subset | Samples /subset | Subset comparisons | | All other comparisons | | t[a] |
|---|---|---|---|---|---|---|
| | | N | Mean ± SE | N | Mean ± SE | |
| Trial I | 48 | 1128 | 19.19 ± 0.36 | 3432 | 20.55 ± 0.21 | 3.26[b] |
| Trial II | 48 | 1128 | 21.77 ± 0.38 | 3432 | 19.70 ± 0.21 | 4.81 |
| Trial I + II | | 2256 | 20.48 ± 0.26 | 2304 | 19.95 ± 0.25 | 1.47 |
| Facial sites | 48 | 1128 | 20.53 ± 0.37 | 3432 | 20.11 ± 0.21 | 1.01 |
| Proximal sites | 48 | 1128 | 29.94 ± 0.35 | 3432 | 19.97 ± 0.21 | 2.39 |
| Facial + Proximal | | 2256 | 20.74 ± 0.25 | 2304 | 19.69 ± 0.26 | 2.88 |
| Person | | | | | | |
| 1 | 24 | 276 | 27.47 ± 0.79 | 4284 | 19.74 ± 0.18 | 9.49 |
| 2 | 24 | 276 | 32.63 ± 0.85 | 4284 | 19.41 ± 0.18 | 15.22 |
| 3 | 24 | 276 | 20.87 ± 0.65 | 4284 | 20.17 ± 0.19 | 1.03 |
| 4 | 24 | 276 | 16.26 ± 0.68 | 4284 | 20.47 ± 0.19 | 5.95 |
| Person 1+2+3+4 | | 1104 | 24.31 ± 0.42 | 3456 | 18.90 ± 0.19 | 11.73 |
| GI score = 0 | 18 | 153 | 23.95 ± 0.99 | 4407 | 20.08 ± 0.18 | 3.84 |
| = 1 | 42 | 861 | 22.33 ± 0.42 | 3699 | 19.72 ± 0.20 | 5.63 |
| = 2 | 36 | 630 | 18.84 ± 0.51 | 3930 | 20.43 ± 0.19 | 2.91 |
| GI 0+1+2 | | 1644 | 21.14 ± 0.31 | 2916 | 19.69 ± 0.22 | 3.81 |
| Person 1 GI 0 | 1 | 0 | | 4560 | 20.21 ± 0.18 | |
| GI 1 | 12 | 66 | 31.38 ± 1.95 | 4494 | 20.05 ± 0.18 | 5.79 |
| GI 2 | 11 | 55 | 25.15 ± 1.67 | 4505 | 20.15 ± 0.18 | 2.97 |
| Person 2 GI 0 | 3 | 3 | 50.00 ± 9.07 | 4557 | 20.19 ± 0.18 | 3.28 |
| GI 1 | 9 | 36 | 33.67 ± 2.35 | 4524 | 20.10 ± 0.18 | 5.75 |
| GI 2 | 12 | 66 | 30.76 ± 1.72 | 4494 | 20.06 ± 0.18 | 6.18 |
| Person 3 GI 0 | 4 | 6 | 28.00 ± 6.87 | 4554 | 20.20 ± 0.18 | 1.13 |
| GI 1 | 15 | 105 | 19.79 ± 1.09 | 4455 | 20.22 ± 0.18 | 0.39 |
| GI 2 | 5 | 10 | 20.20 ± 2.78 | 4550 | 20.21 ± 0.18 | 0.01 |
| Person 4 GI 0 | 10 | 45 | 23.00 ± 1.91 | 4515 | 20.18 ± 0.18 | 1.47 |
| GI 1 | 6 | 15 | 18.53 ± 3.25 | 4545 | 20.22 ± 0.18 | 0.52 |
| GI 2 | 8 | 28 | 15.07 ± 1.74 | 4532 | 20.24 ± 0.18 | 2.96 |
| Person x trial | 12 | 582 | 25.52 ± 0.60 | 4032 | 19.52 ± 0.19 | 9.48 |
| Person x GI score | | 435 | 25.35 ± 0.68 | 4125 | 19.67 ± 0.18 | 8.05 |
| Person x GI x trial | | 213 | 26.88 ± 1.01 | 4347 | 19.88 ± 0.18 | 6.80 |
| Each tooth (both trials) | 4 | 144 | 30.03 ± 1.29 | 4416 | 19.89 ± 0.18 | 7.80 |
| Both teeth same time and person | 4 | 144 | 30.02 ± 1.23 | 4416 | 19.89 ± 0.18 | 8.15 |
| Both teeth same day and person same trials | 8 | 336 | 28.02 ± 0.79 | 4224 | 19.59 ± 0.18 | 10.45 |
| Other site same tooth and time | 2 | 48 | 32.29 ± 2.35 | 4512 | 20.08 ± 0.18 | 5.17 |
| Same site other trial | 2 | 48 | 31.83 ± 2.14 | 4512 | 20.09 ± 0.14 | 5.47 |
| Comparable site other tooth same time | 2 | 48 | 30.19 ± 1.86 | 4512 | 20.11 ± 0.18 | 5.41 |

[a] $t = [(\text{Mean other})-(\text{Mean subset})]/[(\text{subset SE})^2+(\text{other SE})^2]^{1/2}$. Although the values of t have been calculated by a standard formula as if it were a Student's t, the use of the standard t test is not applicable in the present context. The reason is explained in the appendix. The values of t should be interpreted only in the spirit of "exploratory data analysis". While we can safely regard values of t less than 2 as not significant, we cannot safely regard those greater than 2 (or even 3) as statistically significant, but only as suggestive of real differences. The statistical significance of several variables was determined by a simulation calculation described in the text.

