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ORIGINAL ARTICLE

Microflora in oral ecosystems in subjects with radiation-induced hyposalivation

A Almståhl[1], M Wikström[1], B Fagerberg-Mohlin[2]

Departments of [1]Oral Microbiology and [2]Oral and Maxillofacial Surgery, The Sahlgrenska Academy at Göteborg University, Göteborg, Sweden

AIM: To analyse the microbial flora in specific oral sites in 13 dentate subjects, 6–8 months after completed radiation therapy (RT group) and in 13 matched controls.
MATERIAL AND METHODS: The microflora on the tongue, buccal mucosa, vestibulum, supragingival plaque and subgingival region was analysed using duplicate sampling and cultivation technique. A clinical examination was also performed.
RESULTS: *Candida albicans* was found in one or more sites in 54% of the RT subjects and in 15% of the controls. In three RT subjects, *C. albicans* was found at all four sites analysed. An unexpected finding was that enterococci were found in all RT subjects and in high number in 38%. None of the controls harboured enterococci. In supragingival plaque, *Lactobacillus* spp. were detected in 92% of the RT subjects and the number and proportion of *Lactobacillus* spp. were extremely high compared with the controls. Mutans streptococci were detected in high numbers in 31% of the RT subjects, while they were not detected in 23%.
CONCLUSION: The microbial results explain why some RT subjects have an increased susceptibility to oral diseases and stress that site-specific microbial analysis is an important diagnostic tool when planning oral health preventive care for RT subjects.
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Keywords: hyposalivation; radiation therapy; saliva; microflora; oral

Introduction

Radiation therapy in the head and neck region often leads to dramatically reduced saliva production. Among those subjects some develop caries while others do not (Spak *et al*, 1994). Also, for oral mucosal infections, some subjects suffer from recurrent infections, while others do not.

In our previous study, we analysed the oral microflora in rinsing samples in subjects with radiation-induced hyposalivation 6 months after completed radiation therapy (Almståhl *et al*, 2003). Compared with subjects with normal salivary secretion rates, most subjects harboured markedly higher numbers of *Lactobacillus* spp., associated with an acidic milieu and caries development (Corby *et al*, 2005; O'May *et al*, 2005). For mutans streptococci, also associated with caries development (Van Houte, 1994), 29% of the subjects had high numbers in rinsing samples while 29% had undetectable numbers. *Candida albicans* were detected in 79% and *Staphylococcus aureus* in 36% of the subjects.

For the group with hyposalivation caused by primary Sjögren's syndrome, samples from specific sites revealed further differences compared with rinsing samples (Almståhl *et al*, 2001, 2003). In this study, we hypothesized that samples taken from specific oral ecosystems would reveal further differences in oral microflora compared with rinsing samples and explain the differences in susceptibility to caries and mucosal infections seen in subjects with radiation-induced hyposalivation.

Microbial samples were collected 6–8 months after completed radiation therapy from the dorsum of the tongue, buccal mucosa, vestibulum in the molar region, supragingival tooth surface and gingival crevice region. The samples were analysed for the following microorganisms: mutans streptococci, *Lactobacillus* spp., *Actinomyces* spp. associated with the development of caries (Van Houte, 1994), *Fusobacterium nucleatum* and *Prevotella intermedia/Prevotella nigrescens*, associated with plaque accumulation and gingivitis (Moore *et al*, 1982), *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *Porphyromonas gingivalis*, frequently found in elevated numbers in periodontitis sites (Renvert *et al*, 1990), *C. albicans*, *S. aureus*, enteric rods and enterococci, associated with oral mucosal infections (Dahlén *et al*, 1992). The total microbial count was also registered, together with the total number of streptococci,

Correspondence: A. Almståhl, Department of Oral Microbiology, The Sahlgrenska Academy at Göteborg University, Institute of Odontology, Box 450, 405 30 Göteborg, Sweden. Tel: +46 31 786 3266, Fax: +46 31 825733, E-mail: annica.almstahl@odontologi.gu.se
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Streptococcus sanguis/Streptococcus oralis and *Streptococcus salivarius*, associated with good oral health (Socransky and Manganiello, 1971).

