Oral Microbiology Immunology 2009: 24: 183–189
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

The microbiota on different oral surfaces in healthy children

W. Papaioannou[1], S. Gizani[2], A. D. Haffajee[3], M. Quirynen[4], E. Mamai-Homata[1], L. Papagiannoulis[2]

[1]Department of Preventive and Community Dentistry, University of Athens, [2]Department of Paediatric Dentistry, University of Athens, Athens, Greece, [3]Department of Periodontology, The Forsyth Institute, Boston, MA, USA, [4]Department of Periodontology, Catholic University of Leuven, Leuven, Belgium

Papaioannou W, Gizani S, Haffajee AD, Quirynen M, Mamai-Homata E, Papagiannoulis L. The microbiota on different oral surfaces in healthy children. Oral Microbiol Immunol 2009: 24: 183–189. © 2009 John Wiley & Sons A/S.

Introduction: Knowledge of the early oral colonization patterns could provide a better understanding of oral biofilm development and disease initiation that in turn could be the basis for early preventive programmes.
Methods: Microbial samples were collected from five different oral habitats from a total of 93 children (age 3–12 years), attending the Dental School of the University of Athens, who were split into three age groups. A total of 38 microbial species were sought out by the checkerboard DNA–DNA hybridization technique.
Results: All of the test species, except *Parvimonas micra* and *Porphyromonas gingivalis*, differed significantly among sample locations providing quite distinct microbial profiles for the different oral surfaces. Supragingival and subgingival plaque had similar profiles and exhibited higher proportions of *Actinomyces* species and Green complex while soft tissue samples were dominated by streptococci of the Yellow complex. The profiles of the tongue dorsum and saliva were also similar. Many of the species were in similar proportions in all three age groups for a given location. Periodontal pathogens showed increases in proportions with increasing age. Specifically, the Red complex species (*Tannerella forsythia*, *P. gingivalis*, *Treponema denticola*) showed a significant increase in proportion with age ($P < 0.05$) in all sample locations.
Conclusions: The results showed a pattern of colonization in children similar to that previously found in adults. Differences in the profile between age groups suggest a gradual maturation of the oral microbiota, with it being made up of an increasing number of Orange and Red complex species.

Key words: children; oral habitats; oral microbiota; periodontopathogens

William Papaioannou, Department of Preventive and Community Dentistry, University of Athens Dental School, 2 Thivon Street, Athens 115 27, Greece
Tel.: +30 210 7461210;
fax: +30 210 7461132;
e-mail: vpapaio@dent.uoa.gr

Accepted for publication October 20, 2008

The oral cavity is a highly diverse ecosystem with up to 600 different microbial species colonizing the many different habitats. Colonization of the oral cavity begins as early as birth during the passage of the newborn through the birthing canal. Initially the oral microbiota is relatively simple in composition. However, a major ecological differentiation of the microbiota occurs with the eruption of the teeth, which present non-shedding surfaces onto which bacteria can adhere, leading to the formation of the dental plaque biofilm. The presence of the teeth differentiates the oral cavity from other sites of the body. Dental plaque is a complex and organized community of microorganisms that can potentially cooperate and communicate with each other leading to the creation of conditions conducive to the survival of more fastidious bacterial species. Moreover, it has been demonstrated that the bacteria colonizing one oral habitat can 'translocate' to other habitats of the oral cavity (25, 29).

Bacteria found in plaque biofilms are responsible for the aetiology of the two major oral diseases that can ultimately lead to tooth loss: caries and periodontitis. The association of specific pathogenic species with these diseases is the cornerstone of the specific plaque theory. *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus acidophilus* among others, have been associated with the initiation of dental caries whereas suspected periodontal pathogens include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*. These species are, for the most part, indigenous to the oral cavity, but when these pathogens initially colonize the oral cavity is unclear. Knowledge of the early oral colonization patterns could provide a better understanding of oral biofilm development and disease initiation, which in turn could be the basis for early preventive programmes.

The purpose of the present study was to examine the bacterial composition of oral plaque biofilms sampled from five different oral habitats in three different age groups of Greek schoolchildren using checkerboard DNA–DNA hybridization (38).

Materials and methods

Study population

A total of 93 children (age 3–12 years), attending the Dental School of the University of Athens, were selected for the present study. Subjects were in good general health and were not undergoing treatment for systemic or oral diseases (including antibiotic use or prophylactic dental cleaning in the previous 3 months) that may alter the oral microbiota or immune response. Additionally, the children showed no clinical signs of periodontitis as determined by no pockets or attachment loss > 3 mm. Subjects were recruited consecutively and divided into three groups based on age and dentition, with each group comprising 31 children (Table 1). Approval for the study was given by the Committee for Ethics and Research of the Athens Dental School, in accordance with the Helsinki Declaration. Parental informed consent was obtained for all children included in the study.

Clinical examination

The children were clinically examined in the dental clinic by one investigator. Measurements included the simplified Plaque Index (23), where the presence of plaque was assessed on all tooth surfaces to determine the level of oral hygiene, and the Gingival Index simplified (14), to determine gingival inflammation. Microbiological sampling was performed after the Plaque Index measurements and before the Gingival Index measurements.