[b] Underlined t values indicate the subset is less similar (more heterogeneous) than the other possible sample comparisons.

0.02) than for facial sites (mean ± standard error, 0.98 ± 0.04).

Comparisons of samples within people versus those between people reveal that "people" differences account for a major portion of the experimental variation. The samples of persons 1 and 2 were more homogeneous, and those of person 4 were less homogeneous than all other possible sample comparisons. Persons 1 and 2 had, or developed more rapidly, higher mean sample site GI scores than did persons 3 and 4. These observations indicate that a specific, more homogeneous flora may relate to the development of gingivitis.

The statistical significance of differences between the similarities of the samples within and between people was determined by a simulation calculation based on the mean similarity between the two subsets (e.g., person 1 samples and person 2 samples) divided by the mean similarity within subsets to obtain a ratio value, near 1.0, which we called L. Because the simulation analyses used here are not a standard test but were developed by one of us for these data and because they are useful for many other applications if computers are available, they are described here in some detail. For example, the 24 samples from person 1 (subset 1) were compared with the 24 samples from person 2 (subset 2). The mean of these 576 between-subset comparisons was divided by the mean similarity of all 552 possible comparisons within each of the two subsets. All 48 samples were then randomly distributed into two new subsets $10^3$ times and reanalyzed each time to determine the probability of detecting a ratio as low as the observed ratio. (Note: The greater the difference between the two subsets, the lower the mean between-subset similarity and the resulting between/within ratio [L] will be.) The technique will be described in more detail and in general terms elsewhere (I. J. Good, J. Statist. Comput. Simula., in press).

Simulation analyses showed that the small difference in the floras of the two trials was significant at the 0.046 level, the larger difference between floras of proximal and facial sites was significant at the 0.0033 level, and the composition of the flora of each person was significantly different from that of each other person ($P < 0.001$ in all six possible person-to-person comparisons). The relatively large differences in composition of the floras of different people should help to pinpoint species that may relate to the etiology of gingivitis because a smaller portion of the flora was held in common, yet gingivitis developed in all eight person trials.

Simulation analyses showed that the flora of the 18 GI 0 sites was not significantly different from the flora of the 42 GI 1 sites ($P = 0.31$) but was significantly different from that of the 36 GI 2 sites ($P = 0.02$). The flora of GI 1 sites was significantly different from that of the GI 2 sites ($P = 0.01$). These data confirm that, although much of the initial flora remains, flora diversity increases as gingivitis develops, and a further large change is associated with the presence of serum or blood.

The person-GI score and person-trial interactions were tested by a slight elaboration of the simulation just described. For person-trial interactions, the eight between/within ratios (L) for each person in each trial versus all samples in the other trial were calculated (24 such values for person-GI score interaction). The ratios (L's) were then compared as follows (in a self-explanatory notation):

$$\{[L(P_1\ T_1,\ T_2)] - [L(P_2\ T_1,\ T_2)]\} - \{[L(P_1\ T_2,\ T_1)] - [L(P_2\ T_2,\ T_1)]\} = x_1$$

$$\{[L(P_1\ T_1,\ T_2)] - [L(P_3\ T_1,\ T_2)]\} - \{[L(P_1\ T_2,\ T_1)] - [L(P_3\ T_2,\ T_1)]\} = x_2,$$

etc., to

$$\{[L(P_3\ T_1,\ T_2)] - [L(P_4\ T_1,\ T_2)]\} - \{[L(P_3\ T_2,\ T_1)] - [L(P_4\ T_2,\ T_1)]\} = x_6$$

to give six values of $|x_i|$ (the absolute values of $x_i$), comparing each person with each other person in both trials. (There are 18 values of $|x_i|$ for interactions of four people by three GI scores.) The observed sum of the six values of $|x_i|$ was compared with the sums of $|x_i|$ obtained after randomizing the samples only within each trial, not between trials, 100 times. (For person-GI score interactions, the samples were randomly perverted only within each GI score and then redistributed into person-GI score subsets of the initial sizes.) The probability of the observed value was determined after adding 1 to the number of values equal to or lower than the observed value and adding 2 to the total number of simulation values (that is, by Laplace's law of succession). The person-trial interaction had a probability of 0.07.

The person-GI score subsets (Table 8) reflect a similar direction of response in all four people superimposed on the person-to-person differences. These values indicate that the differences between people or disease states were not caused by unusual or unique high or low sample subsets. The person-GI score interaction was not statistically significant ($P = 0.79$). Thus, there is no evidence that the floras of individual people respond differently to the increasing severity of gingivitis.

Comparisons of subsets of samples from the same tooth, both teeth sampled at the same time, and the same tooth sampled in the two trials showed greater similarity of these samples as opposed to all other comparisons (Table 8). These results indicated that the flora of different teeth was relatively homogeneous within each person at a given time and tended to be similar at the same relative time in the repeat trial.

Even greater similarities were evident between sites on the same tooth sampled at one time, the same site sampled at the same time in a replicate trial, and the comparable (facial or proximal) site on the other tooth sampled at the same time. All of these subsets were more homogeneous than the means of all other possible combinations ($P < 0.00033$), indicating (in spite of the small sample size of 30 isolates and

only 48 comparisons) that the results were reasonably repeatable and, therefore, that the changes in composition of the flora may be predictable.

Floras on day 4, 11, or 26 corresponded quite well to the comparisons of sites with each GI score (Table 2). Comparisons of days were omitted from Table 8 because the differences between days were confounded by GI scores which were not uniform on each day. In this respect, days were used only to produce different disease severity and were not considered to be a cause of gingivitis per se.

