Age-related microbiological changes in the salivary and plaque microflora of healthy adults

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Summary. The effect of age on quantitative or qualitative differences in selected bacteria of dental significance and on the carriage of opportunistic pathogens and transient oral species was determined in 79 healthy, non-denture wearing individuals divided into four age groups: 20–39 years (group A), 40–59 years (group B), 60–79 years (group C) and ≥ 80 years (group D). Samples of dental plaque and whole saliva were cultured on appropriate selective and non-selective bacteriological media. The total numbers of viable bacteria in saliva, and the prevalence of mutans streptococci in plaque and saliva were similar in all age groups. Similarly, there was no correlation between the numbers of spirochaetes in plaque and age. In contrast, statistically significantly higher mean proportions (p = 0.004), mean log_{10} viable counts (p < 0.01) and isolation frequencies (p < 0.01) of lactobacilli were found in the saliva of those aged ≥ 70 years compared to subjects in group A. The isolation frequency (p < 0.05) and proportions (p = 0.056) of staphylococci in saliva were also higher in those aged ≥ 70 years. Yeasts were isolated most often and in higher numbers from saliva in those aged ≥ 80 years and the proportion of yeasts was higher after 60 years of age, but these differences were not significant in comparison with results from individuals in group A. Actinomyces spp. were commonly isolated from plaque, but there was a change, with age, in the ratio of the proportions of A. viscosus and A. naeslundii so that A. viscosus predominated in elderly subjects (groups C and D). The results suggest that genuine age-related changes in the oral microflora can be detected, particularly after the age of 70 years, which are not related to denture-wearing or disease.

Introduction

The oral cavity comprises a series of different environments such as the tongue, cheek, palate, tooth surfaces and gingival crevice, which are colonised by different microfloras. These microfloras are influenced by host factors such as tooth eruption or loss of teeth, oral hygiene, and hormonal changes of the host.1 The numbers and types of micro-organisms in the mouth also change with age, particularly in the early years during the development of the dentition.2,3 Similarly, the isolation frequency of black-pigmented gram-negative anaerobic bacilli,4,5 spirochaetes,6–8 and Actinomyces viscosus9 are reported to increase in adolescents and young adults. However, much less is known of the effect of subsequent natural ageing processes on the stability of the resident oral microflora.

The host is able to maintain microbial homeostasis in the mouth by means of specific and innate host defences.10 These defences, together with the commensal microflora, act to prevent colonisation by non-resident species and opportunistic pathogens. Cell-mediated immunity is reported to decline with age,11 but little is known about the effect of age on the oral host defences,12 nor how this may be reflected in changes in the oral microflora. Thus, the aim of the present study was to compare the resident oral microflora of healthy subjects in different age groups. Because of the complexity of the microflora and the known inter-subject variation, only selected species of dental significance were chosen for comparison, together with species considered normally to be present only transiently, thereby acting as indicators of the breakdown of homeostasis.10

Materials and methods

Study population

A total of 79 healthy subjects took part in the study. They were sub-divided into four groups: (A) 20–39 years, mean age 26.8 years, ± 1.03, n = 30; (B) 40–59 years, mean age 50.1 years, ± 2.1, n = 23; (C) 60–79 years, mean age 72.5 years, ± 4.1, n = 16; and (D) ≥ 80 years, mean age 83.8 years, ± 0.9, n = 10. The subjects in groups A and B included staff and patients...
of Guy's Hospital, London, who were attending routine dental examinations, and groups C and D were elderly groups living mainly in sheltered residential accommodation comprising a mixture of houses, apartments and units with access to central dining facilities (Morden College, London). The study population satisfied the following requirements for inclusion within this project: (a) the presence of a minimum number of teeth (seven), including one molar; (b) the absence of active oral disease; (c) the absence of dentures; (d) no recent history of antimicrobial therapy or other drug therapy, including immunosuppressives; and (e) no history of diabetes. A haematological screen was performed on all subjects in groups C and D to exclude latent disease. These subjects also received regular medical checks.

Collection of saliva samples

Unstimulated whole saliva was collected from all subjects by direct expectoration into a sterile container during a 10-min period.