Table 1. Characteristics of the three groups of children

	Age group (yrs) Range (yrs) Mean age (yrs)	Type of dentition	Number (boys/girls)
GR1	3–6 2.83–6.24 5.07	Primary	31 (18/13)
GR2	>6–9 6.50–9.00 7.80	Early mixed	31 (20/11)
GR3	>9–12 9.25–12.43 10.68	Mixed	31 (18/13)

Microbial sampling

Saliva sample

Each child expectorated whole unstimulated saliva into a sterile Petri dish. A 0.2-ml aliquot of the saliva was vortexed with 0.15 ml sterile, filtered Tris–HCl/ethylenediaminetetraacetic acid (EDTA) buffer (TE: 10 mM Tris–HCl, 1 mM EDTA, pH 7.6). A 0.2-ml sample of this mixture was then taken and placed into an Eppendorf tube and 0.1 ml of 0.5 M NaOH was added.

Subgingival plaque sample

Samples of the subgingival plaque were collected separately from either one of the second primary molars of the younger patients, or from one of the first permanent molars of the children older than 5 years with a mixed dentition. After removal of supragingival plaque, the sample teeth were isolated, dried and subgingival plaque samples were taken from the mesial–buccal aspect using sterile curettes. Each sample was placed into an individual Eppendorf tube containing 0.15 ml TE buffer to which 0.1 ml of 0.5 M NaOH was then added.

Tongue sample

Samples were taken from the tongue dorsum using a MasterAmp™ buccal swab brush (Epicentre Technologies, Madison, WI). One square centimetre of the centre of the dorsum of the tongue was brushed for 5 s for each child. The brush was swirled, to remove adhering bacteria, in an individual Eppendorf tube containing 0.15 ml TE buffer; 0.1 ml of 0.5 M NaOH was added to the solution.

Soft tissue sample

Samples from the soft tissues were obtained using a MasterAmp buccal swab brush. The areas that were swabbed were the buccal mucosa, the hard palate, the anterior vestibule and the mucosa of the maxillary and mandibular lips (17). The pooled sample was placed into an individual Eppendorf tube containing 0.15 ml TE buffer to which 0.1 ml of 0.5 M NaOH was then added.

Total supragingival plaque sample

Supragingival plaque was collected by swabbing all the erupted teeth with a MasterAmp buccal swab brush. The plaque from the swab brush was removed by washing, and diluting, in a tube with 1.5 ml TE buffer. From this, 0.2 ml of the solution was removed and placed into an individual Eppendorf tube and 0.1 ml of 0.5 M NaOH was added.

All samples were stored at −60°C until transportation to The Forsyth Institute (Boston, MA) for microbiological assessment using a modification of the checkerboard DNA–DNA hybridization technique (6). Thirty-eight microbial species were sought in each sample.

Microbiological assessment

Briefly, the samples were lysed and the DNA was placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). 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-labelled whole genomic DNA probes to 38 bacterial species were hybridized in individual lanes of the Miniblotter. After hybridization, the DNA probes presented the target DNA using chemifluorescence detection. A computer-linked instrument read the intensity of the fluorescent 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 the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals evaluated were converted to absolute counts by comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero (6).

Statistical analysis

Data available for all subjects were the counts of 38 bacterial species from five different oral habitats in each of 93 children. In addition, the mean proportion (percentage of the DNA probe count) that each species comprised of the total DNA probe count at each sample location was computed. The significance of differences among mean counts and proportions for each species among sample locations was determined using the Kruskal–Wallis test and adjusted for multiple comparisons (37). Subjects were subset into three age groups: 3–6, >6–9 and >9–12 years. The significance of differences in the proportions of the test species among the three age groups was determined using the Kruskal–Wallis test. Cluster analysis was performed using the species proportions at

the different sample locations in the 93 subjects. Similarities were computed using the minimum similarity coefficient (36) and sorted using an average unweighted linkage sort (35). The Kruskal–Wallis test was performed to examine the differences among the three age groups for the clinical data (the simplified Plaque and Gingival Indices). Level of significance was set at $P = 0.05$.

Results

Clinical findings

There was a significant reduction ($P = 0.002$) in oral cleanliness, as determined by the simplified plaque index, with increasing age (Fig. 1) that was accompanied by a significant increase ($P < 0.001$) in gingival inflammation (Fig. 2).