As has been observed by others (S. S. Socransky, personal communication), the frequencies of occurrence of the individual bacterial species were not distributed in a normal manner. They do not even have Poisson distributions. Unless suitable transformations can be made, the non-normal distribution of species in periodontal floras precludes reasonably meaningful information from some standard statistical tests.

There were two apparent discrepancies. The first was the occurrence of a few scattered samples in which many isolates were members of a single species. This might possibly occur from a spreading film of growth in the isolation medium, but our observations do not indicate that this was the cause. Control samples were taken routinely from areas where there were no visible colonies in the plates and tubes that were picked. These samples nearly always failed to detect growth. On the few occasions when they did produce growth, the isolates were recognized as contaminants of several of the colonies that were picked. If, as infrequently happened, two or more neighboring colonies yielded the same organisms in mixed culture with other species, the species in common was counted only once. For a spreading organism to be mistaken as the predominant species, nearly all of the visible colonies that were picked would have to be nonviable, which is most unlikely. Overall, only 3.5% of the colonies failed to grow in pure culture or to survive through identification.

Others have suggested that a colony in the sulcus may be sampled to yield a highly enriched sample. In view of the relative size of the Morse 00 scaler tip, the colony would have to fill the entire quadrant of the sulcus. Whether this is similar to an abscess condition is not known. It does appear that very occasionally the flora of a sulcus is highly enriched with a single species.

The second discrepancy from normal distribution in our data was the appearance of a high proportion of three isolates of individual species among the 30 colonies picked from each sample. It is interesting that, in other equally complex floras (8, 16), the most common species seldom exceed 10 to 12% of the flora. This apparent limit might result from species interdependence. If the limit is real, samples might be expected to contain a disproportionate number of 3's among 30 isolates per sample.

Certainly the biggest problem for statistical analysis results from the complexity of the flora. Although 3,034 isolates were analyzed in this study (with a total of over 150,000 tests), they were distributed among 153 kinds of non-treponemal bacteria and 96 samples, to give an average of only 0.2 isolates per species per sample.

To examine possible relationships of individual species to developing experimental gingivitis, the species were listed according to their relative incidences in sites with GI scores of 0, 1, or 2. In the following tables and text, undescribed species are given a number or letter designation (e.g., *Lactobacillus* D-2), serovars and subspecies are designated by Roman numerals, and phenotypically similar strains of *Actinomyces* that failed to react with specific fluorescent-antibody conjugate are indicated by (−).

Those species whose appearance seems to be negatively correlated with a GI score of 1 (lower incidence than in GI 0 sites) are listed in Table 9. Species whose incidence shows no correlation with a GI score of 1 (higher incidence in sites with scores of 0 and 2) are listed in Table 10. Species whose frequency appears to be positively correlated with the initiation of gingivitis (more prevalent in sites with a GI score of 1 than with a GI score of 0) are listed in Table 11. Species that appear to colonize as a result of gingivitis (observed only in sites with a GI score of 2) are listed in Table 12. It is probable that species listed in Table 12 appeared in response to increased serum and exudates or the products and metabolic reactions of recently established species. Several of these species are of special interest because they have been found in samples from the gingival sulcus of patients with periodontitis. Their appearance is a further indication that the flora changes in a continuing sequence, similar to that seen in mixed floras of other microbial populations. The incidence of the 13 species of treponemes that were detected in this study is shown in Table 13.

To determine which species are most suspect as etiological agents of gingivitis, we made the following assumptions: (i) the concentration of the organism in sites with a GI score of 1 should be nearly equal to or greater than its concentration in healthy (GI 0) sites (Table 11), (ii) it should represent at least 1% of the flora or be detected in at least one-third of the samples from sites with a GI score of 1, and (iii) if there is a common cause of gingivitis, the organism should be present in all four people.

Of the 166 bacterial and treponemal species

TABLE 9. Frequency distribution by gingival index (GI): Species that appear to be negatively correlated with gingivitis

| Species | Gingival index | | | % of total flora |
|---|---|---|---|---|
| | 0 | 1 | 2 | |
| *Actinobacillus actinomycetemcomitans* | 0.18[a] | –[b] | – | 0.03 |
| *Actinomyces* D-1 | 0.18 | – | – | 0.03 |
| *Bacteroides capillosus* | 1.07 | – | – | 0.20 |
| *Bacteroides melaninogenicus* 2381 | 0.18 | – | – | 0.03 |
| *Bacteroides oralis* | 0.53 | – | – | 0.10 |
| *Bacteroides* D-39 | 0.18 | – | – | 0.03 |
| Fac. G-pos. coccus D-19 | 0.18 | – | – | 0.03 |
| Fac. G-pos. coccus SM II | 0.71 | – | – | 0.13 |
| *Fusobacterium* D-12 | 0.18 | – | – | 0.03 |
| *Haemophilus parahaemophilus* | 0.18 | – | – | 0.03 |
| *Selenomonas* D-10 | 0.18 | – | – | 0.03 |
| *Streptococcus* D-25 | 0.18 | – | – | 0.03 |
| *Veillonella atypica* | 0.18 | – | – | 0.03 |
| *Actinomyces meyeri* (–) | 0.18 | 0.08 | – | 0.07 |
| *Bacteroides buccalis* | 0.18 | 0.08 | – | 0.07 |
| *Fusobacterium* D-6 | 0.53 | 0.30 | – | 0.23 |
| *Wolinella* HS | 0.18 | 0.08 | – | 0.07 |
| *Actinomyces viscosus* II | 2.31 | 1.50 | 1.49 | 1.65 |
| *Bacteroides* D-31 | 1.07 | 0.30 | 0.09 | 0.36 |
| *Capnocytophaga gingivalis* | 2.67 | 1.80 | 1.49 | 1.85 |
| Fac. G-pos. coccus SM I | 3.02 | 1.43 | 0.70 | 1.45 |
| *Fusobacterium naviforme* | 1.07 | 0.30 | 0.18 | 0.40 |
| *Streptococcus intermedius* IV | 0.36 | 0.08 | 0.09 | 0.13 |
| *Streptococcus sanguis* I | 15.48 | 6.31 | 2.89 | 6.72 |
| *Streptococcus sanguis* II | 4.27 | 4.06 | 1.34 | 3.00 |
| *Streptococcus* D-7 | 4.45 | 1.05 | 0.18 | 1.35 |
| *Streptococcus* D-16 | 4.09 | 3.53 | 0.70 | 2.57 |
| *Streptococcus* SA | 1.60 | 0.75 | 0.44 | 0.79 |
| *Wolinella recta* | 0.89 | 0.60 | 0.61 | 0.66 |

