*Journal of* Clinical
Periodontology

# Subgingival microbiota of chronic periodontitis subjects from different geographic locations

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## Abstract

**Background:** Most clinical studies assume that the subgingival microbiota is similar from one geographic location to another. The purpose of the present investigation was to examine the composition of the subgingival microbiota in chronic periodontitis subjects from four countries.

**Method:** Subjects with chronic periodontitis ($N$, Sweden = 101; USA = 115; Brazil = 58; Chile = 26) were recruited. Subjects were measured at baseline for plaque, gingivitis, bleeding on probing (BOP), suppuration, pocket depth (PD) and attachment level (AL) at six sites per tooth. Subgingival plaque samples taken from the mesial aspect of each tooth at baseline were individually analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization (total samples = 6036). % DNA probe counts comprised by each species was determined for each site and averaged across sites in each subject. Significance of differences in proportions of each species among countries was determined using ANCOVA adjusting for age, mean pocket depth, gender and smoking status. $p$-Values were adjusted for multiple comparisons.

**Results:** On average, all species were detected in samples from subjects in the four countries. Thirteen species differed significantly in adjusted mean proportions among countries even after adjusting for multiple comparisons. *Porphyromonas gingivalis*, one species that differed in proportions among countries, comprised adjusted means of 7.5, 11.9, 1.6 and 6.6% of the microbiota in subjects from Brazil, Chile, Sweden and USA ($p < 0.001$), while mean proportions of *Treponema denticola* were 6.7, 4.2, 0.8 and 2.3, respectively ($p < 0.001$). In contrast, a key periodontal pathogen, *Tannerella forsythensis*, exhibited mean proportions ranging from 6.2–8.5% and did not differ significantly among countries. Besides these species, prominent species in Brazil were *Actinomyces naeslundii* genospecies 1 and 2 (8.4%, 7.2%) and *Prevotella intermedia* (6.5%); in Chile, *Prevotella melaninogenica* (6.4%) and *Neisseria mucosa* (5.3%); in Sweden *A. naeslundii* genospecies 2 (8.4%), *Capnocytophaga gingivalis* (7.1%) and *Peptostreptococcus micros* (5.0%); in USA *A. naeslundii* genospecies 2 (7.5%), *P. intermedia* (6.8%) and *C. gingivalis* (6.1%).

**Conclusions:** The microbial profiles of subgingival plaque samples from chronic periodontitis subjects in four countries showed surprisingly marked differences. These differences persisted after adjusting for age, mean pocket depth, gender and smoking status.

Studies testing the efficacy of different forms of periodontal treatment are carried out in many institutions and practices in different parts of the world. It has commonly been assumed that results obtained in one geographic location may be directly extrapolated to others. While in a given study, some individuals respond better to a given therapy than others, the variations in clinical response have usually been thought to be due to differences in: patient compliance, host response, or the impact of environmental factors such as cigarette smoking. Thus, the prevailing attitude was that, on average, subjects with similar forms of periodontal disease in different geographic

locations would exhibit essentially the same response to therapy. It had been assumed (if it was ever thought about) that on average, the microbial challenge would be consistent from study to study. That is, the mean subgingival microbial profiles in a given periodontal disease state, would be similar from one geographic location to another. This notion arose partly from an assumed constant microbial challenge. Further, most microbiological studies performed to date have indicated that each tested subgingival species may be found in virtually any geographic location (Cao et al. 1990, Sanz et al. 2000, Yano-Higuchi et al. 2000, Dowsett et al. 2002, Papapanou et al. 2002). The few reports that have directly compared dental plaque microbiotas in individuals from different geographic locations, however, have shown differences in microbial composition (Cao et al. 1990, Sanz et al. 2000). In particular, a series of surveys of the plaque microbiotas obtained from individuals residing in different countries, when taken together, suggest that there may be substantial differences in prevalence and/ or levels of a range of subgingival species (Sanz et al. 2000, Yano-Higuchi et al. 2000, Papapanou et al. 2002).

These findings raised the concern that differences in the subgingival microbial challenge from one geographic location to another might impact on the outcome of studies of periodontal therapy. Advantage was taken of a series of ongoing clinical studies performed in different countries that were designed to evaluate the changes in clinical and microbial parameters brought about by different individual or combinations of therapies. Thus, the purpose of the present investigation was to compare the pre-therapy subgingival microbial profiles in samples obtained from subjects with "chronic periodontitis" who were enrolled in treatment studies in four countries.