Materials and methods

The study was approved by the Ethics Committee at Göteborg University. During 1 year, all subjects 45–70 years old undergoing radiation therapy in the head and neck region with ≥16 own teeth and no removable prosthetic constructions and living in the Göteborg area were invited to participate in the study by author BFM at the Department of Oral and Maxillofacial Surgery, Institute of Odontology, the Sahlgrenska Academy at Göteborg University. The subjects were undergoing curative bilateral radiation therapy with external beam in the head and neck region with or without brachytherapy. Before radiation therapy, a panoramic radiograph and four bitewing radiographs had been taken. If needed, caries lesions were restored, endodontic treatment performed and doubtful teeth extracted. The subjects were instructed to use sodium fluoride gel in custom-made trays for 5 min day^{-1} for 2 months. During radiation therapy, the subjects received tooth cleaning and mucositis treatment one to two times. Subjects who have undergone radiation therapy in the head and neck region are routinely followed for at least 2 years at the Department of Oral and Maxillofacial Surgery for examination of the mucosal membranes and osteoradionecrosis. The subjects fulfilling the inclusion criteria were contacted 6–8 months after completed radiation therapy. Of these, 13 subjects were willing to participate in the study. Each subject was matched according to age, sex and number of teeth with a healthy control with normal salivary secretion rates. Written informed consent was obtained from all subjects. All participants were asked about medication and smoking habits. The subjects who had undergone radiation therapy (RT subjects) were also asked about their use of saliva stimulating agents and fluorides. All subjects were seen twice 10 ± 4 days apart (median 7 days, range 7–21). At both appointments, the unstimulated and the stimulated secretion rates were measured and microbial samples taken. A clinical oral examination was performed at the first appointment. All measurements and examinations were performed between 8 AM and 11 AM by AA, one of the authors.

The clinical variables registered were: status of the mucosal membranes, number of teeth, fillings, crowns and bridges, presence of plaque along the gingival margin, bleeding following probing and periodontal pocket probing depths of ≥5 mm at four sites on each tooth. The unstimulated secretion rate was measured for 15 min. The subject leant forward and passively allowed the saliva to flow into a graduated test-tube. The stimulated secretion rate was measured using paraffin wax. The first saliva was swallowed and then all saliva secreted during 5 min was collected in an ice-chilled test-tube. The pH and buffer capacity were measured in the stimulated saliva using a pH meter (Metrohm 632, Herisau, Switzerland). With the assay used 'normal' pH range between pH 6.3 and 7.2 and 'normal' buffer capacity between pH 5.0 and 7.0 (Ericsson, 1959).

Microbial sampling

Microbial samples were collected after determination of the unstimulated secretion rate and before measuring of the stimulated secretion rate and the clinical examination. Before sampling the dorsum of the tongue, the buccal mucosa and the vestibulum, saliva was wiped off with sterile compresses. This was done to equalize the two groups. For sampling of the dorsum of the tongue, a plastic spatula with a circular hole (diameter 1.5 cm) was placed on the dorsal part of the tongue and the sample was taken with a sterile cotton pellet, which had been immersed in sampling fluid VMGA I (containing mainly gelatine, salts and peptides) (Möller, 1966). First, the pellet was swept along the margins of the hole in the spatula and then back and forth over the area inside the hole. Sterile cotton sticks, which had been immersed in VMGA I, were used to collect the samples from the buccal mucosa (sampled area 6 cm^2) and from the vestibulum in the molar region (sampled area 5 cm^2). The samples collected from the left and right buccal mucosa, centrally in the molar region, were pooled, as were the samples collected from the upper-right and lower-left vestibulum in the molar region. Supragingival plaque was collected with sterile toothpicks. The samples from between the upper-right first and second molar and between the lower-left first and second molar and buccally along the gingival margin on the upper-right and the lower-left first molar were pooled. Four gingival crevicular sites were sampled using the paper point technique (Renvert *et al*, 1990). The sites that were sampled and pooled were mesially on the upper-right first molar, distally on the first premolar, mesially on the lower-left molar and distally on the lower-left premolar. Two paper points were used at each site. All the microbial samples were transported to the laboratory in bottles containing 3.3 ml of transport medium, VMGA III (containing mainly gelatine, salts and peptides) (Möller, 1966) and processed within 4 h.

Cultivation media

Cultivation media used were Brucella agar (BBL Microbiological Systems, Cockeyville, MD, USA) with 50 ml l^{-1} of defibrinated horse blood, 20 ml l^{-1} of haemolysed human blood and 0.5 mg l^{-1} of menadione, Mitis-Salivarius (MS) agar (0298; Difco, Detroit, MI, USA), Mitis-Salivarius-Bacitracin (MSB) agar (Gold *et al*, 1973), Rogosa-SL agar (4080; Difco), CFAT agar plates (Zylber and Jordan, 1982), Staphylococcus agar (Staphylococcus medium 110: 0297; Difco), Drigalski agar (Vahlne, 1945), Enterococcus agar (Isenberg *et al*, 1970), Sabouraud Dextrose agar (Difco) with 100 mg l^{-1} of tetrazoliumchloride (Sabouraud-T) and Trypticase-Soyserum-Bacitracin-Vancomycin (TSBV) agar (Slots, 1982).

Laboratory analysis

Dilutions of the samples were performed in VMGA I (Möller, 1966). For inoculation of TSBV agar plates,

undiluted sample was used. All agar plates were inoculated from dilutions 10^{-1}. Plates with MSB agar, Rogosa agar and CFAT agar were also inoculated from dilution 10^{-3}. Plates with Brucella agar and MS agar were inoculated from dilutions 10^{-2}, 10^{-3} and 10^{-4}. Brucella agar plates were incubated using the hydrogen combustion technique (Möller and Möller, 1961) at 36°C for 5–7 days. Plates with MS agar, MSB agar, Rogosa agar, CFAT agar and TSBV agar were incubated in an atmosphere of 90% CO_2 and 10% N_2 at 36°C for 3–5 days. Plates with Sabouraud T agar, Staphylococcus agar, Drigalski agar and Enterococcus agar were incubated aerobically at 36°C for 3–5 days.