Bacterial profile of oral habitats

Overall, mean total DNA probe counts differed significantly among sample locations. The highest mean counts of bacteria were detected in samples from supragingival plaque (mean ± SD × 10^5: 220.6 ± 107.7) and the tongue dorsum (mean ± SD × 10^5: 216.8 ± 76.7) and the lowest counts were found on the soft tissues (mean ± SD × 10^5: 61.7 ± 40.0). Furthermore, each of the test species examined differed significantly among sample locations (data not shown). Fig. 3 presents the mean proportions of each of the test species at the five different sample locations. All of the test species, with the exception of *Parvimonas micra* (previously *Peptostreptococcus micros*) and *P. gingivalis* differed significantly among sample locations providing quite distinct microbial profiles for the different oral surfaces. Supragingival and subgingival plaque, derived from the non-shedding teeth, had similar profiles and exhibited higher proportions of *Actinomyces* species and Green complex species compared with

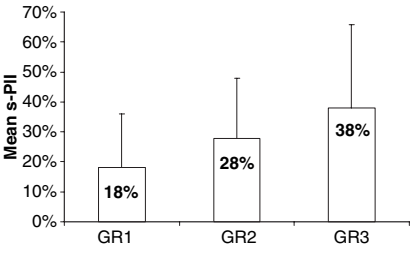

Fig. 1. Mean simplified Plaque Index (s-PlI) according to age group. Differences among the three groups were found to be significant at $P = 0.002$ (Kruskal–Wallis test).

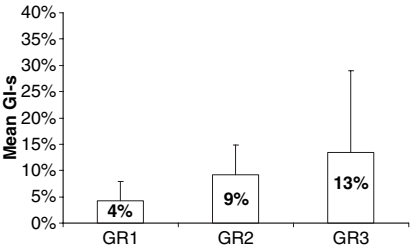

Fig. 2. Mean Gingival Index simplified (GI-s) according to age group. The Kruskal–Wallis test found the differences over the three groups to be significant ($P < 0.001$).

the other sample locations. The soft tissue samples were dominated by streptococci of the Yellow complex, particularly *Streptococcus mitis* (18.7%) and *Streptococcus oralis* (9.5%) as well as *Streptococcus salivarius* (9.5%). The profiles of the tongue dorsum and saliva were also similar and were characterized by high proportions of *Prevotella melaninogenica* (5.7% and 5.6%, respectively) and *S. salivarius* (10.9% and 10.7%, respectively). Not surprisingly, the periodontal pathogens of the Red complex were found at very low counts and proportions at all sample locations. The presence of *A. actinomycetemcomitans* in all children, albeit in low numbers and proportions, was noteworthy. Cluster analysis of the species proportions confirmed the similarity between certain microbial profiles as indicated above (Fig. 4). The supragingival and subgingival profiles clustered with >80% similarity and the tongue and saliva profiles clustered with >90% similarily. The soft tissue profile appeared to be distinct from the teeth, tongue and saliva profiles.

Effect of age on bacterial profiles

Fig. 5 presents the proportion of the test species at each sample location in children subset into the three age/dentition groups. Many of the species were in similar proportions in all three age groups for a given location. However, several species showed changes in proportions with age (Fig. 5). The *Actinomyces* species, particularly *Actinomyces israelii* and *Actinomyces naeslundii* genospecies 1, showed increased proportions with age at the supragingival, saliva and tongue locations. For example, the proportions of *A. israelii* in the three age groups were 1.4%, 1.9% and 2.2%, respectively, with the overall difference among groups being significant as well as between age groups 1 and 2 using a protected least squares significant difference (LSD). The streptococci also increased in proportion with age at most sample locations, particularly S*treptococcus gordonii* and *Streptococcus sanguinis.*

Suspected periodontal pathogens also showed increases in proportions with increasing age. *Campylobacter showae*, a member of the Orange complex showed a significant increase with age both supragingivally and subgingivally, particularly between age groups 2 and 3. Overall, across all sample locations, the Red complex species (*T. forsythia*, *P. gingivalis*, *T. denticola*) as a group showed a significant increase in proportion with age ($P < 0.05$). Specifically, *P. gingivalis* and *T. denticola* demonstrated a clear tendency for an increase in proportions for the five habitats, although these increases were not significant except for *P. gingivalis* in the tongue samples (Fig. 6). *T. forsythia* showed less change among the three age groups, and its levels remained around 1% of the total microbiota.

Table 2 presents the detection frequencies of putative periodontopathic species in the different age groups. *P. gingivalis* showed a higher dissemination in the oral cavity (i.e. more positive habitats) in the older age groups. However, the percentages and numbers of several of the other bacteria remained low, although there was a tendency for an increase with age.

Discussion

The aim of this study was to examine the oral microbiota at five distinct oral locations in healthy Greek children in different age groups. The samples examined included those from the gingival crevice, supragingival plaque, the tongue dorsum, other soft tissue surfaces and saliva. The microbial composition of the microbiota in the biofilm samples from these locations was examined using whole genomic probes to 38 species and the checkerboard DNA–DNA hybridization technique. The composition of the oral microbiota was determined for three different age groups to examine the effect of age and changes in the dental condition on the selected group of microorganisms.