## Material and Methods
### Subject population

Three hundred adult subjects with chronic periodontitis who were enrolled in periodontal treatment studies in Rio de Janeiro, Brazil, Santiago, Chile, Gothenburg, Sweden and two centers in Boston, USA were selected for the study. Human Investigational Review Boards at the five collaborating institutions approved the protocol, including the taking of clinical measurements and plaque samples. All subjects were informed of the nature, potential risks and benefits of study participation and signed informed consent prior to entry into the study. Subjects ranged in age from 24 to 82 years of age. Included subjects were $> 20$ years of age and had at least 14 natural teeth. Subjects exhibited at least four pockets of $\geqslant 4$ mm and at least four sites with attachment level measurements of at least 3 mm or more. Exclusion criteria included pregnancy, nursing, periodontal therapy and antibiotic administration within the previous 3 months as well as any systemic condition that might have affected the progression of periodontitis. Individuals who required antibiotic coverage for routine periodontal procedures were also excluded. No subjects with localized aggressive periodontitis, rapidly progressive periodontitis or acute necrotizing ulcerative gingivitis were included in the study. Demographic parameters as well as smoking histories were obtained using a questionnaire. Members of the study teams reviewed all answers with the subject. The baseline clinical parameters for the subject groups in the four countries are presented in Table 1. The subgingival microbiota of the Chilean subjects has been described previously (Lopez et al. 2003) but is included to permit direct comparison with the subgingival microbiotas detected in subjects from the other three countries.

### Clinical measurements

Measures of plaque accumulation (0/1), overt gingivitis (0/1), bleeding on probing (BOP, 0/1), suppuration (0/1), probing pocket depth (PD) and probing attachment level (AL) were taken at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars at a baseline visit. PD and AL measurements were made using a North Carolina probe in all populations except the Chileans where a Florida probe was employed.

### Microbiological assessment

Subgingival plaque samples were taken at baseline from the mesiobuccal aspect of all teeth (excluding third molars) in the subjects from Chile, Sweden and USA. For the Brazilian subjects, samples were taken from the four deepest sites and three sites with PD $< 4$ mm. Samples were individually analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization. The samples from the USA, Sweden and Chile were analyzed in the same periodontal laboratory at The Forsyth Institute. The samples from Brazil were analyzed in Brazil using the same technique by an individual trained in the Forsyth laboratory. Counts of 40 subgingival species were determined in each plaque sample using

*Table 1.* Mean ( $\pm$ SD) clinical parameters of the subjects in the four geographic locations

| | Brazil | Chile | Sweden | USA |
|---|---|---|---|---|
| *N* | 58 | 26 | 101 | 115 |
| age (years) | $44 \pm 7$ | $45 \pm 8$ | $55 \pm 10$ | $47 \pm 10$ |
| number of missing teeth | NA | $5 \pm 3$ | $12 \pm 2$ | $3 \pm 3$ |
| % males | 38 | 27 | 37 | 66 |
| % sites with | | | | |
| plaque | $83 \pm 15$ | $84 \pm 13$ | $39 \pm 28$ | $58 \pm 28$ |
| gingival redness | NA | NA | $38 \pm 26$ | $75 \pm 23$ |
| BOP | $78 \pm 17$ | $42 \pm 19$ | $56 \pm 28$ | $49 \pm 22$ |
| suppuration | $11 \pm 15$ | NA | $2 \pm 4$ | $3 \pm 5$ |
| Mean PD (mm) | $3.98 \pm 0.81$ | $2.63 \pm 0.50$ | $4.47 \pm 0.67$ | $3.64 \pm 0.71$ |
| Mean AL (mm) | $4.58 \pm 1.00$ | $3.70 \pm 0.77$ | $4.57 \pm 1.36$ | $3.57 \pm 1.13$ |
| % current smokers | 2 | 46 | 62 | 25 |
| Racial/ethnic background | | | | |
| % White | 0 | 0 | 99 | 60 |
| % African descent | 0 | 0 | 0 | 27 |
| % Hispanic* | 100 | 100 | 0 | 10 |
| % Asiatic | 0 | 0 | 1 | 3 |

Statistically significant differences among countries existed for each of these clinical parameters.
*The designation "Hispanic" may be less than optimal to describe racial/ethnic background. For example, the Chilean subjects were selected from the population of Santiago where the subjects were an admixture of Aboriginal American Indians and Spaniards. This admixture may not have been typical of "Hispanic" subjects in Brazil or Boston.
NA, not available; BOP, bleeding on probing; PD, pocket depth; AL, attachment level.