Microbial species identified at the specific sites are shown in Figures 1–5. *A. actinomycetemcomitans* and *P. gingivalis* were analysed in the gingival crevice region. The detection limit was 100 colony-forming units (CFU) ml^{-1} for all species except *A. actinomycetemcomitans* where it was 10 CFU ml^{-1}. If possible, the number of different micro-organisms in a sample was calculated from their numbers on a plate giving 30–300 colonies. The total number of bacteria growing anaerobically and the numbers of *P. intermedia*/*P. nigrescens*, *P. gingivalis* and *F. nucleatum* were calculated from their growth on Brucella agar plates. *P. intermedia*/*P. nigrescens* was identified as black, indole-positive colonies of Gram-negative coccoid rods showing

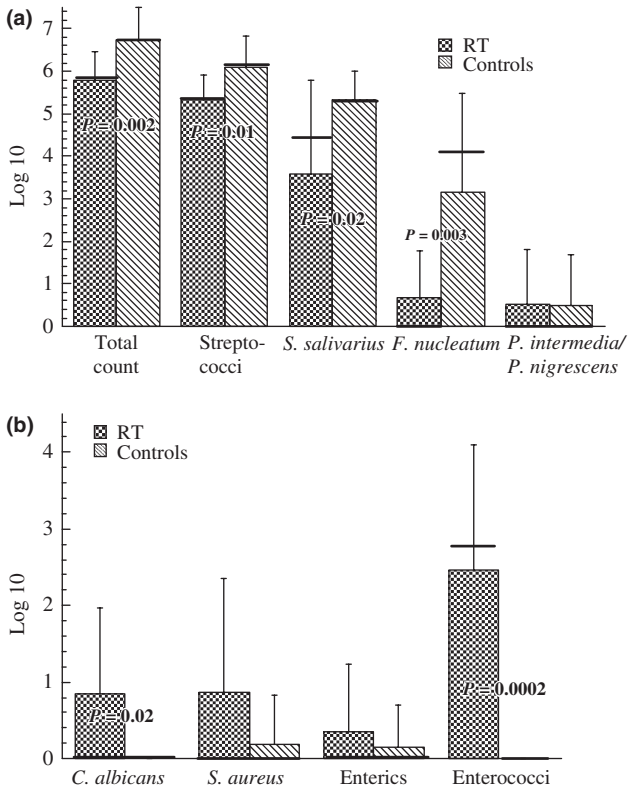

Figure 1 (**a**, **b**) Numbers (mean of two samplings) of micro-organisms on the dorsum of the tongue (the sampled area was 1.8 cm^2) in the RT group (n = 13) and in the controls (n = 13). Mean (bars) + SD and median (lines) values are given

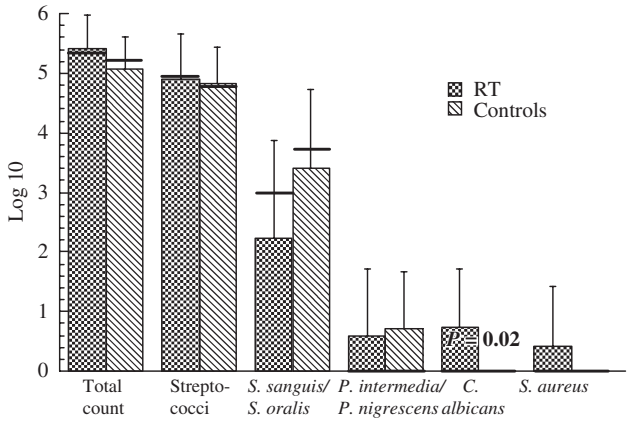

Figure 2 Numbers (mean of two samplings) of micro-organisms on the buccal mucosa (the sampled area was 6 cm^2) in the RT group (n = 13) and in the controls (n = 13). Mean (bars) + SD and median (lines) values are given

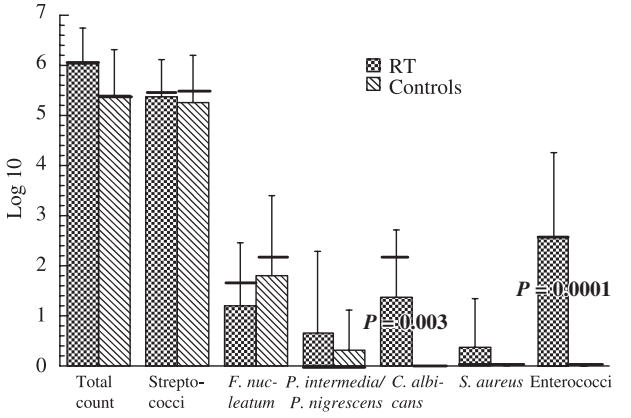

Figure 3 Numbers (mean of two samplings) of micro-organisms on the vestibulum in the molar region mucosa (the sampled area was 5 cm^2) in the RT group (n = 13) and in the controls (n = 13). Mean (bars) + SD and median (lines) values are given