Collection of plaque samples

Supragingival molar plaque was collected above the gingiva on the lower right or left sixth tooth by running a standard dental probe along the buccal and mesio-buccal surfaces. Plaque samples were transferred into saliva and plaque samples were processed within 2-4 h of collection.

Microbiological procedures

Saliva samples were dispersed by vortex mixing for 30 s and 10-fold dilutions were prepared in reduced transport fluid. Samples of 100 μl at dilutions from 10^3 to 10^7 were plated in duplicate on to Columbia blood agar and incubated aerobically and anaerobically in an atmosphere of CO₂ 10% in H₂ at 37°C for 4 days, to determine the total numbers of aerobic and anaerobic cfu. The saliva samples from neat to a dilution of 10^4 were also plated on to a range of selective media to enable key micro-organisms to be enumerated. The media were Sabouraud Agar (Oxoid) for Candida spp., MacConkey Agar (Oxoid) for enteric bacteria, Rogosa Agar (Oxoid) for Lactobacillus spp., Mannitol-Salt Agar (Oxoid) for Staphylococcus spp. and TYC medium (Lab M, Bury) with sucrose 20%, w/v and bacitracin 0-1 units/ml (TYCSB) for mutants streptococci. Sabouraud, MacConkey, and Mannitol-Salt media were incubated aerobically at 37°C for 4 days, TYCSB medium was incubated anaerobically at 37°C for 4 days, and Rogosa medium was incubated in CO₂ 5-10% in air at 37°C for 4 days.

Plaque samples were held under CO₂ and dispersed by vortex mixing for 1 min with 3-mm glass beads to disaggregate any clumps of micro-organisms within the plaque. Plaque samples were treated in the same way as saliva samples except that, in addition to the above media, samples were plated on CFAT medium, which is selective for A. viscosus and A. naeslundii, and incubated anaerobically at 37°C for up to 10 days. Facultatively anaerobic, catalase-positive and catalase-negative isolates were identified as presumptive A. viscosus and A. naeslundii, respectively.

Plaque samples were also plated on Columbia blood agar supplemented with vancomycin 2.5 μg/ml and incubated anaerobically at 37°C for up to 21 days for the isolation of black-pigmented gram-negative anaerobic bacilli. Yeasts were identified with API 20C commercial galleries (API, Basingstoke) according to the manufacturer's instructions. Coagulase-positive colonies on Mannitol-Salt agar were considered to be S. aureus.

Dark field microscopy was used to determine the presence and numbers of spirochaetes in dispersed plaque samples. For each sample, 60 fields were viewed and the number of spirochaetes in each counted and summated. Results were expressed as a percentage of the viable count.

The reproducibility of the bacterial isolation techniques was tested in six volunteers by dividing the saliva samples in two. Subsequently, these were treated as separate samples and serially diluted; inocula were spread on duplicate plates of Columbia blood agar and selective media for yeasts, enteric bacteria, lactobacilli, staphylococci and mutants streptococci.

Statistical procedures

Viable counts were transformed to log₁₀ cfu; results are presented as the mean of the log₁₀ cfu, and were compared by analysis of variance and Student's t test. Isolation frequencies were compared by χ² test, with Yates's correction for small numbers where appropriate. Spearman rank correlations were performed on the whole study population to determine significant correlations between age and percentage viable counts and age and log₁₀ viable counts. Where data were to be log-transformed, and micro-organisms were not detected, the baseline was set as half of the minimum levels of detection. The latter were 10 cfu/ml of saliva or plaque sample for yeasts, lactobacilli and staphylococci, and 10³ cfu per sample for mutants and total streptococci or total cfu. This represented a detection sensitivity of about 1 in 10⁸ cfu.

Results

The mean haemoglobin concentrations of subjects in groups C and D were within the normal range and were not significantly different from those in the younger age groups, implying no latent disease in the elderly.

The mean variation in cfu (percentage varia-
The isolation frequency (table I) and proportions (fig. 1) of lactobacilli in saliva in each age group. A, 20-39 years; B, 40-59 years; C, 60-79 years; D, ≥ 80 years. Group C was subdivided into those aged 60-69 years (C,), and those aged 70-79 years (C,).