[a] Percent of flora of 562 isolates (GI 0), 1331 isolates (GI 1), 1141 isolates (GI 2), and 3034 isolates (total).

[b] –, not detected.

detected, only 17 species (listed in Table 14) met the first two requirements. Together, these species accounted for 37% of the flora in GI 0 sites, 59% of the flora in GI 1 sites, and 55% in GI 2 sites. Only 13 taxa met all three criteria for a common etiology. Only nine of these taxa were not detected in equal numbers in people with healthy gingiva (see Discussion).

(In this paper, strains identified as *Capnocytophaga ochracea*, *Capnocytophaga gingivalis*, and *Capnocytophaga sputigena* had phenotypic characteristics as described for the respective species [23]. Strains of *C. ochracea* had polyacrylamide gel electrophoresis patterns like those of ATCC 27872 and VPI 5569. ATCC 27872 was the reference strain of *Bacteroides ochraceus* and has 77% DNA homology with strain 25, the type strain of *C. ochracea*. VPI 5569 has 86% homology with strain 25 [26]. Strains identified as *C. sputigena* had polyacrylamide gel electrophoresis patterns like strain 4 [type strain] received from Betsy Williams, University of Washington, Seattle. Strains identified as *C. gingivalis* had polyacrylamide gel electrophoresis patterns like strain 27 [type strain] received from Betsy Williams but not like ATCC 33624, the strain deposited with ATCC as the type strain of *C. gingivalis* [22]. We have not yet been able to obtain ATCC 33596 and ATCC 33612, strains deposited with ATCC as the type of *C. ochracea* and *C. sputigena*, respectively [22], for comparison with our strains.

We recently determined that ATCC 33397 [= NCTC 10713], the type strain of *Streptococcus anginosus* [21], and ATCC 27335, the type strain of *Streptococcus intermedius*, have extremely similar phenotypic reactions and polyacrylamide gel electrophoresis patterns. It appears that they are the same species. Since the name *S. anginosus* has priority, it is used in this manuscript and represents lactose-fermenting strains previously identified by us as *S. intermedius*.)

## DISCUSSION

In periodontal microbiology, as in any complex microbial problem, there have been nearly as many proposed theories as there have been investigators. No matter what new results might show, someone is sure to say the findings are not new. Indeed, the present study supports previous suggestions of a logical sequence of progression of bacterial species in and near the gingival crevice (4). Most of the implicated species have properties that have been related to the etiology of gingivitis. The data equally indicate that sev-

TABLE 10. Frequency distribution: Species that show no correlation with gingivitis

| Species | Gingival index | | | % of total flora |
|---|---|---|---|---|
| | 0 | 1 | 2 | |
| *Actinomyces israelii* I | 0.53[a] | 0.38 | 0.88 | 0.59 |
| *Actinomyces meyeri* + | 0.36 | 0.08 | 0.26 | 0.20 |
| *Actinomyces odontolyticus* II | 0.36 | –[b] | 0.18 | 0.13 |
| *Bacillus circulans* | 0.53 | – | 0.09 | 0.13 |
| *Bacteroides* D-26 | 0.36 | 0.15 | 0.35 | 0.26 |
| *Capnocytophaga ochracea* | 0.36 | 0.30 | 0.96 | 0.56 |
| *Eikenella corrodens* | 0.53 | – | 0.18 | 0.16 |
| *Eubacterium saburreum* | 0.89 | 0.68 | 1.14 | 0.89 |
| Fac. G-pos. rod D-5 | 0.18 | 0.08 | 0.26 | 0.16 |
| Did not survive | 5.69 | 1.73 | 4.47 | 3.49 |
| *Staphylococcus epidermidis* | 0.53 | 0.38 | 0.53 | 0.46 |
| *Streptococcus* SM | 3.02 | 1.13 | 1.49 | 1.62 |
| *Streptococcus* D-21 | 0.36 | 0.22 | 0.26 | 0.26 |
| *Veillonella dispar* | 0.71 | – | 0.18 | 0.20 |

[a] Percent of flora of 562 isolates (GI 0), 1331 isolates (GI 1), 1141 isolates (GI 2), and 3034 isolates (total).

[b] –, not detected.