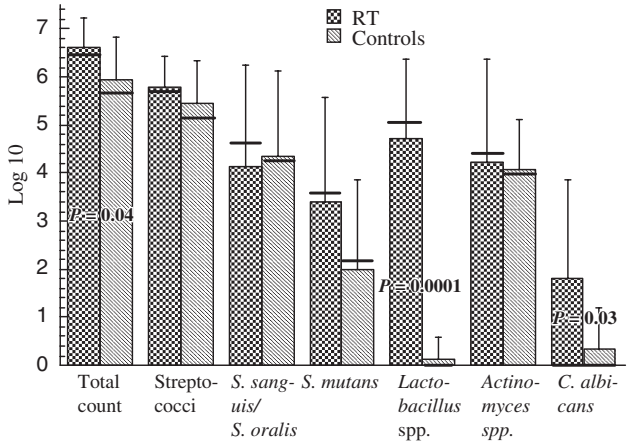

Figure 4 Numbers (mean of two samplings) of micro-organisms on the supragingival tooth surfaces, four sites sampled, in the RT group (n = 13) and in the controls (n = 13). Mean (bars) + SD and median (lines) values are given

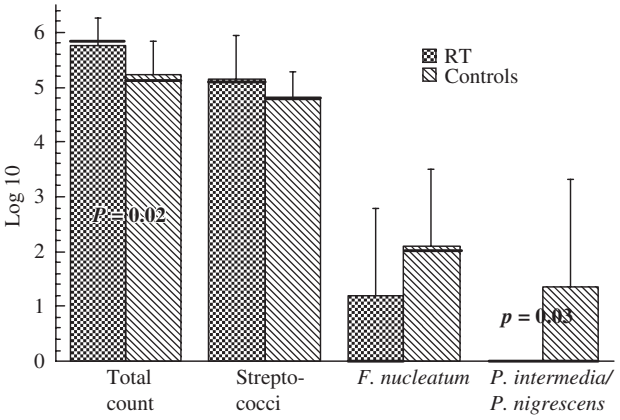

Figure 5 Numbers (mean of two samplings) of micro-organisms in the gingival crevice region, four sites sampled, in the RT group (n = 13) and in the controls (n = 13). Mean (bars) + SD and median (lines) values are given

dark-red fluorescence in long-wave (360 nm) UV light. *P. gingivalis* was identified as greenish-black colonies with Gram-negative coccoid rods not showing fluorescence. *F. nucleatum* was identified as grey colonies with a nacreous appearance of Gram-negative long and slender cells with tapering ends. Representative colonies were subjected to carbohydrate fermentation tests. On MS agar plates, *S. salivarius* was identified as large, light-blue colonies of Gram-positive cocci and colonies that were small, firm, and adherent were identified as *S. sanguis*/*S. oralis*. *Streptococcus mutans* and *S. sobrinus* were identified on MSB agar plates as Gram-positive cocci. *S. mutans* was identified as small, mucoid and irregular colonies and *S. sobrinus* as creamy marzipan-like colonies. *Lactobacillus* spp. were calculated from their growth on Rogosa agar plates and identified as Gram-positive rods. *Actinomyces* spp. were identified as small, yellow and high colonies of Gram-positive rods on CFAT agar plates. *S. aureus* was calculated from its growth on Staphylococcus agar plates. Colonies of *S. aureus* were distinguished from *Staphylococcus epidermidis* by their ability to degrade DNA on DNA agar plates (Difco). The number of *C. albicans* was calculated from its growth on the Sabouraud T agar plates. It was identified as lustreless and creamy whitish-pink, or pink, colonies. In doubtful cases, API biochemical tests (API System; Les Balme de Grottes, Montalieu, France) were used. Enteric rods were identified as large yellow or green colonies of Gram-negative rods on Drigalski agar plates and enterococci on Enterococcus agar plates as small brown colonies of Gram-negative cocci surrounded by a black zone. *A. actinomycetemcomitans* was identified on TSBV agar on the basis of a star-shaped colony morphology of coccoid rods and a positive catalase reaction. In doubtful cases, further identification was performed with API biochemical tests (API System; Les Balme de Grottes).

Statistical methods

To normalize the microbial data, the numbers were logarithmically transformed. Zero counts were treated as 1 CFU ml^{-1}. For analysis of possible differences between the two groups, Student's two-sample (unpaired) t-test was used. Because of the multiple influence aspect, single significances should be interpreted with some care.

