Oral Microbiology Immunology 2008: 23: 7–13
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: an *in situ* study

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Thomas RZ, van der Mei HC, van der Veen MH, de Soet JJ, Huysmans MCDNJM. Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: an in situ *study.*
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Background/hypothesis: Secondary caries has been suggested as the main reason for restoration replacement. We hypothesized that more caries-associated bacteria are found on composite resin restoration material, compared to sound tooth tissue.
Methods: Both restored and unrestored dentin and enamel samples were placed in a full denture of eight subjects for 20 weeks. The microbiological composition of approximal plaque and the association between caries-associated bacteria and red autofluorescence of dental plaque was studied. Every 4 weeks the specimens were microradiographed using transversal wavelength independent microradiography (T-WIM). After 1 and 20 weeks red fluorescence pictures and plaque samples were taken. Samples were cultured for total anaerobic counts, mutans streptococci, lactobacilli, candida and *Actinomyces odontolyticus*.
Results: Lesion depth in the dentin and enamel was positively associated with lactobacilli, and lesion depth in dentin was positively associated with *A. odontolyticus*, whereas no association was found between mutans streptococci and lesion depth. The red-fluorescent bacteria *A. odontolyticus* and lactobacilli did not correlate with red-fluorescent plaque, indicating that red fluorescence is probably not caused by a single species of these bacteria. After 20 weeks, a higher proportion of combined mutans streptococci and lactobacilli was found on restored tissue compared to non-restored tissue ($P = 0.04$).
Conclusion: The higher proportion of caries-associated bacteria on restored tissue indicates that the ecology on the surface of primary lesions differs from that on lesions next to composite, and that secondary caries next to composite may differ from the primary caries process.

Key words: bacterial composition; transversal wavelength independent microradiography (T-WIM); secondary caries

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Accepted for publication February 19, 2007

Composite resin is now the most commonly used dental restoration material. This is in spite of the fact that the median age of composite restorations *in vivo* (8 years) is still shorter than that of amalgam restorations (11 years) (20). Secondary caries has been suggested as the main reason for restoration replacement (20), but it is not a well-defined entity, either clinically or histopathologically, and little information is available on its microbiology (9, 21). Secondary caries traditionally covered both recurrent (new) and residual (old) caries, it is impossible to see the difference clinically. In an attempt to

distinguish between both forms the current definition of secondary and recurrent caries is 'a caries lesion that develops adjacent to a restoration' (5).

Secondary caries next to amalgam has been described as occurring in two regions: an outer lesion formed on the surface of the tooth, and a wall lesion formed between the restoration and the cavity wall (6). The clinical relevance of newly formed wall lesions adjacent to amalgam as developed in *in vitro* models is under discussion (24). Such wall lesions are supposed to be the result of microleakage, but clinical and microbiological studies appear to indicate that this leakage does not lead to active demineralization beneath the restoration (10). Active demineralization adjacent to amalgam has only been found in relationship with large voids or gaps of ≥250 μm (24) or even ≥400 μm (12).

In situ research of secondary caries reported fewer outer lesions next to composite than next to amalgam and an absence of wall lesions next to composite (7). Since composites are adhesively bonded, while amalgam is not, different secondary caries lesion patterns and microbial composition may occur. Recent *in situ* research reported no difference in lesion depth of primary and secondary outer lesions, next to composite (29).

The plaque on different materials may contain high percentages of caries related microorganisms. The percentage of mutans streptococci in *in vivo* plaque was reported to be 13.7% on composite, 4.3% on amalgam and 1.1% on glass ionomer cement (26). These differences might be caused by physical parameters, such as surface roughness, or by the antibacterial effects of the dental materials. Amalgam and glass-ionomer release ions with bacteriostatic properties (22) in contrast to composite. The reported low median age of composite restorations and the high colonization level of composite compared to amalgam suggest a causative relationship, thus it seems clinically relevant to study the levels of mutans streptococci and other caries-associated bacteria in initial plaque on sound tooth enamel and dentin and compare them with the flora on composites.

A study of the microflora of caries underneath restorations found a greater microbial variety and up to eight times more micro-organisms underneath composite restorations compared to amalgam (25). Therefore, evaluation of whether composite has an effect on the microbial composition and on the development of caries lesions is necessary. We hypothesized that caries-associated bacteria increase more in the presence of composite compared to unrestored tooth tissue during the progression of caries lesions.