TABLE 11. Frequency distribution by gingival index: Species that appear to have positive correlation with gingivitis

| Species | Gingival index | | | % of total flora | Species | Gingival index | | | % of total flora |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | | | 0 | 1 | 2 | |
| *Actinomyces* WVa 963 | 0.36[a] | 0.68 | 1.14 | 0.79 | *Leptotrichia buccalis* | –[b] | 0.38 | 0.18 | 0.23 |
| *Actinomyces israelii* (–) | 0.18 | 0.53 | 2.98 | 1.38 | *Leptotrichia* D-22 | – | 0.15 | 0.26 | 0.16 |
| *Actinomyces naeslundii* I | 3.38 | 4.88 | 10.96 | 6.89 | *Selenomonas sputigena* | – | 0.83 | 0.61 | 0.59 |
| *Actinomyces naeslundii* III | 2.49 | 4.21 | 1.14 | 2.74 | *Selenomonas* D-3 | – | 0.45 | 0.26 | 0.30 |
| *Actinomyces naeslundii* (–) | 4.98 | 6.54 | 3.59 | 5.14 | *Selenomonas* D-4 | – | 0.15 | 0.09 | 0.10 |
| *Actinomyces naeslundii-viscosus* | 1.96 | 4.58 | 4.29 | 3.99 | *Selenomonas* D-11 | – | 0.45 | 0.09 | 0.23 |
| | | | | | *Selenomonas* D-12 | – | 0.30 | 1.40 | 0.66 |
| *Actinomyces odontolyticus* I | 1.60 | 4.73 | 3.51 | 3.69 | *Selenomonas* D-13 | – | 0.15 | 0.18 | 0.13 |
| | | | | | *Selenomonas* D-15 | – | 0.30 | 0.88 | 0.46 |
| *Actinomyces odontolyticus* (–) | 1.42 | 1.95 | 1.49 | 1.68 | *Streptococcus bovis* | – | 0.08 | 0.09 | 0.07 |
| | | | | | *Streptococcus constellatus* | – | 0.22 | 0.35 | 0.23 |
| *Actinomyces viscosus* (–) | 0.18 | 0.30 | 0.79 | 0.46 | *Streptococcus intermedius* III | – | 0.08 | 0.35 | 0.16 |
| Anaerobic coccus D-30 | 0.18 | 1.20 | 0.18 | 0.63 | *Streptococcus mutans* | – | 0.08 | 0.44 | 0.20 |
| *Bacteroides oris* | 0.18 | 1.05 | 1.84 | 1.19 | *Streptococcus* D-37 | – | 0.22 | 0.61 | 0.33 |
| *Capnocytophaga sputigena* | 0.36 | 0.68 | 0.61 | 0.59 | *Wolinella* D-X | – | 0.38 | 0.35 | 0.30 |
| *Eubacterium timidum* | 0.36 | 0.38 | 0.70 | 0.49 | *Actinomyces* WVa-963 (–) | – | 0.08 | – | 0.03 |
| *Fusobacterium nucleatum* | 3.56 | 3.68 | 5.70 | 4.42 | *Bacillus* D-3 | – | 0.08 | – | 0.03 |
| *Fusobacterium* D-4 | 0.18 | 0.30 | 0.26 | 0.26 | *Bacteroides buccae* | – | 0.15 | – | 0.07 |
| *Lactobacillus* D-2 | 0.18 | 1.58 | 3.77 | 2.14 | *Bacteroides* D-4 | – | 0.08 | – | 0.03 |
| *Peptostreptococcus micros* | 0.89 | 1.28 | 1.75 | 1.38 | *Bacteroides* D-10C | – | 0.15 | – | 0.07 |
| *Propionibacterium acnes* | 0.18 | 1.20 | 1.05 | 0.96 | *Bacteroides* D-34 | – | 0.22 | – | 0.10 |
| *Streptococcus anginosus* | 4.98 | 6.39 | 4.73 | 5.50 | *Fusobacterium* D-1 | – | 0.38 | – | 0.16 |
| *Streptococcus mitis* | 1.07 | 1.20 | 0.61 | 0.96 | Fac. G-neg. rod D-10 | – | 0.08 | – | 0.03 |
| *Streptococcus sobrinus* | 0.18 | 0.30 | 0.53 | 0.36 | Fac. G-neg. rod D-13 | – | 0.22 | – | 0.10 |
| *Veillonella parvula* | 10.32 | 13.22 | 10.52 | 11.67 | Fac. G-neg. rod D-17 | – | 0.08 | – | 0.03 |
| *Actinomyces* D-8 | – | 0.45 | 0.53 | 0.40 | Fac. G-pos. coccus D-25 | – | 0.08 | – | 0.03 |
| *Actinomyces israelii* II | – | 1.20 | 0.18 | 0.59 | *Haemophilus aphrophilus* | – | 0.08 | – | 0.03 |
| *Bacteroides gracilis* | – | 0.38 | 1.05 | 0.56 | *Lactobacillus* D-7 | – | 0.08 | – | 0.03 |
| *Bacteroides intermedius* 8944 | – | 0.83 | 0.61 | 0.59 | *Lactobacillus* D-9 | – | 0.15 | – | 0.07 |
| | | | | | *Leptotrichia* D-35 | – | 0.08 | – | 0.03 |
| *Bacteroides pneumosintes* | – | 0.08 | 0.09 | 0.07 | *Peptostreptococcus anaerobius* | – | 0.45 | – | 0.20 |
| *Bacteroides socranskii* | – | 0.15 | 0.26 | 0.16 | | | | | |
| *Campylobacter concisus* | – | 0.30 | 0.44 | 0.30 | *Propionibacterium* D-3 | – | 0.08 | – | 0.03 |
| *Eubacterium bracby* | – | 0.15 | 0.09 | 0.10 | *Streptococcus* D-6 | – | 0.08 | – | 0.03 |
| *Eubacterium* D-6 | – | 0.15 | 0.44 | 0.23 | *Streptococcus* D-12 | – | 0.08 | – | 0.03 |
| *Eubacterium* D-8 | – | 0.22 | 0.18 | 0.16 | *Streptococcus* D-31 | – | 0.45 | – | 0.20 |
| Fac. G-neg. coccus D-3 | – | 0.22 | 0.26 | 0.20 | *Streptococcus* D-34 | – | 0.08 | – | 0.03 |
| Fac. G-pos. coccus D-40 | – | 0.08 | 0.18 | 0.10 | *Streptococcus* D-35 | – | 0.08 | – | 0.03 |
| *Lactobacillus* D-10 | – | 0.08 | 0.18 | 0.10 | "Vibrio" D-14 | – | 0.08 | – | 0.03 |