Results

The RT group consisted of nine men and four women with a mean age of 53 ± 8 years (median 52 years, range 42–70 years). For all subjects, the major salivary glands were lying within the radiation field. The dose was radical between 64.6 and 76.6 Gy depending on site and stage of disease. Twelve of the 13 subjects were treated with brachytherapy between 6 and 30 Gy. Tumour size or extent of primary tumour, spread to regional lymph nodes, presence of distant metastasis and stage of cancer as well as its treatment are presented in Table 1. Four subjects used between one and six

Table 1 Sex, age, primary tumour site, tumour type, TNM (T = size or extent of primary tumour, N = spread to regional lymph nodes and M = distant metastasis), stage of cancer, cytostatic treatment, radiation dose and treatment with iridium-implant and dose for 13 subjects with radiation-induced hyposalivation

Sex (M/F)	*Age*	*Primary tumour site*	*Tumour type*	*TNM*	*Stage*	*Cytostatics*	*External radiation dose (Gy)*	*Iridium implant (Gy)*
M	53	Tonsill	SCC	$T_3N_2M_0$	4	Yes	64.6	12
M	71	Tongue	SCC	$T_2N_0M_0$	2	No	40.8	25
M	60	Tonsil	SCC	$T_2N_2M_0$	4	Yes	40.8	20
M	61	Tonsil	SCC	$T_2N_2M_0$	4	Yes	40.8	20
M	47	Tonsil	SCC	$T_2N_2M_0$	4	Yes	40.8	20
M	49	Tonsil	SCC	$T_3N_2M_0$	4	Yes	64.6	12
M	52	Tongue	MEC	$T_2N_0M_0$	2	No	40.8	30
K	46	Tonsil	SCC	$T_4N_1M_0$	4	Yes	64.6	12
K	57	Tonsil	SCC	$T_1N_3M_0$	4	Yes	64.6	12
M	51	Tonsil	SCC	$T_1N_2M_0$	4	Yes	40.8	20
K	42	Tonsil	SCC	$T_3N_1M_0$	3	No	64.6	–
M	48	Tonsil	SCC	$T_2N_0M_0$	2	No	40.8	25
K	47	Nasopharynx	SCC	$T_2N_1M_0$	3	Yes	61.2	6

SCC, squamous cell carcinoma, MEC, mucoepidermoid carcinoma.

medicines, most frequently used were: pain killers (four subjects) and antihypertensives (two subjects). In the control group, three subjects used between one and three medicines; antihypertensives (three subjects) and a blood-thinning medicine (one subject).

None of the controls was a smoker. In the RT group, one subject smoked and five were previous smokers. The time that had elapsed since they quit smoking was 6 months for one subject and varied between 6 and 30 years for the other four subjects. Exclusion of the current smoker and the subject who had quit smoking 6 months earlier from the RT group did not affect the differences in oral microflora detected between the RT group and the control group. Five of the subjects in the RT group had taken antibiotics <3 months prior to the examination. Those five subjects tended to have lower proportions of streptococci of the total count on the tongue and buccal mucosa and a higher number of enterococci on the tongue compared with the other eight RT subjects. None of the subjects in either group showed clinically visible signs of mucosal infection. One RT subject was on anti-fungal medication and in this subject *Candida* could not be detected in any of the four sites analysed. Six RT subjects and five controls had sites with periodontal pocket probing depths of ≥5 mm (data not shown). For these subjects, the number of sites with pocket probing depths of ≥5 mm varied between 1 and 5. The mean pocket probing depths of these sites were 5.6 ± 0.7 mm in the RT group and 5.3 ± 0.4 mm in the controls. In the RT group, nine subjects used fluoride rinse or fluoride gel daily, one subject every second day and one used a spray with sodium fluoride. Of these 11 subjects, three in addition used tablets or chewing gums with fluoride daily. The two other RT subjects not using fluoride gel or rinse, reported that they only used water to relieve their dry mouth problems. The only source of fluorides in the control group was fluoridated toothpaste.

The salivary secretion rates, pH and buffer capacities are shown in Table 2. Only two subjects in the RT group had a detectable unstimulated secretion rate. The stimulated secretion rate was >0.7 ml min^{-1} for only one subject in the RT group, 1.2 ml min^{-1}. The pH in the stimulated saliva was below normal for five RT subjects and normal for all controls. Nine RT subjects and two controls had a final pH ≤ 5.0, indicating a low buffering capacity. The final pH for these subjects varied between 3.3 and 5.0 in the RT group and was 4.0 and 4.8 respectively in the controls. For those nine RT subjects, the numbers of mutans streptococci, *Lactobacillus* spp. and *Candida* were comparable to those found in the four RT subjects with normal buffer capacity.

In the RT group, the median total counts per cm^2 were 4×10^5 on the tongue, 4×10^4 on the buccal mucosa and 9×10^5 in the vestibulum. The corresponding numbers for the controls were 3×10^6, 3×10^4 and 5×10^4.