Carious dental tissue shows red autofluorescence (13, 14, 16) when tooth material is illuminated with violet light and viewed through a high-pass filter. Red fluorescence of dental plaque has been associated with both caries and gingivitis (8). In recent studies, single species of caries-associated bacteria were related both positively (15) and negatively (31) with red autofluorescence of plaque.

The aim of this *in situ* study was to investigate the microbiological composition of approximal plaque in relation to primary and secondary caries lesions next to composite restoration material as well as the association between caries-associated red-fluorescent bacteria and red autofluorescence of dental plaque.

Material and methods

Subjects

Twenty-one preselected patients wearing full dentures (living in Groningen, aged <75 years, healthy patients of the University Medical Centre of Groningen using no medication) were asked to take part in the study. Five female and three male volunteers agreed to participate by written informed consent. At the end of the study the subjects were between 54 and 70 years old. The study protocol was approved by the institutional review board of the University Medical Centre of Groningen (reg. number 2002/217).

Preparation of the specimens and specimen holders

Freshly extracted sound human molars and premolars, provided by local dentists, were stored in a 0.2% thymol solution. The surface of the tooth was flattened and smoothed using grinding paper (220 grit; Siawat Abrasives, Bern, Switzerland). Thirty-two enamel blocks (size 3.2 × 3.2 × 1.5 mm) and 32 enamel sections of half that size (1.6 × 3.2 × 1.5 mm) were cut from the crown areas. Two sets of 32 dentin sections of similar sizes were cut from the root areas. The half-sized sections were restored with an adhesively bonded composite resin restoration resulting in restored whole-sized sections of dentin/enamel and composite (Clearfill photoposterior; Kuraray Medical Inc, Okayama, Japan). Both enamel and dentin were treated with the adhesive procedure as described previously (29). The surface to be exposed to the caries challenge was polished using 1200-grit grinding paper. This resulted in four conditions (n = 32) of unrestored enamel (control), unrestored dentin (control), restored enamel and restored dentin samples. Randomly, two samples from each group were positioned on either side of a simulated approximal space and embedded in an acrylic (De Trey, Self-cure Acrylic, UK) specimen holder. The composite part of the restored samples was oriented towards the coronal side, to simulate an approximal box restoration. Thus, each specimen holder contained eight specimens (two of each group) (Fig. 1A,B). The specimen holder measured about 20 × 4.7 × 7 mm. Ethylene oxide gas sterilization of the specimen holders was performed at WIMAC (Kliniekdiensten B.V., Rotterdam, the Netherlands) according to ISO 9001:2000 and EN 13485:2003.

Experimental protocol

The subjects received a copy of their lower denture (experimental denture) with two sample holders, placed in the left and the right molar area (Fig. 1A). The experimental denture was worn for 20 weeks; 24 h a day, being removed only twice a day for cleaning with water and soap or fluoride-free toothpaste (Urtekram, Mariager, Denmark). Subjects were instructed to keep to their normal diet and not to clean the sample holders. After 4, 8, 12, 16 and 20 weeks the subjects were recalled and the sample holders were taken out. After plaque removal with a microbrush [Omnident blue (diameter ∼1.5 mm), Rodgau, Germany] the sample holders were microradiographed using the transversal wavelength independent microradiography (T-WIM) technique (28). After 1 week and after 20 weeks, *in situ* red fluorescence pictures were taken (see below), and subsequently plaque was sampled. Plaque sampled at 20 weeks was thus undisturbed for 4 weeks.