The healthy children of the current investigation present an interesting group of subjects in whom to examine the composition of the oral microbiota because they had not experienced significant levels of oral diseases. However, the age groups examined in the present study have had time to develop a stable oral ecosystem. The subjects showed a decline in the level of oral hygiene as age increased with a concomitant increase in gingival

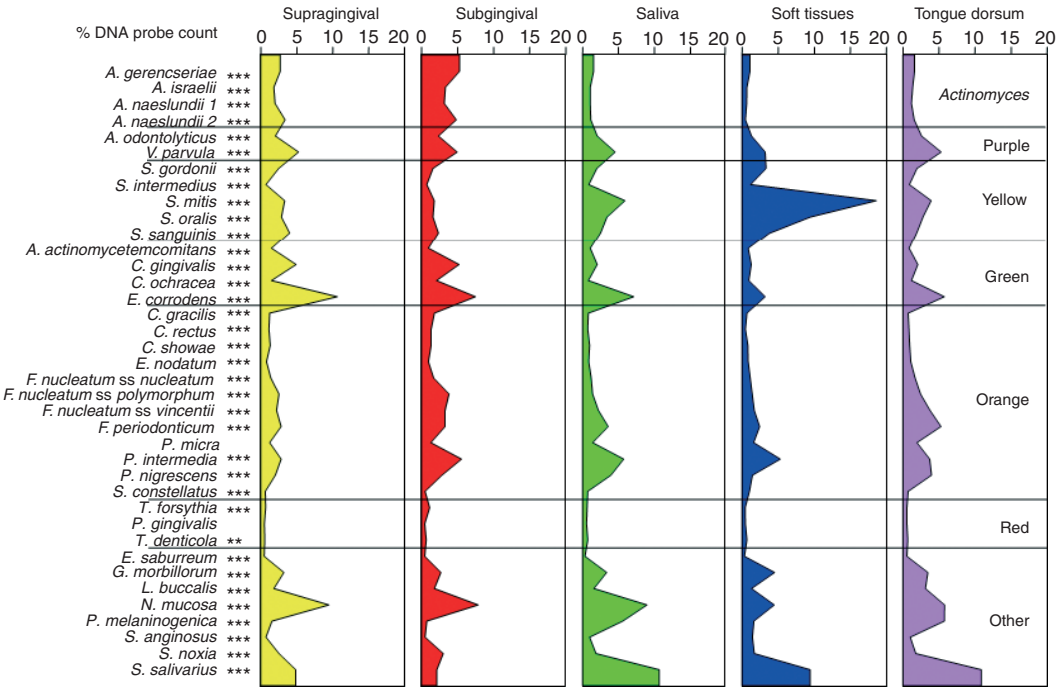

Fig. 3. Composition of microbiota in five oral habitats of young Greek schoolchildren according to the proportions of the DNA counts for the 38 bacterial species. Significance of differences between surfaces was determined by Kruskal–Wallis test adjusted for multiple comparisons: $^{**}P \leq 0.005$, $^{***}P \leq 0.001$.

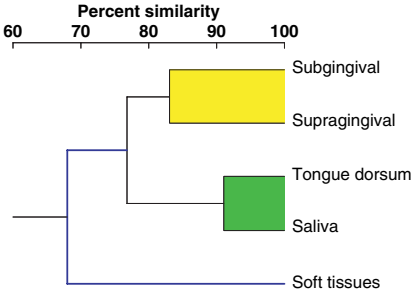

Fig. 4. Dendrogram of hierarchical cluster analysis for the mean species proportions from the five sample locations.

inflammation, both of which were found to be significant. However, this increase in plaque levels and inflammation did not lead to any significant periodontal disease.

The microbiological results showed that there were major differences in the composition of the microbiota on the different surfaces sampled. The soft tissue samples were dominated by streptococci. These species were also found at higher concentrations in the saliva and tongue samples compared to the tooth-associated samples. These findings are in agreement with previous studies that also found *S. mitis*, *S. oralis* and *S. salivarius* as the predominant species in saliva and soft tissue samples from children (16, 34). Moreover, *S. salivarius* has been found to be one of the primary early colonizers of the oral cavity (5, 34).

Unlike the soft tissues, which are constantly shedding the superficial cell layers, the hard tissues offer non-shedding surfaces that allow the development of more complex and mature biofilms. *Actinomyces* species were found in higher concentrations in both supragingival and subgingival samples compared to the soft tissues and saliva. This is in agreement with results from adult subjects (17). It was generally seen that there was an increase in species that form a bridge between the initial, or early, colonizers and the late colonizers. Early colonizers are primarily the streptococci that are unique in their abilities to coaggregate bacteria of the same genus as well as species of other genera (9, 10). Species in the genus *Fusobacterium*, which were found commonly in both supragingival and subgingival samples, act as bridging species between early and late colonizers (9–11, 44). Moreover, *Fusobacterium nucleatum* subspecies have an ability to adhere to mammalian cells, such as fibroblasts and epithelial cells, which can explain their presence in higher numbers on the tongue. *F. nucleatum* has been found in the oral cavities of infants during their first year of life, even before the eruption of teeth (12). Although it is present in periodontally healthy subjects, its presence in higher numbers has been associated with periodontal disease (19). In the current study, levels of Red complex pathogens, *P. gingivalis*, *T. forsythia* and *T. denticola*, remained very low, with values around or lower than 1% of the total microbiota, for all age groups and all habitats. Nevertheless, a slight shift towards the more pathogenic complexes in the subgingival region, especially in the older children, was observed especially for *P. gingivalis* and *T. denticola*, although these differences did not reach significant levels (Fig. 6).