Dorsum of the tongue

The mean total microbial count and the numbers of streptococci, *S. salivarius* and *F. nucleatum* were significantly lower in the RT group than in the control group (Figure 1a). The proportion of streptococci of the total count was slightly higher in the RT group than in the controls (mean ± SD: 45 ± 34% and median: 34% compared with 35 ± 31% and 21%). *F. nucleatum* was detected in four RT subjects and in 10 controls and *P. intermedia*/*P. nigrescens* in two RT subjects and in two controls. The proportion of *F. nucleatum* was lower in the RT group than in the controls ($P < 0.05$), while the number and proportion of *P. intermedia*/*P. nigrescens* were similar in the two groups. The number of *C. albicans* and enterococci were significantly higher in the RT group than in the controls (Figure 1b). Seven of the RT subjects but none of the controls harboured at least two of the following species: *C. albicans*, *S. aureus*, enterococci and enteric rods. Of these four species, enterococci were most frequently detected, in 10 subjects followed by *C. albicans*, *S. aureus* and enteric rods. *C. glabrata* was detected in one RT subject.

Buccal mucosa

The number of *S. sanguis*/*S. oralis* (Figure 2) and the proportion of *S. sanguis*/*S. oralis* of the total number of

Table 2 Salivary secretion rates (mean of two measurements) and clinical features in 13 RT subjects and 13 controls matched for age, sex and number of teeth

Subjects	*Unstimulated secretion rate (ml min^{-1})*	*Stimulated secretion rate (ml min^{-1})*	*pH*	*Buffer capacity*	*Number of teeth*	*Number of crowned teeth*	*Number of filled surfaces*	*Surfaces with plaque along gingival margin (%)*	*Surfaces with bleeding on probing (%)*
Mean ± s.d.									
RT	0.005 ± 0.02	0.32 ± 0.32	6.4 ± 1.0	4.4 ± 0.9	28 ± 2	5 ± 5	49 ± 20	51 ± 22	26 ± 18
Controls	0.4 ± 0.2	2.6 ± 1.0	7.6 ± 0.1	5.8 ± 0.8	27 ± 3	3 ± 4	49 ± 22	32 ± 20	19 ± 10
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS	NS	NS	NS	NS
Median									
RT	0	0.23	7.0	4.7	28	3	48	56	20
Controls	0.4	2.1	7.6	5.9	27	1	40	28	19
Range									
RT	0–0.05	0.06–1.2	4.9–7.5	3.1–5.9	24–32	0–16	10–91	13–82	3–67
Controls	0.2–0.9	1.5–4.8	7.4–7.8	4.0–6.7	21–31	0–10	19–81	13–91	7–42

RT, radiation therapy ; NS, no significant difference.

streptococci tended to be higher in the RT group ($P = 0.06$ and 0.07). In the RT group, *C. albicans* was detected in five subjects, *C. glabrata* in one subject and *S. aureus* in two subjects. None of the controls harboured either *Candida* or *S. aureus*.

Vestibulum
The total count tended to be higher in the RT group ($P = 0.06$). The numbers of *C. albicans* and enterococci were significantly higher in the RT group than in the controls (Figure 3). The mean proportion of streptococci of the total count tended to be lower in the RT group than in the controls, 42 ± 32% and 61 ± 30%, while the median proportion was 50% for both groups. *F. nucleatum* was detected in seven RT subjects and in eight controls and *P. intermedia/P. nigrescens* in two RT subjects and in two controls. Seven RT subjects, but none of the controls, harboured at least one of the following species: *C. albicans*, *S. aureus* and enterococci. *C. albicans* was found in seven RT subjects, *C. glabrata* in two subjects (one subject harboured both *C. albicans* and *C. glabrata*), *S. aureus* in two subjects and enterococci in 11 subjects. Enteric rods were not detected in any subject in either group.

Supragingival plaque
The numbers of *Lactobacillus* spp. and *C. albicans* were significantly higher and the number of *S. mutans* tended to be higher in the RT group than in the controls (Figure 4). The proportion of *Lactobacillus* spp. of the total count was 10 ± 19% (median 2.7%) in the RT group and 0.004% in the one control with detectable *Lactobacillus* spp. The mean proportion of *S. mutans* of the total number of streptococci tended to be higher in the RT group, 6.2 ± 5.9% than in the controls, 3.6 ± 11% and the median proportion was markedly higher, 6.1% compared with 0.2% in the controls. *S. mutans* was detected in 10 RT subjects and in 12 controls and *Actinomyces* spp. in 11 and 13 subjects respectively. *Lactobacillus* spp. were more frequently detected in the RT group, in 12 subjects compared with one control. *S. sobrinus* was not detected in any subject in neither group, while *C. albicans* was detected in six RT subjects and in two controls. The proportion of *C. albicans* tended to be higher in the RT group than in the controls, 0.9 ± 2.9% and 0.0004 ± 0.001% respectively. Two of the RT subjects harboured *C. glabrata*; of these, one subject harboured both *C. albicans* and *C. glabrata*. One RT subject harboured *C. glabrata* both in the vestibulum and in the supragingival plaque and one RT subject in all four sites analysed.