a modification (Haffajee et al. 1997) of the checkerboard DNA–DNA hybridization technique (Socransky et al. 1994). In brief, after the removal of supragingival plaque, subgingival plaque samples were taken using individual sterile Gracey curettes from the mesial aspect of each tooth. The samples were placed in separate Eppendorf tubes containing 0.15 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and 0.15 ml of 0.5 M NaOH was added. The samples were stable in this solution at room temperature for at least 3 months and thus could be sent to Forsyth without freezing or drying. All samples were received for analysis well within the 3 month window. On arrival at Forsyth, the samples were lysed and the DNA placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA, USA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to 40 subgingival species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes detected using antibody to digoxigenin conjugated with alkaline phosphatase and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL, USA) and were read using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA), a computer-linked instrument that read the intensity of the fluorescence signals resulting from the probe-target hybridization. Two lanes in each run contained standards at concentrations of $10^5$ and $10^6$ cells of each species. The sensitivity of the assay was adjusted to permit detection of $10^4$ cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the Storm Fluorimager and converted to absolute counts by comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero. A total of 6036 subgingival samples were evaluated.

### Data analysis

The % of the total DNA probe count was determined for each species at each site in each subject and averaged within subjects in the different geographic

locations. The data from the two Boston locations, Boston University and The Forsyth Institute, were combined, since there were no significant differences in clinical and microbial parameters between the subjects sampled in the two institutions. In the analyses that did not include the Brazilian subjects, the data from all sampled sites were utilized. In the analyses that included the Brazilian subjects, only the data from the four deepest sampled periodontal pockets and the three shallowest pockets of the Chilean, Swedish and American subjects were employed. Samples from these sites were used in order to correspond to the sampling strategy employed in the Brazilian study. Significance of differences for each species across geographic locations was sought using ANCOVA, adjusting for age, mean pocket depth, gender and smoking status. *p*-Values were adjusted for multiple comparisons as described previously (Socransky et al. 1991).

### Results

Since the majority of subjects came from either Sweden or the USA, the first analysis compared the subgingival microbiota of subjects from these two countries (Fig. 1). Mean proportions of nine of the 40 test species differed significantly after adjusting for multiple comparisons. Species found in significantly higher proportions in Swedish subjects were *Streptococcus gordonii, Streptococcus sanguis, Campylobacter gracilis, Campylobacter rectus, Peptostreptococcus micros, Eubacterium saburreum, Propionibacterium acnes* and *Streptococcus anginosus. Porphyromonas gingivalis* was found in significantly higher proportions in the American subjects.

The Chilean subjects also provided samples from the mesio-buccal surface of all teeth excluding third molars and the samples were processed at The Forsyth Institute. Fig. 2 presents a comparison of data from the Chilean, Swedish and American subjects. *Actinomyces naeslundii* genospecies 2 and *C. gracilis* were found at significantly lower percentages in the Chilean than Swedish or American subjects, while *S. gordonii, Actinobacillus actinomycetemcomitans, Eubacterium nodatum, Fusobacterium periodonticum, P. gingivalis, Treponema denticola* and *Treponema socranskii* were found at highest

proportions in the Chilean individuals. The most striking differences in bacterial composition among countries was that observed for *P. gingivalis*. The Chilean subjects exhibited an adjusted mean percentage of 11.7, the Americans, 5.9 and the Swedes 0.9 (Fig. 2).

Fig. 3 presents the adjusted mean proportions of subgingival species in samples from the four deepest and three shallowest sampled periodontal pockets in the Brazilian, Chilean, Swedish and American subjects. Thirteen of the 40 species examined differed significantly among subjects in the four countries after adjusting for multiple comparisons. The Brazilian subjects exhibited the highest mean proportions of *Actinomyces naeslundii* genospecies 1*, S. gordonii, S. sanguis, Streptococcus intermedius, Streptococcus constellatus, E. nodatum* and *T. denticola*. The Chilean subjects exhibited the highest mean proportions of *P. gingivalis* and *F. periodonticum*. the Swedish subjects exhibited the highest mean proportions of *Capnocytophaga gingivalis, C. gracilis, P. micros* and *Leptotrichia buccalis*.

There were marked differences in the adjusted mean proportions of red complex species in samples taken from the seven sampled sites in the four populations that are highlighted in Fig. 4. *P. gingivalis* was found in highest proportions in the Chilean subjects, *T. denticola* in the Brazilian subjects. There was no significant difference among countries in the adjusted mean proportions of *T. forsythensis*. The high proportions of *Tannerella forsythensis* and somewhat lower proportions of *P. gingivalis* and *T. denticola* in the Swedish subjects were particularly striking.