There were no clear age-related trends in the isolation of staphylococci from saliva. Although the highest proportions were found in those aged 70-79 years, and their proportions were higher in those aged ≥ 60 years (fig. 3), but these findings were not statistically significantly different from those in group A, mainly because of the large variation in counts between individuals. The majority of yeasts were identified as Candida albicans and this species was isolated from 19 (24%) of 79 subjects; 4 (5%) of 79 subjects had C. tropicalis and 3 (4%) of 79 subjects had C. parapsilosis. There was no discernible trend in the prevalence of mutants streptococci in saliva, although the highest proportions were found in those aged ≥ 80 years (group D) (fig. 4a). In 10 (13%) of 79 subjects salivary counts of mutants streptococci were > 10^6 cfu/ml. No enteric bacteria were isolated from the saliva of any subject (table I).

There were no significant differences in the total number of viable cells (cfu/ml) in saliva samples from individuals in the four age groups, although the lowest mean count was in those aged ≥ 80 years. The mean viable counts for age groups A, B, C and D were 9.22, 9.51 and 8.60 log,, cfu/ml. In contrast, statistically significant differences were found in the prevalence of certain groups of micro-organism in saliva. The isolation frequency (table I) and proportions (fig. 1) of lactobacilli in saliva were significantly higher (p = 0.02; group B, < p = 0.001, p=0.004, respectively). In the series as a whole there was a significant correlation between age and the presence of lactobacilli, expressed as either log,, cfu or percentage viable count (table IV).

The isolation frequency of staphylococci from saliva was also significantly higher (p < 0.05) from those in groups C and D (table I) than from group A; again the increases occurred primarily in those aged ≥ 70 years (p < 0.05). The mean log,, viable count (table II) and proportions (fig. 2) of staphylococci were highest in groups C and D, and in group D, respectively, although these findings were not significantly different from those in group A. The higher proportions of staphylococci in saliva of those aged ≥ 70 years did, however, approach statistical significance (p < 0.056). A mean of 49% of all staphylococcal isolates were coagulase positive and this proportion was similar in each age group. The isolation frequency (table I) and mean viable counts (table II) of yeasts in saliva were highest in those aged ≥ 80 years, and their proportions were higher in those aged ≥ 60 years (fig. 3), but these findings were not statistically significantly different from those in group A, mainly because of the large variation in counts between individuals. The majority of yeasts were identified as Candida albicans and this species was isolated from 19 (24%) of 79 subjects; 4 (5%) of 79 subjects had C. tropicalis and 3 (4%) of 79 subjects had C. parapsilosis. There was no discernible trend in the prevalence of mutants streptococci in saliva, although the highest proportions were found in those aged ≥ 80 years (group D) (fig. 4a). In 10 (13%) of 79 subjects salivary counts of mutants streptococci were > 10^6 cfu/ml. No enteric bacteria were isolated from the saliva of any subject (table I).

There were no clear age-related trends in the

Table I. Frequencies of isolation of selected micro-organisms from saliva (S) or plaque (P) as a function of age

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>A (n = 30)</th>
<th>B (n = 23)</th>
<th>C (n = 16)</th>
<th>D (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>P</td>
<td>S</td>
<td>P</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>.1</td>
<td>.4</td>
<td>.3</td>
<td>.0</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>.0</td>
<td>.0</td>
<td>.0</td>
<td>.0</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>3.0</td>
<td>1.0</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>3.0</td>
<td>1.0</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>BPGNAB</td>
<td>.7</td>
<td>.5</td>
<td>.7</td>
<td>.5</td>
</tr>
<tr>
<td>Mutans</td>
<td>.7</td>
<td>.5</td>
<td>.7</td>
<td>.5</td>
</tr>
<tr>
<td>streptococci</td>
<td>.4</td>
<td>.5</td>
<td>.4</td>
<td>.5</td>
</tr>
<tr>
<td>A. viscosus</td>
<td>.4</td>
<td>.5</td>
<td>.4</td>
<td>.5</td>
</tr>
<tr>
<td>A. naeslundii</td>
<td>.5</td>
<td>.6</td>
<td>.5</td>
<td>.6</td>
</tr>
</tbody>
</table>