Gingival crevicular region
The total count was significantly higher and the number of *P. intermedia/P. nigrescens* significantly less in the RT group than in the controls (Figure 5). No significant differences in the proportions of the micro-organisms analysed could be detected between the groups. *F. nucleatum* was detected in seven RT subjects and in 10 controls and *P. intermedia/P. nigrescens* in none of the RT subjects and in five controls. *P. gingivalis* and *A. actinomycetemcomitans* were not detected in any of the subjects in neither group.

Micro-organisms analysed in more than one site
F. nucleatum was detected in at least two of the three sites analysed in five RT subjects and in 11 controls. *P. intermedia/P. nigrescens* was detected in at least one of the three sites sampled in three subjects in the RT group compared with seven controls. *C. albicans* was found in at least one of the four sites analysed in seven of the RT subjects and in two controls (in one site, the supragingival plaque). In three RT subjects, *C. albicans* was found in all four sites analysed. *S. aureus* was detected in at least one of the three sites analysed in four subjects in the RT group and in one control (tongue). Enteric rods were only detected in two RT subjects on the tongue. The enteric rods were identified as *Pseudomonas aeroginosus* and *Serratia liquefaciens*. One control also harboured enteric rods (*Yersinia*) on the tongue. Enterococci were detected in all 13 RT subjects and in both sites analysed in eight subjects while none of the controls harboured enterococci.

Discussion

The RT subjects in this study were recruited during one year among the 50–100 persons with cancer in the head and neck region referred to the Department of Oral and Maxillofacial Surgery in Göteborg. Thirteen subjects fulfilling the inclusion criteria were willing to participate in the study. For microbial analysis of the oral samples, cultivation technique was used. The use of cultivation technique enabled us to compare the results obtained for the RT group with those obtained for subjects with hyposalivation caused by primary Sjögren's syndrome and subjects with hyposalivation caused by medicines or of unknown origin (Almståhl *et al*, 2001; Almståhl and Wikström, 2005). Cultivation technique also made it possible to calculate proportions of different species in the samples and to save strains for further characterization, like fermentation patterns and resistance to antibiotics and antimicrobial agents.

Brachytherapy
Brachytherapy has been used at Sahlgrenska university hospital for treatment of various oral cancers for about 20 years. Using brachytherapy, a higher radiation dose can be given to the tumour with minimal damage to the normal tissue. To our knowledge, this is the first study on the oral microbial flora in subjects who have been treated with brachytherapy.

Mucosal samples
An increased incidence of candidiasis before and during radiation therapy, as well as several years after completed radiation therapy has, been reported (Redding *et al*, 1999; Schwarz *et al*, 1999). In this study, 6–8 months after completed radiation therapy, we found no clinical signs of mucosal infections in the RT group. Risk factors for candidiasis in subjects with radiation-induced hyposalivation are smoking, alcohol abuse and

presence of prosthesis (Epstein *et al*, 1993). Only one of our RT subjects reported smoking and none wore prosthesis and it is our impression that they were not alcohol abusers, which might explain the low prevalence of candidiasis in our study. It should also be noted that in 46% of our RT subjects *Candida* could not be found in any of the four sites analysed. In clinical practice, it is common that subjects with inflammatory symptoms in the oral mucosal tissues are treated with anti-fungals, without preceding microbial confirmation. It is experienced that anti-fungal treatment is effective for some subjects while for others the symptoms remain. *S. aureus*, enteric rods and enterococci, all known to be frequently involved in nosocomial infections (Emori and Gaynes, 1993), have also been associated with oral mucosal infections (Dahlén *et al*, 1992). The detection frequencies of *S. aureus* and enteric rods were low in the RT subjects in this study as well as in subjects with hyposalivation caused by primary Sjögren's syndrome (Almståhl *et al*, 2001) and in subjects with hyposalivation caused by medicines or of unknown origin (Almståhl and Wikström, 2005). An unexpected finding was that all RT subjects harboured enterococci and 65% of them both on the tongue and on the vestibular mucosa. The high frequency of enterococci could be because of factors such as contact with hospital environments during the cancer treatment, periods of decreased immuno-defence and hyposalivation, factors known to enhance enterococcal colonization (Emori and Gaynes, 1993). This stresses the importance of taking microbial samples at a suspected mucosal infection to identify the micro-organism or micro-organisms involved.

Antibiotic treatment might influence on the composition of the oral microflora (Brismar *et al*, 1993; D'Antonio *et al*, 1996). No controls who had taken antibiotics <3 months prior to the examination were included. In the RT group, five subjects had used antibiotics <3 months prior to the study. It is well known from clinical practice that the subjects in this category are susceptible to infections especially in close connection with their cancer treatment. Since antibiotic treatment might influence on the oral microbial flora we thought it was important to include also subjects with recent antibiotic treatment. In those five subjects, a tendency towards lower proportions of streptococci of the total count on the tongue and buccal mucosa and tendency to a higher number of enterococci on the tongue compared with the other eight RT subjects. It is interesting to note the higher number of enterococci in the RT subjects with recent antibiotic. Enterococci are naturally resistant to many antibiotics (Emori and Gaynes, 1993) and might therefore cause opportunistic infections in these subjects.