### Discussion

The data from the present investigation indicate that the mean proportion of bacterial species in subgingival plaque samples taken from subjects with chronic periodontitis differed in different geographic locations. On average, the 40 test species could be detected in all populations examined. However, there were major differences in the proportions of many of the test species, particularly those thought to be associated with periodontal disease initiation and progression. In particular, the red complex species *T. forsythensis, P. gingivalis* and *T. denticola* showed quite different proportions in the four populations (Figs 3 and 4). Adjusted
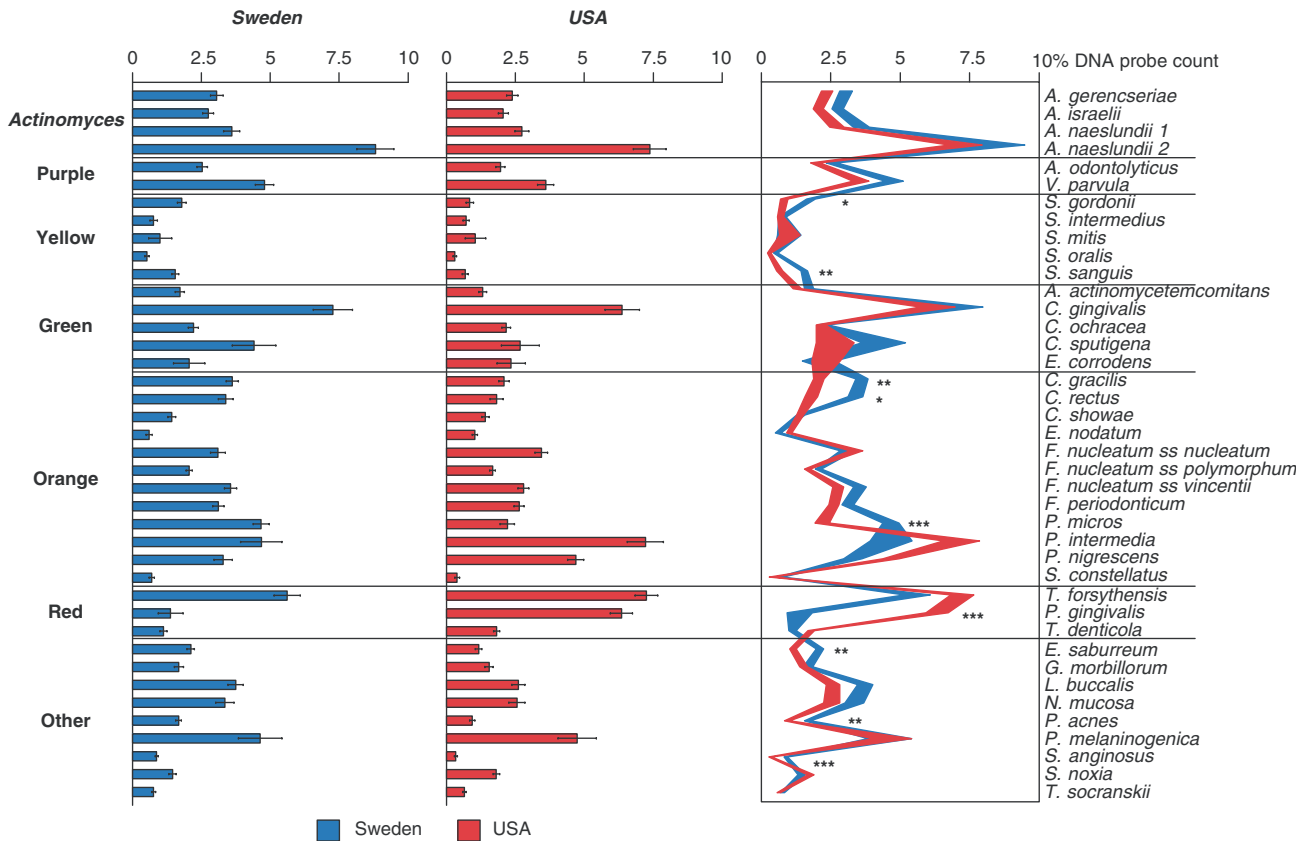
*Fig. 1.* Bar charts of adjusted mean percents (± SEM) of the total DNA probe count of 40 bacterial species in baseline subgingival plaque samples taken from 101 Swedish and 115 American chronic periodontitis subjects. The bars (left and middle panels) represent the mean percents after adjusting for age, mean pocket depth, gender and smoking status. The whiskers indicate the adjusted standard error of the mean. The profiles in the right panel represent the adjusted standard errors of the mean for each species in the subjects in each country. Mean percents of each species were computed by averaging up to 28 samples in each subject, and then averaging across subjects in the two countries. Significance of differences between groups was sought using ANCOVA adjusting for age, mean pocket depth, gender and smoking status; $*p<0.05$, $**p<0.01$, $***p<0.001$ after adjusting for multiple comparisons. The species were ordered and grouped according to the complexes described by Socransky et al. (1998). In order to facilitate country-to-country species comparisons, the right panel presents the microbial profiles as ''bands'' in which the width of the band is depicted as the adjusted means ± SEMs of each species. The blue profile represents the mean data for the Swedish subjects and the red profile represents the adjusted mean data for the American subjects.