[a] Percent of flora of 562 isolates (GI 0), 1331 isolates (GI 1), 1141 isolates (GI 2), and 3034 isolates (total).

[b] –, not detected.

eral other proposed theories and species are probably irrelevant.

If the taxa listed in Table 14 are, as they appear to be, primary agents of adult gingivitis, it is reasonable to postulate the roles that each may play. The physical association of certain *Streptococcus* and *Actinomyces* species with the tooth surface may be a necessary prerequisite to colonization by veillonella, fusobacteria, and treponemes that produce stronger irritants such as propionic and butyric acids or antigens or both. Physical associations between actinomyces or streptococci and other bacteria, including veillonella and fusobacteria, have been demonstrated by Bladen et al. (2), Kelstrup and Funder-Nielson (10), Peros and Gibbons (18), and many other authors cited in a review by Cisar (3). In addition to their dual physical association with the tooth surface and with other bacteria, streptococci, lactobacilli, and actinomyces produce lactic acid, a preferred substrate for veillonella, which convert lactic acid to propionic acid. Both *Peptostreptococcus micros* and *S. anginosus* are found in infections of other body sites, and they may have pathogenic activity in periodontal sites.

The essential role of streptococci and actinomyces is indicated by established knowledge that strict control of the initial flora (primarily actinomyces and streptococci) prevents gingivitis. However, the presence of relatively high numbers of actinomyces and cocci in healthy sites (GI 0) in this experiment and in separate studies of gingival floras of periodontally healthy adults (unpublished data) indicate that the actinomyces and streptococci alone usually do not

TABLE 12. Frequency distribution: Species that appear to be present as a result of gingivitis

| Species | Gingival index | | | % of total flora |
|---|---|---|---|---|
| | 0 | 1 | 2 | |
| *Actinomyces* D-6 | –[a] | – | 0.09[b] | 0.03 |
| *Actinomyces naeslundii* X | – | – | 0.35 | 0.13 |
| *Actinomyces propionica* | – | – | 0.09 | 0.03 |
| *Bacillus* D-1 | – | – | 0.09 | 0.03 |
| *Bacteroides bivius* | – | – | 0.09 | 0.03 |
| *Bacteroides intermedius* 4197 | – | – | 0.09 | 0.03 |
| *Bacteroides loescheii* | – | – | 0.35 | 0.13 |
| *Bacteroides* D-12 | – | – | 0.09 | 0.03 |
| *Bacteroides* D-19 | – | – | 0.26 | 0.10 |
| *Bacteroides* D-23 | – | – | 0.26 | 0.10 |
| *Bacteroides* D-27 | – | – | 0.09 | 0.03 |
| *Bacteroides* D-38 | – | – | 0.09 | 0.03 |
| *Bifidobacterium* D-3 | – | – | 0.09 | 0.03 |
| Fac. G-pos. coccus D-22 | – | – | 0.09 | 0.03 |
| *Eubacterium nodatum* | – | – | 0.09 | 0.03 |
| *Eubacterium* D-4 | – | – | 0.09 | 0.03 |
| *Eubacterium* D-14 | – | – | 0.09 | 0.03 |
| *Eubacterium* D-23 | – | – | 0.18 | 0.07 |
| *Fusobacterium* D-3 | – | – | 0.09 | 0.03 |
| *Fusobacterium* RD | – | – | 0.09 | 0.03 |
| Fac. mtl. G-neg. rod D-11 | – | – | 0.26 | 0.10 |
| Fac. G-neg. rod D-4 | – | – | 0.09 | 0.03 |
| Fac. G-neg. rod D-8 | – | – | 0.09 | 0.03 |
| Fac. G-neg. rod D-15 | – | – | 0.18 | 0.07 |
| Fac. G-pos. rod D-7 | – | – | 0.09 | 0.03 |
| *Lactobacillus minutus* | – | – | 0.53 | 0.20 |
| *Lactobacillus* D-8 | – | – | 0.18 | 0.07 |
| *Peptostreptococcus anaerobius* II | – | – | 0.26 | 0.10 |
| *Peptococcus magnus* | – | – | 0.09 | 0.03 |
| *Selenomonas* D-2 | – | – | 0.70 | 0.26 |
| *Selenomonas* D-6 | – | – | 0.18 | 0.07 |
| *Selenomonas* D-7 | – | – | 0.09 | 0.03 |
| *Selenomonas* D-17 | – | – | 0.09 | 0.03 |
| *Staphylococcus haemolyticus* | – | – | 0.09 | 0.03 |
| *Staphylococcus saprophyticus* | – | – | 0.09 | 0.03 |
| *Streptococcus* D-2 | – | – | 0.18 | 0.07 |
| *Streptococcus* D-36 | – | – | 0.09 | 0.03 |
| *Streptococcus* D-38 | – | – | 0.09 | 0.03 |

[a] –, not detected.

