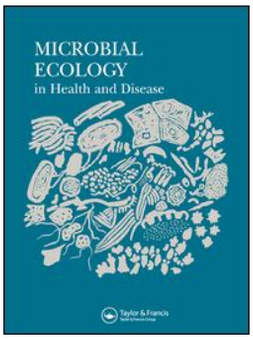

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Tongue Microflora in Edentulous Geriatric Denture-Wearers

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The aim of this study was to investigate the bacterial composition of tongue plaque of healthy edentate geriatric individuals wearing dentures. One male of 67 years-old and four females ranging from 68 to 73 years-old were involved. Plaque was obtained from an area of 10 mm^2 on the dorsal surface at the anterior two-thirds of the tongue. Total numbers of colony-forming units (CFU) were determined and the predominant bacteria were isolated for identification. Significantly more bacteria ($p < 0.05$) were recovered after anaerobic incubation (mean; 2.0×10^7 CFU/mg) than after aerobic (mean; 1.1×10^7 CFU/mg) or microaerophilic (mean; 1.0×10^7 CFU/mg) incubation. Out of 210 predominant strains isolated, 33% were obligate anaerobes and 66% were facultatively anaerobic. *Veillonella* (8% of total isolates) and *Streptococcus* (35% of total isolates) were the most major genera identified among obligate and facultative anaerobes, respectively. *Actinomyces* strains represented 27% of total isolates. Gram negative anaerobic rods and asaccharolytic *Eubacterium* were also detected, comprising 14% and 3% of total isolates, respectively. The bacterial composition of the tongue was somewhat similar to that of saliva and denture plaque reported in geriatric edentulous persons, suggesting that tongue plaque in geriatric edentulous persons who wear dentures may function as a major bacterial reservoir. *Key words*: tongue plaque, microflora, edentulous, geriatric.

INTRODUCTION

The oral microflora (1–5) is known to undergo dramatic change, both quantitatively and qualitatively, with age, especially in edentulous individuals. In an edentulous oral cavity, bacteria tend to colonize mainly the tongue, oral mucous membranes and denture surfaces, and are transferred to other sites by the saliva. The tongue, which occupies one-third of the oral cavity, has papillary surfaces on the dorsum that easily retain numerous bacteria. Previous studies reported that the tongue is a virtual cornucopia of bacteria especially those isolated from saliva (6), and that these microorganisms could, in turn, directly influence the composition of bacteria attaching to tooth surfaces or other sites in the oral cavity (7, 8). Indeed, previous studies have reported that *Streptococcus salivarius*, which is known to reside commonly on the tongue, is one of the predominant species in both denture plaque (9) and saliva (10, 11) of healthy edentulous persons, while it is predominant in the saliva, but not the dental plaque, gingival crevices, or cheeks of dentate persons (1, 6). These results support our contention that tongue plaque may be the predominant reservoir of oral bacteria, particularly in edentulous individuals.

In spite of its importance as a bacterial source, the microflora of the tongue has received little attention from the medical/dental community, with only two full bacteriological studies ever having been carried out, in dentate adults (12) and children (13). Other investigations of the tongue microflora have been more specific in their objectives, and focussed only on selected bacteria, such as periodontopathic (5, 8) or cariogenic organisms (14).

This study was aimed at investigating, more thoroughly, the bacterial composition of tongue plaque, in healthy, geriatric edentulous persons, with emphasis on strictly anaerobic bacteria, which may not always be cultivated by conventional methods. Since the bacterial composition of the salivary flora was described previously by our research group (11), we opted to limit the present analysis to the microbial elements present on the surfaces of the tongue, and to correlate these results with those for saliva, by taking samples from the same subjects.

MATERIAL AND METHODS

Subjects and samples

The edentulous individuals in this study (one male and four females) (Table I), ranged in age from 67 to 73 years-old. The bacterial composition of the saliva of the same persons was reported previously (11). Three of these individuals had been in their present edentulous state for more than 20 years, and the others for 4 and 6 years. Loss

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of dentition was due to carious lesions (two subjects), periodontitis (two subjects), and, in one patient, a combination of both conditions. None had a history of chronic systemic illness, nor had they taken antibiotics within the 6 months prior to the bacteriological sampling. Their complete dentures were in good condition and oral mucosae were clinically healthy. They had neither atrophic change nor fissuring on the dorsal surfaces of the tongue clinically. The aim, merits, and potential problems of this study were explained to the subjects, in detail, before the commencement of preliminary examinations, and the patients agreed to join.