mean proportions of *T. forsythensis* ranged from 6.2% to 8.5% of the microbiota with the highest proportions being found in the Chilean subjects. Adjusted mean proportions of *P. gingivalis* ranged from 1.6% to 11.9% of the microbiota with the Chilean and Swedish subjects providing the highest and lowest proportions, respectively. Adjusted mean proportions of *T. denticola* ranged from about 1–7% of the microbiota with the highest proportions being detected in the Brazilian subjects.

The reasons for the differences in mean subgingival microbial profiles are only partially understood. Some of the differences in mean proportions might be due to differences in pocket depth of the sampled sites. The Swedish subjects had the deepest mean pocket depth and the Chilean subjects had the shallowest mean pocket depth, although pocket

depth was measured in this latter group using a Florida probe. Nonetheless, the adjusted mean proportions of *P. gingivalis* and *T. forsythensis* were highest in the Chilean subjects (Fig. 4), suggesting that pocket depth might not be the sole or even the major factor controlling the level of colonization of these species. A second contributor to the differences might be previous exposure to periodontal therapy and/or systemically administered antibiotics. The majority of the Chilean and Brazilian subjects had received no or very limited periodontal therapy prior to their inclusion in the studies. On the other hand, the majority of Swedish and American subjects, had received periodontal therapy in the past, particularly SRP, and in some instances systemically administered antibiotics or even periodontal surgery. Although differences in prior treatment is an attrac-