Table II. Viable counts of selected micro-organisms in saliva as a function of age

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Mean log,, (SEM) viable count from group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (n = 30)</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>1.76 (0.30)</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>2.71 (0.34)</td>
</tr>
<tr>
<td>Mutans</td>
<td>4.48 (0.20)</td>
</tr>
<tr>
<td>streptococci</td>
<td>1.40 (0.24)</td>
</tr>
</tbody>
</table>

*Group A v D, NS (p = 0.06); group A v C, p = < 0.001.
†Group B v C, p = < 0.02; group B v D, p = < 0.05.
‡No significant differences.
Table III. Viable counts of selected micro-organisms in dental plaque as a function of age

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Mean percentage count (SEM) in group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (n = 30)</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>0.01 (0.01)</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>0.75 (0.47)</td>
</tr>
<tr>
<td>Yeasts</td>
<td>0.43 (1.55)</td>
</tr>
<tr>
<td>Spirochaetes ($\times 10^6$)</td>
<td>142 (97)</td>
</tr>
</tbody>
</table>

isolation frequency (table I) or proportions (table III) of yeasts, lactobacilli or staphylococci from dental plaque. The cell counts of spirochaetes appeared to decrease with age on a group basis (table III) but this was not statistically significant. However, on an individual basis in the whole series, there was a significant negative correlation between age and $\log_{10}$ percentage viable count ($r = -0.245$, $n = 79$, $p < 0.05$), although there was no obvious correlation between spirochaetes and the gingival index at the sample site or in the mouth as a whole. Black-pigmented gram-negative anaerobic bacilli were isolated infrequently (but more often from group A; table I) and in low numbers from dental plaque, irrespective of age. No enteric bacteria were isolated from plaque samples (table I). In contrast, Actinomyces spp. were commonly detected in plaque; the majority of isolates resembled A. viscosus and A. naeslundii (table I). In the younger age groups (A and B), A. naeslundii was in the highest proportions whereas the situation was reversed in those aged $\geq 60$ years, in whom A. viscosus predominated (fig. 5). The ratios of A. viscosus to A. naeslundii in age groups A, B, C and D were 0.76, 0.25, 2.04 and 2.46, respectively. There were also lower proportions of mutans streptococci in plaque from the most elderly subjects, but this trend was not statistically significant (fig. 4b).

Discussion

There is little information about the effect of natural ageing processes on the stability of the resident human microflora. Such information could be of value to determine the treatment needs for this increasing sector of the population, and may identify individuals at risk of certain infections.

There are several problems in the design of studies which attempt to correlate changes in the microflora with age. These include lack of a universally accepted definition of any age group; clearly, the chronological age of a person does not necessarily equate with their "physiological" age. Similarly, individuals in population groups generally have to satisfy certain minimum requirements for inclusion in a study. These may relate to the presence of a certain number of teeth, the absence of dentures or of active disease, or no recent history of medical treatment. Such requirements may be met easily by most of those in the younger age groups but a study population which involves the elderly may be skewed by the inclusion of a disproportionate number of so-defined "healthy" individuals.

Table IV. Correlation between age and percentage viable count and $\log_{10}$ viable count

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Spearman rank correlation with percentage viable count</th>
<th>Spearman rank correlation with $\log_{10}$ viable count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age rs yeast</td>
<td>0.352</td>
<td>0.971</td>
</tr>
<tr>
<td>Age rs lactobacilli</td>
<td>1.966*</td>
<td>3.182†</td>
</tr>
<tr>
<td>Age rs staphylococci</td>
<td>0.428</td>
<td>0.733</td>
</tr>
<tr>
<td>Age rs mutans streptococci</td>
<td>0.899</td>
<td>1.212</td>
</tr>
</tbody>
</table>

*p = 0.05.
†p = 0.002.
who are regarded as "normal" but may not be representative of the elderly. Finally, even if quantitative differences in the microflora do occur with age, the known wide inter-site and inter-subject variations can make it difficult to establish these changes in a cross-sectional survey. Our inclusion of only healthy individuals, even in the most elderly age groups, was intended to prevent any confusion with changes in the oral microflora which might be due to disease or to any indirect age-associated factors, such as medication or denture-wearing, and is a unique feature of this study.