After the dentures were removed and the mouth rinsed with water to remove the saliva and any loose debris, the dorsum of the tongue was wiped with sterile gauze to absorb excess moisture. The predetermined 10 mm square area in the window made by white plane paper at the anterior two-thirds of the dorsal surface of the tongue was then firmly scraped by an excavator to obtain samples of plaque. Sampling was carried out at approximately the same time in the morning for each subject.

Bacterial isolation and identification

Samples were transported in vials with tight, screw-on caps, under anaerobic condition, weighed by the balance (LIBROR AEL-40SM, Shimazu, Kyoto, Japan) and transferred within several minutes to an anaerobic glove box (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. While in the box, each sample was suspended in 1.0 ml sterile solution of 40 mM potassium phosphate (pH 7.0) and 1 mM EDTA, and dispersed with a motor-drive homogenizer (TISSUE-TEAROR, Biospec Products, Bartlesville, OK, USA) and a glass homogenizer. After serial 10-fold dilution with the same buffer, 0.1ml aliquots of each dilution were spread on surfaces of triplicate brain heart infusion (BHI)-yeast extract-blood (sheep) agar plates (15) and incubated in the anaerobic glove box, at 37°C. Plates, media, buffer solution and experimental instruments were kept in the box, for at least 24 hours before use. To ensure strictly anaerobic conditions in the glove box, the reduction of methylviologen (-446 mV) was carefully checked, whenever experiments were carried out.

The same dilutions were also incubated aerobically in air containing 30% carbon dioxide, and microaerophilically under 5% oxygen with 80% nitrogen, 7.5% carbon dioxide and 7.5% hydrogen, at 37 °C. The total number of colony-forming units (CFU) detected after anaerobic incubation was significantly higher (paired t-test, $p < 0.05$) than was found after either aerobic or microaerophilic incubation (Table II). Therefore, the only colonies selected for further investigation were those in which plates were incubated

Table I

Some clinical features of the subjects in this study

Subject No.	Age	Sex	Number of years being edentulous	Main reasons of toothlessness
1	67	M	4	Caries
2	71	F	>20	Periodontitis
3	70	F	>20	Caries
4	73	F	6	Caries, Periodontitis*
5	68	F	>20	Periodontitis

*: Posterior teeth were extracted due to caries, and anterior teeth were periodontitis.

Table II

Bacterial recovery from tongue plaque of geriatric edentulous persons wearing dentures

Subject No.	Anaerobic incubation	Aerobic incubation	Microaerophilic incubation	*Candida*
				(colony-forming units/mg)
1	3.4×10^7	2.4×10^7	2.0×10^7	0
2	6.4×10^6	2.8×10^6	1.7×10^6	0
3	6.5×10^6	4.2×10^6	4.2×10^6	0
4	2.2×10^7	1.2×10^7	1.5×10^7	0
5	2.9×10^7	1.1×10^7	9.0×10^6	6.0×10^0
Mean	2.0×10^7*,**	1.1×10^7*	1.0×10^7**	1.2×10^0

*,**: Significant difference between mean anaerobic and aerobic or microaerophilic counts. ($p<0.05$; paired t-test)

anaerobically. After a 7-day incubation period, the colonies totalling < 100 from each of the suitably diluted samples were isolated, and subcultured in the anaerobic glove box, with the help of a stereomicroscope ($\times 30$), if needed. No new colonies appeared on the plates, during the additional 7-day incubation period.

The undiluted, homogenized samples (0.1 ml) were also cultured aerobically on selective media for *Candida* (GS media, Eiken, Tokyo, Japan).