[b] Percent of flora of 562 isolates (GI 0), 1331 isolates (GI 1), 1141 isolates (GI 2), and 3034 isolates (total).

produce gingivitis. An increase in certain associated species is probably required. Preliminary comparisons of the incidence of the 13 suspect species in 14 samples from sites in people with healthy gingiva (unpublished data) show that only nine of the suspect taxa were present in greater proportions in GI 1 samples than in the healthy floras. By percentage of the flora, *S. anginosus* was 28.8 times as prevalent; *A. odontolyticus* I, 21.3 times; *A. odontolyticus* (–), 4.4 times; *Lactobacillus* D-2, 7.1 times; *V. parvula*, 3.1 times; *Fusobacterium nucleatum*, 1.8 times; *A. naeslundii* (–), 1.4 times and *A. naeslundii* III, 1.3 times. *Treponema* A was not detected in people with healthy gingiva. The association of *V. parvula* and *F. nucleatum* with GI 1 sites supports previous indications that butyric and propionic acids (primarily produced by *F. nucleatum* and *V. parvula*) may be potent gingival irritants, as demonstrated in dogs (20). The presence of *Treponema* A has not been reported previously. The contribution of this species is not known, but its association with the initiation of gingivitis appears to be clear.

Other pathogenic mechanisms that also may be involved include bacterial production of antigens and polyclonal B-cell activators for specific and nonspecific immunological mediation of disease. Gram-negative bacteria generally are more active polyclonal B-cell activators than are gram-positive species (1; S. L. Donaldson, P. H. Bick, G. A. Miller, W. E. C. Moore, R. R. Ranney, J. A. Burmeister, and J. G. Tew, submitted for publication). Some strains of *F. nucleatum* are especially active polyclonal B-cell activators (1).

Several species that are reported in periodontitis appeared to colonize the gingival crevice as a result of gingivitis (Table 12). This probably reflects the environmental change and indicates that a further sequential progression of species is to be expected in subsequent disease states. The

TABLE 13. Incidence of spirochetes and mycoplasma in experimental gingivitis

| Organism | GI = 0 Samples + (of 18) | GI = 1 Samples + (of 42) | GI = 2 Samples + (of 36) |
|---|---|---|---|
| Treponeme A | 2 | 9 | 12 |
| Treponeme A1 | –[a] | 1 | 3 |
| Treponeme C | – | 3 | 3 |
| Treponeme E | – | 1 | 2 |
| Treponeme F | – | – | 1 |
| Treponeme J | – | 2 | 1 |
| Treponeme K | – | – | 3 |
| Treponeme L | – | – | 1 |
| Treponeme M | – | 1 | – |
| Treponeme N | – | 1 | – |
| Treponeme P | – | 1 | 1 |
| Treponeme R | – | 1 | – |
| Treponeme S | – | – | 1 |
| Any spirochetes seen or cultured | 3 | 12 | 20 |
| Mean count when present | $10^{1.5}$ | $10^{2}$ | $10^{2.8}$ |
| Mycoplasma | – | – | 1 |
| Campylobacter[b] | – | – | – |

[a] –, not detected.

[b] *C. sputigena* was not detected with the campylobacter selective media. However, *Campylobacter concisus* was detected with medium D4 (see Table 11).

TABLE 14. Number of people (of 4) in which each suspect species was detected in 1 or more sites with each GI score

| Species | GI score | | |
|---|---|---|---|
| | 0 | 1 | 2 |
| *Actinomyces israelii* II | 0 | 3 | 1 |
| **Actinomyces naeslundii* I | 3 | 4 | 4 |
| **Actinomyces naeslundii* III | 2 | 4 | 2 |
| **Actinomyces naeslundii* (−) | 3 | 4 | 4 |
| **Actinomyces naeslundii-viscosus* | 4 | 4 | 3 |
| **Actinomyces odontolyticus* (−) | 2 | 4 | 3 |
| **Actinomyces odontolyticus* I | 3 | 4 | 4 |
| *Bacteroides oris* | 1 | 3 | 2 |
| Coccus D-30 | 1 | 2 | 1 |
| **Fusobacterium nucleatum* | 2 | 4 | 4 |
| **Lactobacillus* D-2 | 1 | 4 | 3 |
| **Peptostreptococcus micros* | 3 | 4 | 3 |
| *Propionibacterium acnes* | 1 | 3 | 4 |
| **Streptococcus anginosus* | 2 | 4 | 4 |
| **Streptococcus mitis* | 4 | 4 | 2 |
| **Veillonella parvula* | 4 | 4 | 4 |
| **Treponema* A | 2 | 4 | 4 |

*Present in all 4 people in sites with a GI score of 1.

presence of *Actinobacillus actinomycetemcomitans* in a site with a GI score of 0 suggests that this species is ubiquitous as an opportunistic pathogen. Our results are consistent with reports of many other investigators, but they also suggest that some previously indicated species in the genera *Capnocytophaga* and *Bacteroides* may not play primary roles in the etiology of gingivitis.