Cluster analysis of species proportions revealed similarities between the microbial profiles of supragingival and subgingival biofilms at >80%, and between the tongue dorsum and saliva samples at >90%. This is in agreement with the results from adults (17). However, the level of similarity between the hard tissue profiles and those of the tongue dorsum and saliva was relatively high at over 75%. This would seem to support the use of samples from saliva or the tongue as being fairly representative of the oral microbiota. Indeed, saliva has been used to detect and estimate the oral levels of both periodontopathic (18, 24, 42) as well as cariogenic bacteria (15). The importance of the tongue as a

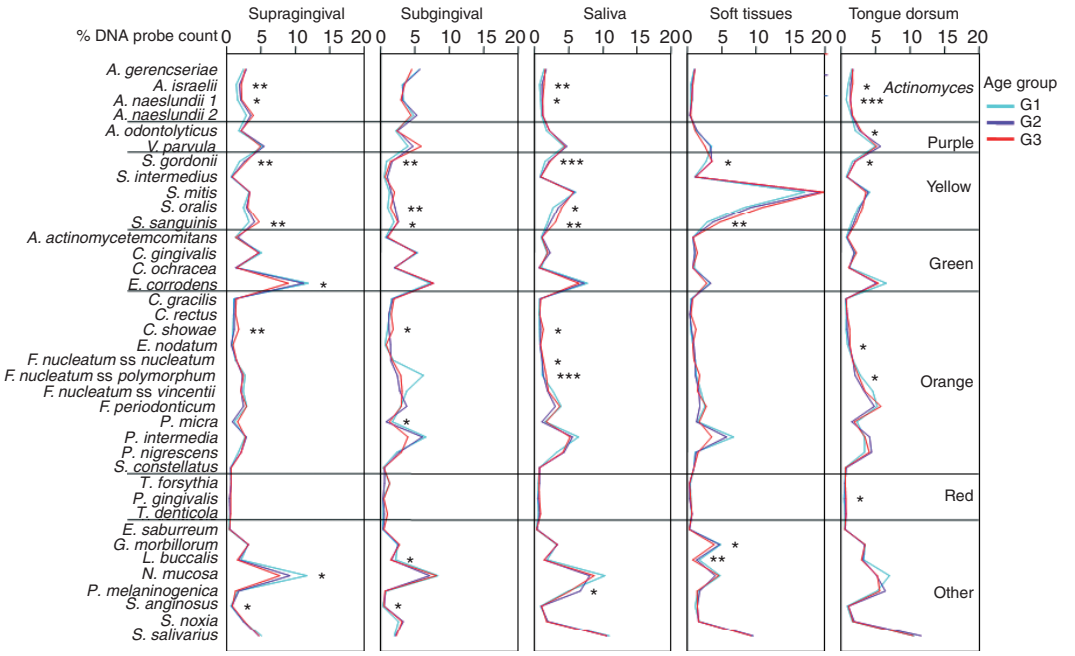

Fig. 5. Composition of microbiota in three groups of young Greek schoolchildren according to the proportions of the DNA counts for the 38 bacterial species. Significance of differences between age groups are denoted: *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.001$.

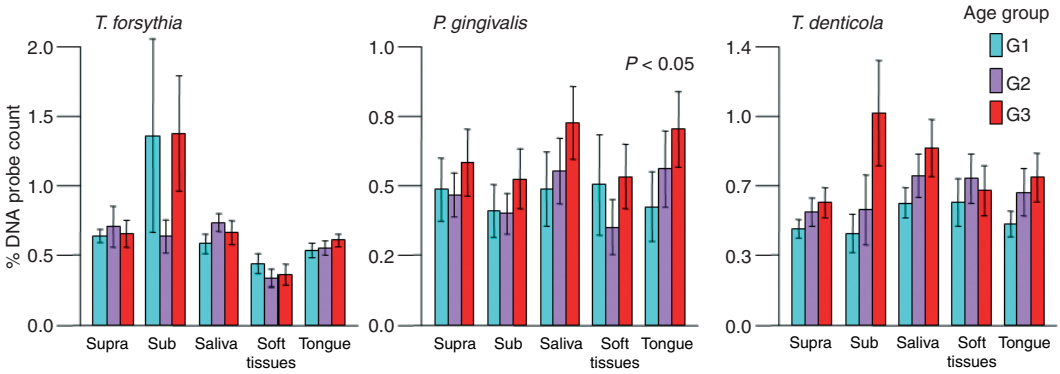

Fig. 6. The mean proportions of the specific Red complex bacteria, *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola*, for the three age groups.