Microbial genera and species were identified according to criteria set forth in the VPI manual (15, 16), supplemented with information relating to the following genera: *Acidaminococcus*, *Actinomyces*, *Bacteroides*, *Eubacterium*, *Fusobacterium*, *Leptotrichia*, *Peptostreptococcus*, *Prevotella*, *Propionibacterium*, *Stomatococcus*, *Streptococcus*, *Veillonella* and *Wolinella* (17, 18). The following characteristics were examined: gram staining, iodine staining, motility, haemolysis; acid production from adonitol, amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, galactose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose and xylose; hydrolysis of esculin and starch; liquefaction of gelatin; production of indole; reduction of nitrate; growth in the presence of NaCl (6.5%), lactate and threonine; catalase and urease activities; ammonia liberation from arginine. Volatile n- and iso- fatty acids (C_2 to C_8), acetoin, diacetyl, alcohols (C_2 to C_8), and free acids and/or methyl derivatives of formic, lactic, succinic, phenyl acetic, phenyl lactic, phenyl propionic, as well as phenyl pyruvic acid, produced in peptone-yeast extract-glucose broth (PYG) (15), were assayed by gas chromatography. In certain cases, the following characteristics were examined: acid production from inulin; growth in the presence of bile; growth stimulation in the presence of Tween 80, formate-fumarate mixture and horse serum; propionate production from lactate and threonine; requirements for factor V and X.

In addition to the tests described above, streptococcal isolates were differentiated by their ability to produce detectable levels of α-L-fucosidase, β-N-acetylgalactosaminidase, β-N-acetylglucosaminidase and sialidase with 4-methylumbelliferyl-linked fluorogenic substrates (19–21).

In this study, obligate anaerobes were defined as those bacteria which grew only under anaerobic conditions, and facultative bacteria, as those which could also grow in air containing up to 30% carbon dioxide. It was confirmed in three separate tests, that obligate anaerobes could not grow in air containing 30% carbon dioxide.

Statistical analysis

Bacterial count-data were analyzed using the paired t-test.

RESULTS

From the dorsal surface of tongue we recovered 10^8 CFU/cm^2 bacteria after anaerobic incubation. Bacterial counts in this instance were significantly higher; –(mean 2.0×10^7, range $6.4 \times 10^6 - 3.4 \times 10^7$ CFU/mg) (paired t-test, $p < 0.05$)– than those for either aerobic (mean 1.1×10^7, range $2.8 \times 10^6 - 2.4 \times 10^7$ CFU/mg) or microaerophilic (mean 1.0×10^7, range 1.7×10^6-2.0×10^7 CFU/mg) incubation, as shown in Table II. When we compared aerobic with microaerophilic recovery data, there was no statistically significant difference in bacterial recovery.

In each sample, there were from 32 to 54 colonies per sample recovered as predominant isolates after anaerobic incubation. Thus, they were defined as the predominant bacteria. A total of 210 isolates were studied further (Table III). Of those, 70 (33%) were identified as being obligate anaerobes, while 138 (66%) were facultatively anaerobic bacteria. Unfortunately, the remaining 2 (1%) were lost before their atmospheric requirements could be determined. Gram-positive cocci (83 isolates, 40%) and gram-positive rods (65 isolates, 31%) constituted the majority of the isolates. Gram-negative rods (28 isolates, 13%) and gram-negative cocci (17 isolates, 8%) were also found.

The predominant genera, among the obligate anaerobes we were able to isolate, were the *Veillonella* (16 isolates, 8% of total isolates) and the *Prevotella* (15 isolates, 7% of total isolates). *Veillonella* was detected in every sample, and constituted the major portion of isolates in 3/5 cases (Subject No. 1, 3, 5, Table III). The genus *Prevotella* was also regularly isolated (4/5 subjects). The other obligate anaerobes that were isolated included *Eubacterium* (7 isolates, 3% of total isolates), *Wolinella* (7 isolates, 3% of total isolates), *Peptostreptococcus* (4 isolates, 2% of total isolates), *Leptotrichia* (3 isolates, 1% of total isolates), *Streptococcus* (3 isolates, 1% of total isolates), *Bacteroides* (2 isolates, 1% of total isolates), *Acidaminococcus* (1 isolates, 0.5% of total isolates), and *Fusobacterium* (1 isolate, 0.5% of total isolates), as shown in Table III. Eleven other obligate anaerobes, including 6 gram-positive cocci, one gram-positive rod and one gram-negative rod, could not be identified, because of their inactivity in most biochemical tests. The proportion of obligate anaerobes varied from 13 to 54% of total isolates examined (Table III).