In the young, changes in the oral microflora that have been related directly to age are reasonably well established and are usually associated with major perturbations to the habitat (e.g., tooth eruption, hormonal changes, etc.). In adults, the resident microflora is believed to remain relatively stable unless the environment is disturbed, as occurs during disease, but there have been few studies of the oral microflora of healthy elderly subjects. Qualitative differences in the microbial composition of plaque between young and elderly individuals were inferred from the biochemical composition and enzyme activity of pooled samples, but a detailed bacteriological analysis was not reported.

The isolation frequencies of \( A. viscosus \), black-pigmented gram-negative anaerobic bacilli, and spirochaetes are reported to increase with age in adolescents and young adults. In the present study, no significant age-associated changes in both the prevalence or proportions of spirochaetes or black-pigmented gram-negative anaerobic bacilli were found; the presence of both groups of organism appeared to be independent of gingival inflammation. \( Actinomyces \) spp. were commonly isolated, but there was a marked shift in the ratio of the predominant species associated with ageing. \( A. naeslundii \) was in the highest proportions in the younger groups (A and B) whereas this was reversed in those aged \( \geq 60 \) years (groups C and D) in whom \( A. viscosus \) predominated.

There were no clear age-related differences in either the isolation frequency or in the proportions of mutans streptococci from saliva or plaque of subjects in our study; these organisms were isolated commonly and sometimes in high numbers, even in the most elderly age group. In contrast, the recovery, absolute viable counts, and proportions of lactobacilli in saliva increased with age, particularly in those aged \( \geq 70 \) years. A high carriage rate for cariogenic bacteria was also reported in a study of elderly Swedish individuals (mean age 85 years) and elderly Americans (range 60–87 years, median 68 years). In one of these studies, analyses revealed that lactobacilli were more prevalent in individuals wearing dentures, whereas our results showed higher levels of these bacteria in elderly dentate subjects proving this to be a genuine age-related observation. The combined findings from these studies suggest that those who retain natural teeth remain at risk of both enamel and root surface caries with increasing age. Many of the elderly people from these studies had \( > 10^6 \) cfu of mutans streptococci/ml of saliva and could also act, therefore, as vectors in the transmission of these potentially cariogenic bacteria.

The prevalence of yeasts in saliva also increased with age, with higher isolation frequencies and viable counts in those aged \( \geq 80 \) years, and higher proportions in those aged \( \geq 60 \) years; although, due to the wide inter-subject variation in counts between subjects, these differences were not statistically significant. Denture-wearers were excluded from our study so that the increased prevalence of \( Candida \) spp. in the elderly was probably due to ageing and may reflect changes...
oropharyngeal microflora was also low in another range of enterobacteria (e.g., study of physiologically normal subjects but rose no trends with age had emerged. In other studies, a prevalence of gram-negative bacilli among the individuals became more debilitated and hospitalised. Previously, although pseudomonads had been isolated from saliva, alterations in the integrity of the host defences may lead to the increased likelihood of colonisation by potentially pathogenic species. These studies may help in the recognition of at-risk elderly subjects.

In summary, the isolation, viable counts and proportions of lactobacilli in saliva increased with age, as did the isolation of potential opportunistic pathogens and other non-resident oral micro-organisms (yeasts and staphylococci). The ratio of the prevalence of Actinomyces spp. also shifted with age. Future work will attempt to determine the levels of the innate and specific host defences in the healthy subjects in different age groups described here, and then compare them with those of patients of a similar age but with various diseases. In certain individuals, alterations in the integrity of the host defences may perturb the stability of the resident oral microflora and lead to the increased likelihood of colonisation by potentially pathogenic species. These studies may help in the recognition of at-risk elderly subjects.

This research was generously supported by a grant from the Hayward Foundation, London. We thank Mr N. Girdler, Miss S. van Oudgaarden and Miss Y. Essami for collecting the samples, and the Trustees, staff and residents of Morden College, Blackheath, London SE3, for their help and co-operation throughout the study.

References
14. van Palenstein Helderman WH, Ijsseldijk van Oudgaarden and Miss SE3, for their help and co-operation throughout the study.