Table 2. Percentage of patients, according to total number of sites (five habitats sampled), positive for specific putative periodontopathogens in each age group ($n = 31$ per group)

	Age group 1 (3–6)				Age group 2 (>6–9)				Age group 3 (>9–12)				
	Number of positive habitats				Number of positive habitats				Number of positive habitats				
Habitats	5	4	3	2	5	4	3	2	5	4	3	2	1
Aggregatibacter actinomycetemcomitans	87.1	12.9	–	–	93.5	6.5	–	–	90.3	6.5	3.2	–	–
Tannerella forsythia	58.1	22.6	12.9	6.5	51.6	38.7	9.7	–	45.2	25.8	22.6	6.5	–
Porphyromonas gingivalis	48.4	25.8	19.4	6.5	61.3	22.6	12.9	3.2	67.7	12.9	19.4	–	–
Treponema denticola	71.0	19.4	6.5	3.2	74.2	22.6	3.2	–	64.5	19.4	16.1	–	–
Prevotella intermedia	93.6	6.5	–	–	96.8	3.2	–	–	77.4	226	–	–	–
Parvimonas micra	96.8	3.2	–	–	87.1	12.9	–	–	93.5	6.5	–	–	–
Campylobacter rectus	51.6	22.6	12.9	12.9	48.4	25.8	22.6	3.2	45.2	25.8	16.1	12.9	3.2

reservoir of microorganisms, especially periodontopathic bacteria, should not be overlooked when attempting to reduce the oral bacterial load (4). Moreover, during the last decade the importance of the tongue and its complex microbiota in the aetiology and treatment of halitosis has generated great interest (30, 31).

The detection frequencies of the periodontopathogens in the children in the current study were high in the majority of the oral habitats. This is not a common

finding in the literature. One explanation for these high detection frequencies is the microbiological technique employed in this study. Studies comparing the detection sensitivity and specificity of checkerboard DNA–DNA hybridization with culture techniques and polymerase chain reaction (PCR) (27, 32, 33, 40, 43) reported a clearly higher detection frequency for *P. gingivalis* and *A. actinomycetemcomitans* for the two molecular tests, compared with culture. Another possibility is that false positives may have occurred because of binding of DNA probe or antibody conjugate to protein or other substances in large biofilm samples such as those for the supragingival and tongue samples. However, the detection frequencies in the present study were high even when small samples, such as those from the gingival crevice area, were examined.

Studies of the periodontal pathogen *A. actinomycetemcomitans* found low numbers of healthy young subjects positive for the presence of this organism using primarily cultural methods. For example, Ashley et al. (3) found only 18 of 89 subjects (20%), aged 14–15 years of age, positive for *A. actinomycetemcomitans.* Similar findings were reported by Alaluusua and Asikainen (2) for even the younger children, where 13% of a group of 55 Finnish children 4–7 years old were sampled. With more specific tests such as slot immunoblotting the prevalence of this bacterium was found to reach 75% (20). Studies using more sensitive PCR techniques gave results that ranged from around 5% up to and exceeding 50% depending on the sampling method and study population (8, 13, 22). In the current investigation, *A. actinomycetemcomitans* was found in at least one of the sample locations in all children.

T. forsythia, *P. gingivalis* and *T. denticola* are species of interest because of their association with periodontitis. Relatively high detection levels have been reported with molecular methods for *P. gingivalis* in even very young healthy children. One study reported around 40% of 6- to 36-month-old children (13) and another study reported 68.8% of children 18–48 months old harboured this species (45). Umeda et al. (42) examined Japanese children of an older age (mean age 8.3 years) and reported a detection level of 8.9% for *P. gingivalis*, while detection levels for *T. forsythia* and *T. denticola* were 42.9% and 48.2%, respectively.

It was believed that pathogenic microorganisms are detected infrequently in periodontally healthy subjects. The findings of the present study, however, show that 'pathogenic' species are more common in the oral cavity of young people, which is in agreement with a growing number of studies examining young people (13, 26, 41, 42, 45). There are indications that the presence of certain oral pathogens may be transient in nature for healthy children. Indeed, Ooshima et al. (24) using PCR concluded that pathogens such as *P. gingivalis*, *P. intermedia* and *T. denticola* appear to be transient oral colonizers. Lamell et al. (13) concluded the same for *A. actinomycetemcomitans* and *P. gingivalis*, although colonization by the latter was more stable in the later teenage years.

The present study found a significant effect of age on the proportions of the Red complex species as a whole, but not for the Orange complex. Tanner et al. (41) examined the microbiota of children aged 6–36 months and reported the detection of periodontopathic bacteria, such as *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans*, in even the youngest children of their study and with a tendency for higher detection in the older children. The increase in the proportions of *P. gingivalis* with age seen in the present study could possibly be linked with the increase in plaque and gingival inflammation levels. Nakagawa et al. (21) reported a positive correlation of increase in age, until puberty, and an increase in serum immunoglobulin G antibody levels against *P. gingivalis*, which in turn were correlated with oral levels of the organism. Similar findings were also reported by Morinushi et al. (20). In addition, a significant connection between clinical signs and inflammation and the levels of spirochaetes is known to exist (3). This in turn may explain the increase in the levels of *T. denticola*, especially in the subgingival habitat.