Of 138 (66%) facultative anaerobes, 73 (35% of total isolates) were assigned to the genus *Streptococcus*, which constituted the major portion of isolates in all cases (Table III). These were further differentiated into *S. parasanguis*, *S. salivarius*, *S. oralis*, *S. mutans*, *S. sanguis* or *S. sobrinus*, on the basis of their biochemical activity (Table IV). The second most predominant genus was *Actinomyces* (57 isolates, 27% of total isolates). The other genera of facultative anaerobes isolated included *Stomatococcus* (3 isolates, 1% of total isolates) and *Propionibacterium* (1 isolate, 0.5% of total isolates), as shown in Table III. Four other isolates could not be identified.

Table III

Bacterial isolates from tongue plaque of geriatric edentulous persons wearing dentures

Subject No.	1	2	3	4	5	Total
Number of isolates	32	49	34	54	41	210 (100%)
Obligate anaerobes	8	24	9	7	22	70 (33%)
(% of total isolates)	(25%)	(49%)	(26%)	(13%)	(54%)	
Veillonella	4	4	3	1	4	16 (8%)
Prevotella	2	7	–	2	4	15 (7%)
Eubacterium	–	3	–	1	3	7 (3%)
Wolinella	1	2	–	–	4	7 (3%)
Peptostreptococcus	–	2	1	–	1	4 (2%)
Leptotrichia	1	–	–	–	2	3 (1%)
Streptococcus	–	–	3	–	–	3 (1%)
Bacteroides	–	–	2	–	–	2 (1%)
Acidaminococcus	–	–	–	1	–	1 (0.5%)
Fusobacterium	–	1	–	–	–	1 (0.5%)
Unidentified	–	5	–	2	4	11 (5%)
Facultatives	24	23	25	47	19	138 (66%)
(% of total isolates)	(75%)	(47%)	(74%)	(87%)	(46%)	
Streptococcus	17	12	15	32	11	73 (35%)
Actinomyces	7	11	10	8	7	57 (27%)
Stomatococcus	–	–	–	3	–	3 (1%)
Propionibacterium	–	–	–	–	1	1 (0.5%)
Unidentified	–	–	–	4	–	4 (2%)
Lost	–	2	–	–	–	2 (1%)

Candida was detected at 6.0×10^0 CFU/mg, in only one out of 5 subjects, which corresponded to 2×10^{-5}% of the total plaque CFU (Table II).

DISCUSSION

In the present study, plaque samples of tongue were taken from the same geriatric edentulous persons from whom saliva had been examined microbiologically using the same methods (11). Denture plaque of different subjects was also examined with the same methods in the same institution and reported previously (9). Our investigations revealed that as many as 10^7 CFU/mg, or 10^8 CFU/cm^2 of bacteria can be recovered from the tongue by means of anaerobic procedures (Table II) in agreement with that of dentate adults (22).

Without exception, anaerobic incubation of tongue plaque in an anaerobic glove box yielded significantly higher bacterial recovery than did either 30% carbon dioxide-based aerobic incubation or microaerophilic incubation (Table II). Viable bacterial counts under aerobic conditions amounted to between 38 and 71% of those determined under anaerobic conditions. This result indicates that the microflora, present on the surface of the tongue in geriatric edentulous persons, consists mainly of bacteria highly adapted to anaerobic conditions. This is consistent with previous studies of the microbial composition of saliva (11) and complete denture plaque (9) of geriatric edentulous subjects which also reported that anaerobic incubation contributes to a higher bacterial recovery rate. These results indicate that, even in edentulous mouths, as well as in dentate mouths, the environment can suit the growth of anaerobic bacteria. However, complete loss of teeth may eliminate sites with a strict anaerobic environment from oral cavity, resulting in a reduction of obligate anaerobes. A previous study cited figures indicating that the proportion of obligate anaerobes in complete denture plaque (49%) (9) was lower than in the dental plaque of dentate adults (72%) (23). The obligate anaerobe-count in the saliva (28%) of geriatric edentulous persons (11) is also lower than that seen in dentate adults (38%) (6). In the present study, obligate anaerobes made up one third of the total cultivable bacteria present on the tongues of geriatric edentulous persons. This figure is even lower than that of dentate adults (36%) (12).