tive explanation for the observed microbial differences, there were marked differences in the subgingival microbial profiles between the Swedish and American subjects (Fig. 1) even though both groups may have had similar ''dental backgrounds''. These differences were still present even when the analyses were restricted to a comparison between white American subjects and white Swedish subjects (data not shown). Other factors that might lead to differences in the composition of the subgingival microbiota include differences in genetic background, diet, culture, health care practices, socio-economic status, oral hygiene procedures and access to dental care. The South American subjects in this study were from lower socio-economic groups with less access to dental and medical care than the Swedish or American subjects.
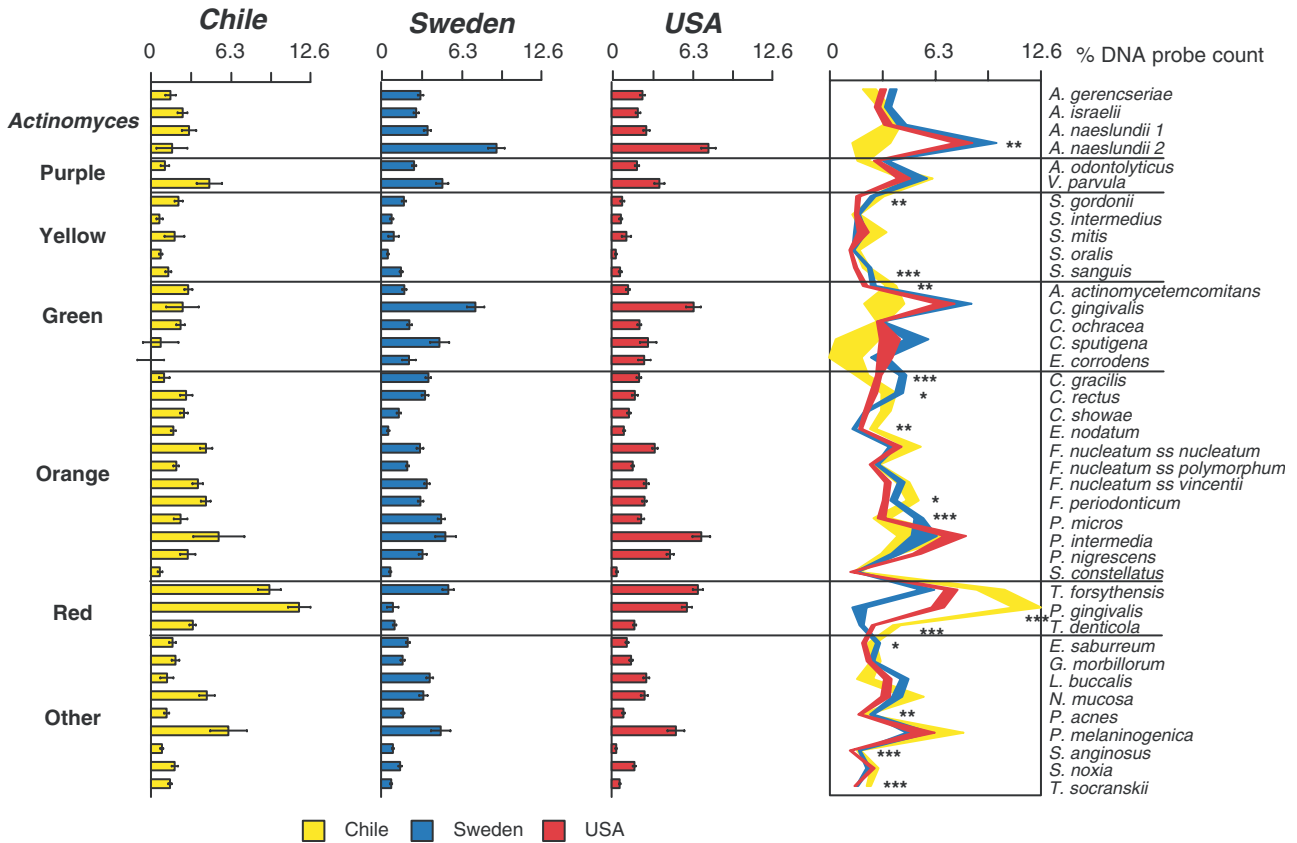
*Fig. 2.* Bar charts of adjusted mean percents (± SEM) of the total DNA probe count of 40 bacterial species in baseline subgingival plaque samples taken from 26 Chilean, 101 Swedish and 115 American chronic periodontitis subjects. The presentation of the data and the statistical analysis were as described for Fig. 1.

One conceptually intriguing factor that might affect the composition of the subgingival microbiota is the so-called ''herd effect''. In veterinary microbiology, it has been recognized that animals living in tight proximity often share similar diseases and microbiotas. The source of an individual's microbiota is, in large part, one's relatives and close neighbors. Thus, it would not be surprising that individuals living in the same community might, over time, develop subgingival or other microbiotas that were quite similar in composition to those of other individuals living in the same community. With the passage of time, it is likely that the ''mean'' microbiotas in different communities might ''drift'' apart leading to the type of differences described in the present or other studies (Cao et al. 1990, Sanz et al. 2000). It might be surmised that subjects living in cities with a major influx and efflux of individuals from different regions, such as cities with large university populations, might show a homogenization of the colonizing microbiotas. This proposition has not been tested.

The results of the present investigation were in accord with other investigators who found differences in the composition of the subgingival microbiota in samples obtained from individuals in different geographic locations. Sanz et al. (2000) used cultural techniques to compare the subgingival microbiota in samples obtained from adult subjects in Spain and The Netherlands who were selected on the basis of demonstrating comparable levels of chronic periodontitis. They found a higher prevalence of *P. gingivalis* in the Spanish subjects and a higher prevalence of *A. actinomycetemcomitans* and *P. micros* in the Dutch subjects. Cao et al. (1990) compared the predominant cultivable microbiota in supra and subgingival plaque samples taken from age-, gender- and periodontal disease-matched Caucasian subjects from Minneapolis, USA and citizens of China who were visiting that city. They found higher mean proportions of spirochetes, motile rods *Fusobacterium* species and ''dark-pigmented'' *Bacteroides* in supragingival plaque samples from the Chinese subjects and higher mean proportions of

*Streptococcus* and *Actinomyces* species in the Caucasian subjects. Differences in the subgingival microbiota were less marked.