What is known about the microbiota in periodontal health and periodontitis has been derived principally from studies in adults, and these have focused on the biofilms that form on the teeth (1, 7, 26, 28). Mager et al. (17) examined the composition of the microbiota of the teeth, soft tissues and saliva in a large group of systemically healthy adults. However, to our knowledge, the current investigation is the first study to examine five different oral habitats for the levels and proportions of 38 bacterial species in a relatively large group of children using the checkerboard DNA–DNA hybridization technique. Other studies in children using similar microbiological techniques focused on tooth and tongue samples (41, 45) or examined a smaller number of bacterial species, limited primarily to periodontopathogens (18, 22, 42). The current study should provide information on the oral bacterial colonization pattern(s) of children that can have an impact on disease prevention strategies. Although colonization patterns of *S. mutans*, a major caries pathogen, have been mapped to a certain degree (39), our knowledge regarding the colonization patterns of periodontopathogens and the risk of future periodontal disease development is limited. This study may add to this knowledge by delineating the normal situations in a highly specific manner. Moreover, this would be the first study of its kind to examine a population of Greek children and it can lay the foundation for comparative studies with other groups of children that comprise the ever diversifying population of Greece, and specifically Athens.

The majority of the test species were found to be present from even an early age. Specifically, periodontal pathogens were present in almost all healthy children with high detection frequencies in all sites sampled. Although differences were found in the proportions of the bacteria colonizing the different habitats, certain locations showed greater similarities in their microbial profiles than others. Differences in the profile between age groups suggest a gradual maturation of the oral microbiota, but total proportions of the pathogenic species remained low. These findings would suggest that children from the age of 3 years already have an (early) acquired microbial community and that the oral microbiota is made up of an increasing number of Orange and Red complex species, with increasing age. To promote oral health, maintenance of a health-related oral microbiota is essential. Preventive efforts should therefore focus on preventing changes in the parameters responsible for the overgrowth of pathogenic bacteria, including oral hygiene, diet and host-related factors. Furthermore, the tongue and soft tissues may act as a reservoir for tooth-borne pathogens and require attention in therapeutic and preventive interventions.

Acknowledgments

The authors would like to acknowledge the invaluable assistance provided by E. Mandelou and T. Yaskell in the preparation and analysis of the samples. They are also grateful to S. Poulopoulou and Associate Professor U. Dafni for their statistical advice. The study was partially funded

by the University of Athens Special Account for Research Grants: 'Kapodistrias' programme (#70/4/8887).