The microflora of the tongue qualitatively resembled that found in any other area of the oral cavity (12). *Streptococcus* and *Veillonella* constitute the majority of facultative and obligate anaerobes recovered. This result agrees with previous findings of the tongue microflora in dentate adults (12) and preschool children (13), and might possibly be a bacteriological characteristic common to all age groups. *S. parasanguis*, *S. salivarius* and *S. oralis* were found most frequently. Although *S. parasanguis* was recently discovered to be a new species (19), *S. salivarius* is known to be the predominant resident of the tongue (1, 12, 13). Oral *Veillonella* is classified as *V. atypica*, *V. dispar*

and *V. parvula* (24, 25). *V. parvula* is predominant in dental plaque (24) and denture plaque (9). *Veillonella* isolates in this study have been further identified by Sato et al. (26), as being *V. atypica* and *V. dispar*, in agreement with the report by Hughes et al. (24), by employing the method of restricted fragment-length polymorphism (RFLP) analysis of 16S rDNA, amplified by polymerase chain reaction (PCR). Oral *Veillonella* species isolated from the tongue are reported to coaggregate with certain strains of *S. salivarius*, suggesting that this coaggregation plays a critical role in the bacterial ecology of the tongue (24).

Interestingly, *Streptococcus* and *Veillonella* are predominant genera in the denture plaque and saliva of geriatric edentulous persons, as well (Table V) (9. 11). In edentulous subjects, the bacterial composition as well as the distribution of streptococcal isolates, are nearly the same, whether obtained from the surface of the tongue, or from saliva (Tables V and VI) (11), indicating that salivary bacteria are mainly derived from the tongue (6). *S. salivarius* is, by far, the most common streptococcal isolate, and the predominant species of denture plaque in geriatric edentulous individuals (Tables V and VI) (9). In the dentate, however, *S. salivarius* is usually limited to the tongue and saliva, appearing only rarely in dental plaque, gingival crevices or the cheeks (1, 6). These results indicate that some bacteria, including *S. salivarius*, that occur in the denture plaque of edentulous persons, also originate from the tongue.

Prevotella, *Wolinella*, and asaccharolytic *Eubacterium* were detected often, comprising 14% of all isolates. Predominant in the periodontal pockets of patients with periodontitis (27), they are thought to be associated with the aetiology of periodontal disease. In two subjects (Table III, Subject No. 2 and 5), where loss of dentition was mainly the result of periodontitis, these bacteria were detected at a higher percentage of total isolates than from the other three subjects (Table III, Subject No. 1, 3, and 4). Asaccharolytic *Eubacterium* is new group which has been recently isolated often from various oral sites. In this study, conventional phenotypic tests revealed 3 isolates resembling *Eubacterium timidum*. They could be differentiated from *E. timidum*, however, by PCR-amplified restricted fragment-length polymorphism analysis of 16S rDNA (28). They will therefore be reclassified as a new species in the future.

In this study, very few *Candida* were detected (Table II), attesting to its reportedly limited presence in denture (29) and palatal (30) plaque and saliva (31) of patients with denture-induced stomatitis, and in edentulous individuals (9, 11, 32).

Bacteria which collect on the tongue, especially gram-negative anaerobic rods, are considered the most important agents in the occurrence of halitosis (22), denture stomatitis (30, 31), or bacterial pneumonia (33), and such bacteria were isolated from tongue of geriatric patients in the present study. Bacterial pneumonia, an ailment common in the elderly, is the cause of far more morbidity and mortality in that segment of the population, than in younger adults. It usually results from the aspiration of either bacteria present in saliva, upper airway secretions or gastric contents. Some of the implicated bacteria might derive from tongue plaque. The resident florae of the tongue sometimes have harmful effects in edentulous persons, not only on the oral health of the elderly, but on their general physical conditions. Any major shift in the proportions of the resident oral bacteria might therefore induce other oral diseases or opportunistic infections, in such immunocompromised individuals. A knowledge of the microbial ecology of geriatric edentulous persons, including tongue plaque as determined in the present study as well as saliva (11) and denture plaque (9), may help to design comprehensive, effective methods of preventing, and treating these diseases.