The notion that the subgingival microbiota differs in composition in subjects in different geographic locations is also supported by a series of reports describing differences in the prevalence of various subgingival species using cultural (Sanz et al. 2000) or other techniques (Papapanou et al. 1997, 2002, Yano-Higuchi et al. 2000, Choi et al. 2000, Colombo et al. 2002, Dowsett et al. 2002). The weakness of these data is that subject selection, sample collection, disease status, the number of sampled sites/subjects and importantly, microbiological techniques, differed quite markedly among studies. Despite these reservations, marked differences in the prevalence of species, could be observed even for studies using similar microbiological techniques.

A major strength of the present investigation was that it overcame the concern outlined in the previous paragraph that geographic differences in subgingival plaque composition might
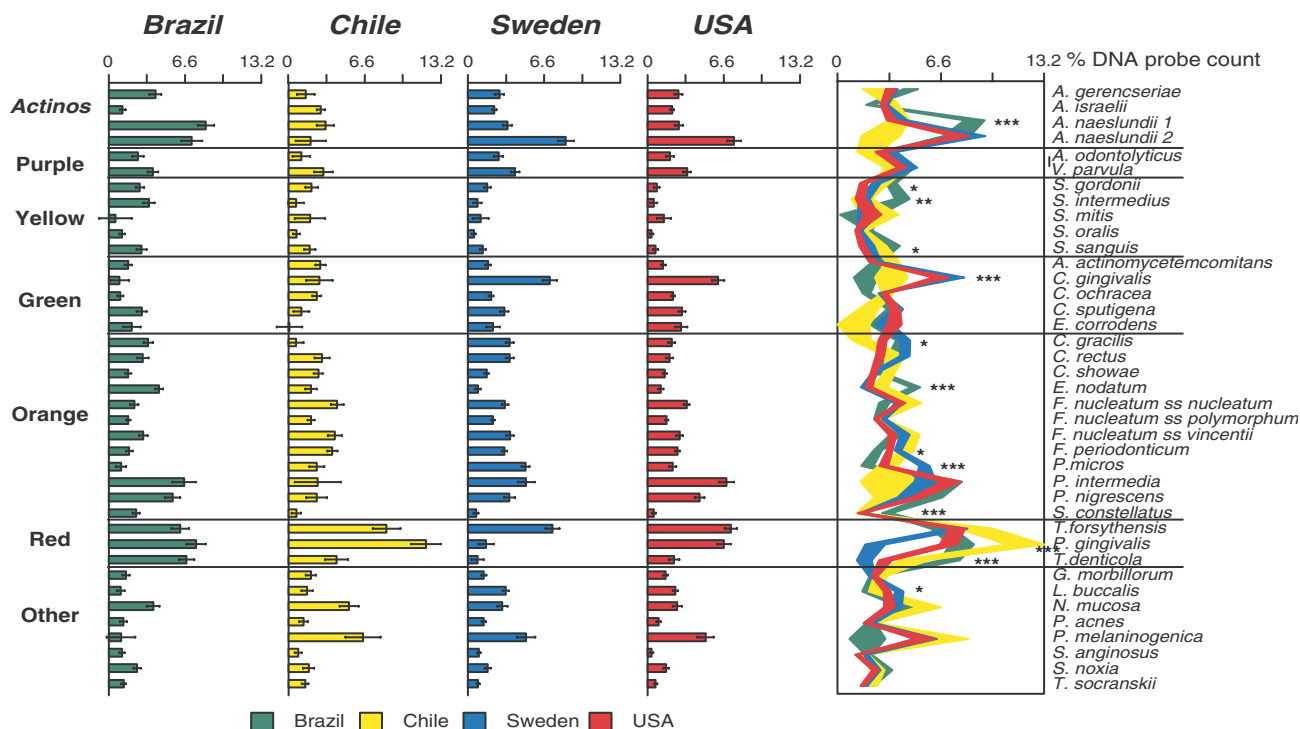
*Fig. 3*. Bar charts of adjusted mean percents ( ± SEM) of the total DNA probe count of 40 bacterial species in baseline subgingival plaque samples taken from 58 Brazilian, 26 Chilean, 101 Swedish and 115 American chronic periodontitis subjects. The presentation of the data and the statistical analysis were as described for Fig. 1 except that samples were taken from the four deepest and three shallowest sampled periodontal pockets of all subjects.
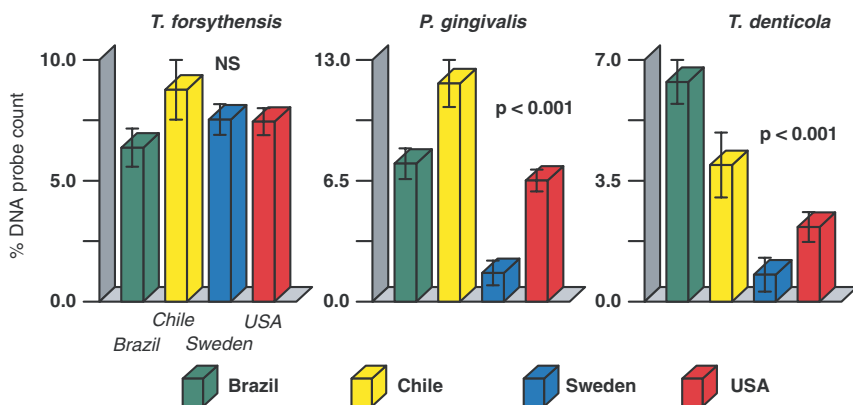