References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 2005: **43**: 5721–5732.
2. Alaluusua S, Asikainen S. Detection and distribution of *Actinobacillus actinomycetemcomitans* in the primary dentition. J Periodontol 1988: **59**: 504–507.
3. Ashley FP, Gallagher J, Wilson RF. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis*, *Bacteroides intermedius* and spirochaetes in the subgingival microflora of adolescents and their relationship with the amount of supragingival plaque and gingivitis. Oral Microbiol Immunol 1988: **3**: 77–82.
4. Bollen CML, Vandekerckhove BNA, Papaioannou W, Van Eldere J, Quirynen M. Full- versus partial-mouth disinfection in the treatment of periodontal infections, a pilot study: long-term microbiological observations. J Clin Periodontol 1996: **23**: 960–970.
5. Carlsson J, Grahnen H, Wilkner S. Early establishment of *Streptococcus salivarius* in the mouth of infants. J Dent Res 1970: **49**: 415–418.
6. Haffajee AD, Cugini MA, Dibart S, Smith C, Kent RL Jr, Socransky SS. The effect of SRP on the clinical and microbiological parameters of periodontal disease. J Clin Periodontol 1997: **24**: 324–334.
7. Herrera D, Contreras A, Gamonal J et al. Subgingival microbial profiles in chronic periodontitis patients from Chile, Colombia and Spain. J Clin Periodontol 2008: **35**: 106–113.
8. Kimura S, Ooshima T, Takiguchi M et al. Periodontopathic bacterial infection in childhood. J Periodontol 2002: **73**: 20–26.
9. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 2000: **54**: 413–437.
10. Kolenbrander PE, Andersen RN, Moore LV. Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. Appl Environ Microbiol 1990: **56**: 3890–3894.
11. Kolenbrander PE, London J. Adhere today, here tomorrow: oral bacterial adherence. J Bacteriol 1993: **175**: 3247–3252.
12. Könönen E, Saarela M, Karjalainen J, Jousimies-Somer H, Alaluusua S, Asikainen S. Transmission of oral *Prevotella melaninogenica* between a mother and her young child. Oral Microbiol Immunol 1994: **9**: 310–314.
13. Lamell CW, Griffen Al, McClellan DL, Leys EJ. Acquisition and colonization stability of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in children. J Clin Microbiol 2000: **38**: 1196–1199.
14. Lindhe J. Text book of clinical Periodontology, 1st edn. Copenhagen: Munksgaard, 1981: 327–352.
15. Li Y, Caufield PW, Dasanayake AP, Wiener HW, Vermund SH. Mode of delivery and other maternal factors influence the acquisition of *Streptococcus mutans* in infants. J Dent Res 2005: **84**: 806–811.
16. Lucas VS, Beighton D, Roberts GJ. Composition of the oral streptococcal flora in healthy children. J Dent 2000: **28**: 45–50.
17. Mager DL, Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Distribution of selected bacterial species on intraoral surfaces. J Clin Periodontol 2003: **30**: 644–654.
18. Mättö J, Saarela M, Alaluusua S, Oja V, Jousimies-Somer H, Asikainen S. Detection of *Porphyromonas gingivalis* from saliva by PCR by using a sample-processing method. J Clin Microbiol 1998: **36**: 157–160.
19. Moore WEC, Moore LVH. The bacteria of periodontal diseases. Periodontol 2000 1994: **5**: 66–78.
20. Morinushi T, Lopatin DE, Van Poperin N, Ueda Y. The relationship between gingivitis and colonization by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in children. J Periodontol 2000: **71**: 403–409.
21. Nakagawa S, Machida Y, Nakagawa T et al. Infection by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, and antibody responses at different ages in humans. J Periodontal Res 1994: **29**: 9–16.
22. Okada M, Hayashi F, Nagasaka N. Detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in dental plaque samples from children 2 to 12 years of age. J Clin Periodontol 2000: **27**: 763–768.
23. O'Leary T, Drake RB, Nayer JE. The plaque control record. J Periodontol 1972: **43**: 38–40.
24. Ooshima T, Nishiyama N, Hou B et al. Occurrence of periodontal bacteria in healthy children: a 2-year longitudinal study. Community Dent Oral Epidemiol 2003: **31**: 417–425.
25. Papaioannou W, Quirynen M, van Steenberghe D. The effect of periodontal parameters on the subgingival flora around implants in partially edentulous patients. Clin Oral Impl Res 1995: **6**: 179–204.
26. Papapanou PN. Population studies of microbial ecology in periodontal health and disease. Ann Periodontol 2002: **7**: 54–61.
27. Papapanou PN, Madianos PN, Dahlen G, Sandros J. Checkerboard vs. culture: a comparison between two methods for identification of subgingival microbiota. Eur J Oral Sci 1997: **105**: 389–396.
28. Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
29. Quirynen M, Papaioannou W, van Steenberghe D. Intra-oral transmission and the colonization of oral hard surfaces. J Periodontol 1996: **67**: 986–993.
30. Quirynen M, Zhao H, van Steenberghe D. Review of the treatment strategies for oral malodour. Clin Oral Investig 2002: **6**: 1–10.
31. Roldán S, Herrera D, Sanz M. Biofilms and the tongue: therapeutical approaches for the control of halitosis. Clin Oral Investig 2003: **7**: 189–197.
32. Siqueira JF, Rocas IN, Souto R, Uzeda M, Colombo AP. Microbiological evaluation of acute periradicular abscesses by DNA–DNA hybridization. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: **92**: 451–457.
33. Siqueira JF, Rocas IN, De Uzeda M, Colombo AP, Santos KR. Comparison of 16S rDNA-based PCR and checkerboard DNA–DNA hybridisation for detection of selected endodontic pathogens. J Med Microbiol 2002: **51**: 1090–1096.
34. Smith DJ, Anderson J, King WF, van Houte J, Taubman MA. Oral streptococcal colonization of infants. Oral Microbiol Immunol 1993: **8**: 1–4.
35. Sneath PHA, Sokal RR. Numerical taxonomy. The principles and practice of numerical classification. San Francisco: W.H. Freeman & Co. 1973.
36. Socransky SS, Tanner ACR, Goodson JM et al. An approach to the definition of periodontal disease syndromes by cluster analysis. J Clin Periodontol 1982: **9**: 460–471.
37. Socransky SS, Haffajee AD, Smith C, Dibart S. Relation of counts of microbial species to clinical status at the site. J Clin Periodontol 1991: **18**: 766–775.
38. Socransky SS, Haffajee AD, Smith C et al. Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems. Oral Microbiol Immunol 2004: **19**: 352–362.
39. Straetemans MM, van Loveren C, de Soet JJ, de Graaff J, ten Cate JM. Colonization with mutans streptococci and lactobacilli and the caries experience of children after the age of five. J Dent Res 1998: **77**: 851–855.
40. Sunde PT, Tronstad L, Eribe ER, Lind PO, Olsen I. Assessment of periradicular microbiota by DNA–DNA hybridization. Endodont Dent Traumatol 2000: **16**: 191–196.
41. Tanner ACR, Milgram PM, Kent R Jr et al. The microbiota of young children from tooth and tongue samples. J Dent Res 2002: **81**: 53–57.
42. Umeda M, Miwa Z, Takeuchi Y et al. The distribution of periodontopathic bacteria among Japanese children and their parents. J Periodontal Res 2004: **39**: 398–404.
43. Watson PS, Sissons CH, Devine DA et al. Minimizing prior risk without compromising the microbial composition of biofilms grown in vivo in a human plaque model. Lett Appl Microbiol 2004: **38**: 211–216.
44. Whittaker CJ, Klier CM, Kolenbrander PE. Mechanisms of adhesion by oral bacteria. Annu Rev Microbiol 1996: **50**: 513–552.
45. Yang EY, Tanner AC, Milgrom P et al. Periodontal pathogen detection in gingiva/tooth and tongue flora samples from 18- to 48-month-old children and periodontal status of their mothers. Oral Microbiol Immunol 2002: **17**: 55–59.