The flora of person 4, a postdoctoral student in Periodontics, was more diverse than those of the other subjects, yet he appeared (Table 2) to be an ideal subject and to develop gingivitis in textbook fashion. In separate studies (unpublished data), we compared the flora of healthy sites in dentists with that of healthy sites in older people of different occupations. The floras of dentists in these studies were also more heterogeneous than those of other people. These observations suggest that in people with special motivation to maintain superior hygiene, the absence of threshold levels of actinomyces and streptococci prevents early establishment of a normal sequence of species association. This also suggests that such people might not represent the majority of the population in studies of gingivitis. After this relationship was noticed, we examined the records of subject 4, which showed that he had the lowest plaque index and gingival index scores of any of the subjects at the first pre-trial examination.

Differences in sample collection, bacterial identification schemes, and statistical analyses complicate comparison of our results with those from previous reports concerning the bacterial flora of samples from experimental gingivitis. For example, in the most extensive cultural study previously reported, Loesche and Syed (14, 24) cultured all of the visible plaque present at the gingival margin of the test site, whereas in the present study the accumulated supragingival plaque at the sites to be sampled was removed as completely as possible (with sterile toothpicks) before the sample to be cultured was obtained from the plaque approximating the gingival margin and in the sulcus. Some comparisons, however, are possible.

In their work, Syed and Loesche (24) found that the viable count does not increase with time after day 7, even though scores for gingival and plaque indices do increase after day 7. Indeed, Loesche and Syed observed that viable counts significantly decrease in sites with a plaque index of 2 as the gingivitis score increases from 0.5 to 1.0. They postulate that either length of exposure of the gingival tissues to the plaque accumulation or development of more virulent bacteria in the flora, not plaque size or bacterial numbers per se, is responsible for the development of gingivitis. Our results support this observation, for we found no increases in the subgingival viable count with increasing GI scores. Each sample was taken in a similar manner with similar instruments throughout each trial. Therefore, a more abundant population should have provided a larger sample and greater counts, yet no consistent increase was observed (Table 7). We confined our samples to the areas in and immediately adjacent to the gingival crevice. Because of the close proximity of this flora to the gingiva, the flora is expected to have the greatest influence on gingivitis. Products of the more distant supragingival flora might also diffuse to the gingiva, but acid irritants and other products could be neutralized, diluted, or removed by saliva before they come in direct contact with the gingiva.

Increased plaque index scores in the absence of increased viable counts in the present study and in Syed and Loesche's study (24) indicate that many of the bacterial cells in plaque are dead. This suggests that microscopy studies may overestimate the proportion of gram-negative cells, because old or dead cells of most gram-positive species stain gram-negative. The dead cells might possibly be a source of antigens and irritants that are released from cellular debris.

The data here are consistent with widely ac-

cepted clinical observations and several of the proposed theories concerning the causes of gingivitis and can be summarized as follows: The subgingival flora of persons with healthy gingiva who keep their teeth fastidiously clean is a complex flora of species in many different genera, especially gram-negative facultative species that are often associated with the nose and throat. The subgingival flora associated with moderately clean teeth and generally healthy gingiva is relatively simple and contains primarily *Actinomyces, Streptococcus*, and *Veillonella* species. These appear to be a prerequisite to colonization and increases in the proportions of specific *Actinomyces, Streptococcus, Fusobacterium, Veillonella*, and *Treponema* species (listed above) that are reproducibly associated with clinical signs of early gingivitis. When gingivitis progresses, additional species appear to colonize the subgingival flora in response to bleeding or increased serum. They further increase the complexity of the flora and include many species that have been found in sites with periodontitis. The data also indicate that the progression of bacterial species is required, rather than a simple increase in the quantity of the same initial species, and that *Capnocytophaga* and pigmented *Bacteroides* species are not important in the initiation of gingivitis. There is no evidence that the floras of different people respond differently to increasing severity of gingivitis.

## APPENDIX

**The need for an informal interpretation of the value of *t* in Table 8.** As mentioned in the main body of this paper, the values of *t* in Table 8 must be interpreted in the spirit of "exploratory data analysis," because their distributions are not the familiar ones of Student's *t* test. To see this, imagine a somewhat analogous situation in which we were comparing two groups of vectors, $x^{(1)}, x^{(2)}, \ldots, x^{(m)}$ and $y^{(1)}, y^{(2)}, \ldots, y^{(n)}$, by comparing the "within" sum

$$W = W_1 + W_2 = \Sigma_{i,j}^{i<j} [x^{(i)} - x^{(j)}]^2 + \Sigma_{i,j}^{i<j} [y^{(i)} y^{(j)}]^2$$

with the "between" sum

$$B = \Sigma_{i=1}^{m} \Sigma_{j=1}^{n} [x^{(i)} - y^{(j)}]^2.$$

It would certainly not be correct to regard this comparison as one between 1/2 $[m(m-1) + n(n-1)]$ independent expressions on the one hand and $mn$ independent expressions on the other. In fact, for example (6):

$$\Sigma_{i,j}^{i<j} [x^{(i)} - x^{(j)}]^2 = m \Sigma_i [x^{(i)} - \bar{x}]^2,$$

where $\bar{x} = \frac{1}{m} \Sigma_i x^{(i)}$, so that the $m(m-1)/2$ expressions in the sum over all pairs have only $m - 1$ degrees of freedom. Moreover, it is an algebraic identity that $B = W_1 + W_2 + 4m^2(\bar{x} - \bar{z})^2$ when $m = n$ and where $\bar{z}$ is $[\Sigma x^{(i)} + \Sigma y^{(i)}]/(m + n)$. Thus, $B - W_1 - W_2$ has only one degree of freedom.

When similarities are used instead of sums of squares, exact mathematical inferences about the distribution probably are intractable other than by a simulation calculation.

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