Microbiological analysis

Plaque was sampled after 1 week and after 20 weeks from all four sample groups, from each artificial interproximal area of the right specimen holder (IV quadrant), with a disinfected microbrush (Omnident blue). The plaque samples were immediately stored in 1 ml reduced transport fluid (NaCl 0.9 g/l, $(NH_4)_2SO_4$ 0.9 g/l, KH_2PO_4 0.45 g/l, $MgSO_4$ 0.19 g/l, K_2HPO_4 0.45 g/l,

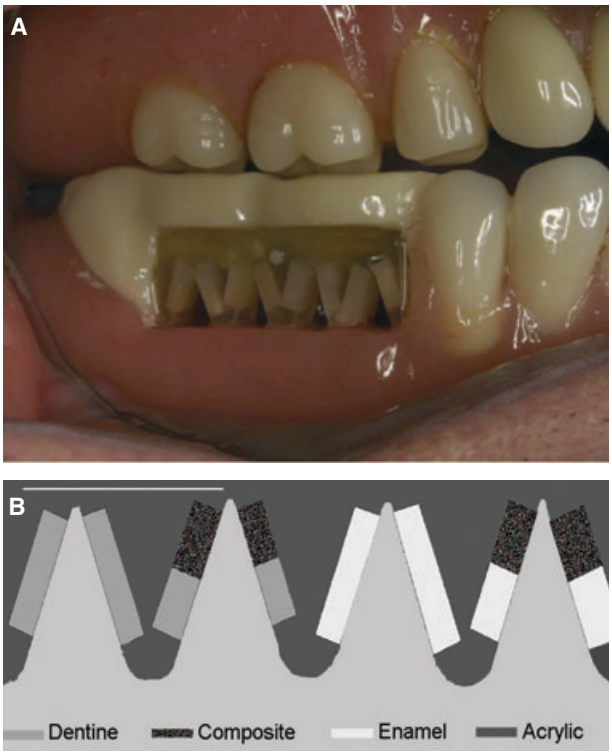

Fig. 1. (A) Photograph of a specimen holder with artificial approximal areas placed in the lower denture of an edentulous subject. Plaque samples were taken from each approximal area of the specimen holder from the fourth quadrant. (B) Schematic drawing of the specimen holder with four simulated approximal spaces at baseline. Two specimens of one condition (restored or unrestored enamel or dentin) are placed bordering one approximal space. From left to right the specimen holder contains two dentin specimens, two dentin/composite, two enamel and two enamel/composite specimens. White size bar indicates 0.5 cm.

Na_2EDTA 0.37 g/l, L-cysteine HCl 0.2 g/l, pH 6.8). Subsequently the samples were sonicated 10 times for 1 s each on ice, and appropriate 10-fold dilutions were inoculated (100 µl) on to different media cadmium sulphate–fluoride–acridine trypticase (CFAT) agar (34) plates for *Actinomyces* species, Chromagar-candida (BBL, Bedford) plates for the detection of *Candida* species, Rogosa plates for the detection of *Lactobacillus* species; TYCSB plates (comprising trypticase, yeast extract, cysteine, sucrose, bacitracin) for the detection of mutans streptococci (32) and 5% horseblood agar plates (supplemented with 5 mg/l haemin and 0.5 mg/l menadion) for detection of total anaerobic bacteria. These media were anaerobically (80% N_2, 10% H_2 and 10% CO_2) incubated at 37°C; TYCSB and Rogosa for 4 days and blood agar and CFAT for 7 days, while Chromagar plates were incubated aerobically for 4 days. Counts on selective plates were based on colony morphology and verified by Gram-stain and cell appearance using light microscopy. On CFAT agar, the *Actinomyces odontolyticus* colonies were distinguished from other *Actinomyces* colonies by colony morphology, esculin hydrolysis and haemolysis.

Red fluorescence

Autofluorescence of plaque was assessed using Quantitative Light-induced Fluorescence (QLF; camera QLF/Clin, Software QLFPATIENT version 3.0.0.4, Inspektor Research Systems BV, Amsterdam, the Netherlands). QLF uses violet–blue light from a xenon lamp filtered using a 370 nm band-pass filter and sent via a liquid light guide to the camera hand-piece, resulting in light with a wavelength peak around 405 nm (1). To filter out all the reflected and back-scattered light, fluorescence is observed through a yellow high-pass filter ($\lambda > 520$ nm). Four QLF images were captured. One image was captured of specimens 1 to 4 and one of specimens 5 to 8, from both the buccal and lingual surfaces of the specimen holder. Images were captured according to the normal procedure of the system. Analysis of red-fluorescent plaque on 128 surfaces (eight lingual and eight buccal surfaces per specimen holder) was performed using the INSPEKTOR-PRO, version 2.0.0.37 software (Inspektor Research Systems BV). The relative change in red fluorescence ratio (%) is defined as the average relative change in red : green pixel ratio with respect to the red : green pixel ratio of a healthy and plaque-free surface, i.e. bright green fluorescing, reference area inside the same image. The averaging was calculated over all the pixels inside the analysed area with a relative change exceeding 20% with respect to the green pixel ratio of the reference area (30). For comparison purposes the relative changes in the red fluorescence of the two buccal and two lingual surfaces on either side of the artificial interdental areas were averaged. Subsequently all the results for each condition were averaged to yield one relative change in red fluorescence for a condition.