*Fig. 4*. Bar charts of adjusted mean percents (± SEM) of the total DNA probe count of the red complex species, *Tannerella forsythensis*, *Porphyromonas gingivalis* and *Treponema denticola* in baseline subgingival plaque samples taken from the four deepest and three shallowest, sampled periodontal pockets in 58 Brazilian, 26 Chilean, 101 Swedish and 115 American chronic periodontitis subjects. The bars represent the mean percents after adjusting for age, mean pocket depth, gender and smoking status. The whiskers indicate the standard error of the mean. Significance of differences among groups for each species was sought using ANCOVA adjusting for age, mean pocket depth, gender and smoking status. The p values were adjusted for 40 comparisons.

be due to differences in the microbiological techniques used in different studies. The plaque samples from Sweden, Chile and the USA were processed at the same time in the same laboratory at The Forsyth Institute by the same personnel using a common set of DNA probes and reagents. Further, an individual who had 3 years of training at Forsyth processed the samples from the Brazilian subjects in Brazil. Although every effort was made to standardize the taking of subgingival plaque samples, it was recognized that small differences in the amount of the plaque samples might have occurred in the different institutions. For this reason, proportions of individual species were used in the analyses rather than counts in order to overcome possible differences in microbial sample size. The combination of the DNA probe analysis of samples from the three countries being analyzed in one laboratory and the use of proportions of species rather than counts limited the likelihood that the observed differences in microbial profiles were due to differences in sample taking or microbiological technique.

There were certain limitations to this study. The subjects in each geographic location were not randomly selected and, thus, it is unclear what populations they represented. As described earlier, the subjects were self-selected since they sought enrollment in the various periodontal treatment studies. There were also significant differences among subject groups in terms of the baseline clinical and demographic features. Although all subjects were considered to have chronic periodontitis, subjects from Sweden had the deepest mean full

mouth pocket depths and the Chilean subjects the shallowest mean pocket depths. The US subjects had the least attachment loss based on mean full-mouth attachment level measurements. Swedish subjects had the highest mean age and the highest proportion of current smokers. Adjustment was made for these differences by using age, gender, mean pocket depth and smoking status as covariates in the analyses. However, these adjustments may not have been adequate. It is worth noting that statistical analyses performed without adjusting for the employed covariates exhibited as many or more ''statistically significant'' differences in species proportions than the analyses that adjusted for these factors. Lastly, different numbers of samples were taken in Brazil than in the other three countries, although the four deepest and three shallowest site samples in all countries were used in the analyses involving the Brazilian subjects (Figs 3 and 4).

An important consequence of these findings is the fact that the microbial differences can impact on therapeutic response. The subjects in the present study provide an example of this situation. Each subject was enrolled in a clinical study in one of the four countries, that was designed to examine the effect of one or more periodontal therapies on clinical and microbiological outcomes. The subject inclusion criteria were slightly different in each geographic location, but all subjects were considered to exhibit ''chronic periodontitis''. In spite of having the same disease diagnosis, there were clear differences in the composition of the subgingival microbiota from one geographic location to another. It is highly likely that subjects with different subgingival microbial profiles will exhibit different clinical and microbial responses to a given periodontal therapy. Thus, studies evaluating periodontal therapies using identical inclusion criteria and protocols, that are performed in different geographic locations, may produce conflicting results due to differences in the subgingival microbiotas rather than other factors including differences in host response, operator skills or subject compliance. A second consequence of these findings is that differences observed in periodontal disease severity in epidemiological studies in different regions of the world may be due in large part to differences in the nature of the colonizing subgingival microbiota. Thus, the data in the present investigation underscore the need to recognize and take into account potential differences in the composition of the subgingival microbiota when designing and interpreting clinical trials involving subjects in geographically different regions.

## Acknowledgments

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