The present study revealed the tongue to be a primary reservoir of oral bacteria in this segment of the population, although the number of subjects was limited. It

Table IV

Identification of isolates from tongue plaque of geriatric edentulous persons wearing dentures

Total isolates = 210			
Obligate anaerobes	(n = 70)	Facultatives	(n = 138)
Veillonella	16	*Streptococcus*	
Prevotella		*S. parasanguis*	31
P. oralis	9	*S. salivarius*	23
P. ruminicola		*S. oralis*	14
ss. Ruminicola	6	*S. mutans*	3
Eubacterium		*S. sanguis*	1
E. timidum-like	3	*S. sobrinus*	1
E. spp	4	*Actinomyces*	
Wolinella		*A. odontolyticus*	33
W. succinogenes	7	*A. israelii*	10
Peptostreptococcus		*A. meyeri*	9
P. anaerobius	1	*A. spp*	5
P. micros	1	*Stomatococcus*	
P. parvulus	1	*S. mucilaginosus*	3
P. spp	1	*Propionibacterium*	
Leptotrichia		*P. acnes*	1
L. buccalis	3	Unidentified	4
Streptococcus			
S. morbillorum	3		
Bacteroides			
B. spp	2		
Acidaminococcus			
A. fermentans	1		
Fusobacterium			
F. nucleatum	1		
Unidentified	11	Lost	2

Table V

Oral microflora of geriatric edentulous persons wearing dentures

Total isolates (% of total isolates)	Tongue Plaque 210 (100%)	Saliva (11) 151 (100%)	Denture plaque (9) 185 (100%)
Obligate anaerobes	70 (33%)	43 (28%)	90 (49%)
Veillonella	16 (8%)	26 (17%)	45 (23%)
Prevotella	15 (7%)	7 (5%)	–
Eubacterium	7 (3%)	1 (1%)	5 (3%)
Wolinella	7 (3%)	–	–
Peptostreptococcus	4 (2%)	–	1 (1%)
Leptotrichia	3 (1%)	–	–
Streptococcus	3 (1%)	–	–
Bacteroides	2 (1%)	3 (2%)	–
Fusobacterium	1 (0.5%)	1 (0.7%)	–
Acidaminococcus	1 (0.5%)	–	–
Anaerohabdus	–	1 (0.7%)	–
Mitsuokella	–	1 (0.7%)	–
Actinomyces	–	1 (0.7%)	9 (5%)
Lactobacillus	–	1 (0.7%)	10 (5%)
Bifidobacterium	–	–	14 (7%)
Propionibacterium	–	–	2 (1%)
Ruminococcus	–	–	1 (0.5%)
Unidentified	11 (5%)	1 (0.7%)	3 (2%)
Facultative anaerobes	138 (66%)	99 (66%)	95 (51%)
Streptococcus	73 (35%)	51 (34%)	57 (31%)
Actinomyces	57 (27%)	20 (13%)	3 (2%)
Stomatococcus	3 (1%)	12 (8%)	–
Propionibacterium	1 (0.5%)	3 (2%)	–
Enterococcus	–	9 (6%)	3 (2%)
Lactobacillus	–	4 (3%)	31 (16%)
Staphylococcus	–	–	1 (0.5%)
Unidentified	4 (2%)	–	–
Lost	2 (1%)	9 (6%)	–

Table VI

Distribution of streptococcal isolates in geriatric edentulous persons wearing dentures

Total isolates (% of total isolates)	Tongue Plaque 210 (100%)	Saliva (11) 151 (100%)	Denture plaque (9) 185 (100%)
Streptococcus	73 (35%)	51 (34%)	57 (31%)
S. parasanguis	31 (15%)	20 (13%)	–
S. salivarius	23 (11%)	11 (7%)	29 (16%)
S. oralis	14 (7%)	16 (11%)	–
S. mutans	3 (1%)	2 (1%)*	16 (9%)*
S. sanguis	1 (0.5%)	–	–
S. sobrinus	1 (0.5%)	–	–
S. intermedius	–	1 (0.7%)	–
S. mitis	–	1 (0.7%)	–
S. milleri	–	–	11 (6%)*
S. mitior	–	–	1 (0.5%)

*: atypical characteristics

further demonstrates that bacteria present on the tongue are somewhat similar to those found in saliva (11) and denture plaque (9).

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