Supragingival samples

In a study by Spak *et al* (1994), the mean number of new carious lesions was 2.9 ± 6.8 despite fluoride gel treatment 6 months after radiation therapy. However, 14 of the 37 subjects had not developed new caries lesions one year after radiation therapy (Spak *et al*, 1994). This is in line with our results. Caries lesions were rarely seen in our RT subjects and in about one-third, mutans streptococci could not be revealed. Lack of compliance with fluoride gel treatment might contribute to a high caries frequency in RT subjects (Epstein *et al*, 1996). Epstein *et al* (1996) found that only 73% of RT subjects still used fluoride gel ≥6 months after completed radiation therapy. In our study two of the 13 subjects had stopped using fluoride rinse or gel. One of these had no detectable mutans streptococci. It is possible that fluoride gel treatment affects the oral microflora and that the differences seen between the RT group and the controls were due to differences in fluoride use. In the study by Epstein *et al* (1996), however, only a minor difference in the number of mutans streptococci and no difference in *Lactobacillus* spp. counts were detected between RT subjects using and not using fluoride gel. It is therefore likely that hyposalivation have a larger effect on the oral microflora than fluoride use.

Lactobacillus spp. are also associated with caries (Van Houte, 1994). *Lactobacillus* spp. were detected in 92% of the subjects in the RT group. The corresponding figures for the pSS and Unknown groups were 70% and 55% respectively. The mean number of *Lactobacillus* spp. in the supragingival plaque was markedly higher in the RT group than in the pSS and Unknown groups, which is in congruence with the results from rinsing samples (Almståhl *et al*, 2003). *Lactobacillus* spp. are aciduric and acidogenic and might therefore enhance other acid-tolerant species such as mutans streptococci and *C. albicans*. *Lactobacillus* species can be divided into homofermentative species producing lactic acid and heterofermentative species producing lactic acid and acetic acid. The prevalence of homofermentative and heterofermentative *Lactobacillus* spp. in supragingival plaque has been analysed in RT subjects (Brown *et al*, 1975). A shift towards a higher proportion of homofermentative species after radiation therapy was seen. The most prevalent homofermentive species were *Lactobacillus casei*, *Lactobacillus salivarius* and *Lactobacillus plantarum* and the most prevalent heterofermentative species *Lactobacillus fermenti* and *Lactobacillus cellobiosus* (Brown *et al*, 1975). However, since then several of the *Lactobacillus* spp. has been reclassified and more accurate identification methods have been developed. Studies on the frequency, number and proportion of *Lactobacillus* species in specific oral sites in subjects with hyposalivation as a result of radiation therapy, primary Sjögren's syndrome, medicines or of unknown origin are in progress.

Subgingival samples

Periodontal pocket probing depths of ≥5 mm are rarely detected in subjects with radiation-induced hyposalivation (Joyston-Bechal *et al*, 1992; Leung *et al*, 1998). This is in agreement with the results for our RT group. Low frequencies of periodontal pathogens have also been reported in RT subjects (Leung *et al*, 1998; Al-Nawas and Grötz, 2006). The detection frequencies of *F. nucleatum* and *P. intermedia*/*P. nigrescens* were higher in the pSS and unknown groups than in the RT group.

Taken together, the results from this and previous studies indicate that radiation-induced hyposalivation does not increase the risk of periodontal disease. The reason might be the aciduric and acidogenic supragingival plaque and low oral pH, which are unfavourable for periodontal pathogens.

Some patients who have been subjected to radiation therapy in the head and neck region develop periodontitis in teeth in the irradiated bone (Epstein *et al*, 1998). The periodontal breakdown was more advanced in subjects with inadequate oral hygiene (Epstein *et al*, 1998). However, the subgingival microflora was not analysed in their study. We did not find increased levels of periodontal pathogens in our RT subjects; however, other micro-organisms might be involved in periodontitis in those RT subjects as in subjects with peri-implantitis such as *S. aureus* and enteric rods (Leonhardt *et al*, 1993).

In conclusion, on group level, the RT group had more pronounced differences in the oral microflora than in the group with hyposalivation caused by primary Sjögren's syndrome and the group with hyposalivation because of medicines or of unknown origin. *S. aureus* and enteric rods were rare findings in the RT group, while *Candida* was detected in 54% and all subjects harboured enterococci, which might therefore, more often than generally expected, be involved in mucosal infections in RT subjects. *Lactobacillus* spp were detected in 92% of the RT subjects and in extremely high numbers compared to the controls. Twenty three percent of the RT subjects had undetectable levels of mutans streptococci, which might explain the clinical finding that some RT subjects develop caries while others do not. The results obtained in this study six months after completed radiation therapy, stress that site-specific microbial analysis is an important diagnostic tool when planning oral health preventive care for RT subjects. A 3-year follow-up study is in progress.

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