Statistical analysis

Total colony-forming units (CFU) per sample were transformed to $\log_{10}$ to normalize the distributions. The detection limit for bacteria in samples was 100 CFU. Means of the restored and unrestored tissue (enamel and dentin) were compared using one-sided paired *t*-tests. For each sample the specific bacteria were expressed as a proportion of the total flora and averages were calculated. These were compared using one-sided paired *t*-tests. The association of bacteria and red fluorescence with lesion depth was evaluated using linear and non-linear curve fitting. All analyses were performed using EXCEL (version 10, MICROSOFT OFFICE EXCEL, 2003). A P value of 0.05 was considered statistically significant.

Results

Seven (out of eight) subjects completed the study successfully for the duration of 20 weeks, one male subject completed 16 weeks. Only for this subject, the second plaque sampling took place at 16 weeks, and the results were included in the study (as 20-week results).

At 20 weeks significantly higher levels of total flora (factor 10), lactobacilli (factor 100) and red fluorescence (factor 2.3) were found in plaque than at 1 week (Table 1). One-week-old plaque shows a complex flora with a small percentage (<15%) of lactobacilli and mutans streptococci. After 20 weeks *in situ*, between 30 and 60% of the total viable count consists of these

Table 1. Bacterial composition from plaque of restored and non-restored dentin and enamel after 1 week and 20 weeks *in situ*

	Dentin (n = 8)		Dentin/composite (n = 8)		Enamel (n = 8)		Enamel/composite (n = 8)	
	1 week Mean (SE)	20 weeks Mean (SE)	1 week Mean (SE)	20 weeks Mean (SE)	1 week Mean (SE)	20 weeks Mean (SE)	1 week Mean (SE)	20 weeks Mean (SE)
Lesion depth (μm)[1,2]	68.8 (2.0)	533.0 (106.4)	65.8 (4.3)	498.1 (121.0)	58.4 (10.1)	211.9 (43.5)	60.9 (4.9)	200 (46.4)
Total CFU	7.34 (0.28)	8.28 (0.3)	7.11 (0.3)	8.1 (0.29)	6.63 (0.46)	8.21 (0.26)	6.67 (0.50)	8.15 (0.31)
Mutans streptococci	4.40 (0.38)	4.95 (0.95)	4.03 (0.34)	4.91 (0.98)	3.91 (0.46)	4.79 (0.96)	4.02 (0.34)	4.91 (0.94)
Lactobacilli	4.56 (0.53)	6.72 (0.67)	4.23 (0.42)	6.69 (1.81)	4.47 (0.5)	6.6 (0.67)	4.67 (0.57)	6.62 (0.66)
Candida	2.98 (0.51)	3.18 (0.62)	2.96 (0.51)	2.80 (0.61)	2.87 (0.54)	3.24 (0.69)	2.89 (0.37)	3.26 (0.66)
A. odontolyticus	4.38 (0.59)	4.59 (0.82)	4.13 (0.57)	4.83 (0.70)	3.97 (0.59)	5.57 (0.61)	4.06 (0.67)	4.32 (0.72)
Red fluorescence(%)	7.8 (0.6)	18.2 (2.5)	7.7 (0.7)	18.4 (2.5)	7.0 (0.7)	17.5 (2.7)	8.5 (0.6)	16.8 (2.1)

Data show total CFU, mutans streptococci, lactobacilli, candida, *Actinomyces odontolyticus* (as $\log_{10}$).
[1]One week and 16 weeks data from Thomas et al., in press (29).
[2]Lesion depth (μm) data are at 16 weeks instead of 20 weeks.

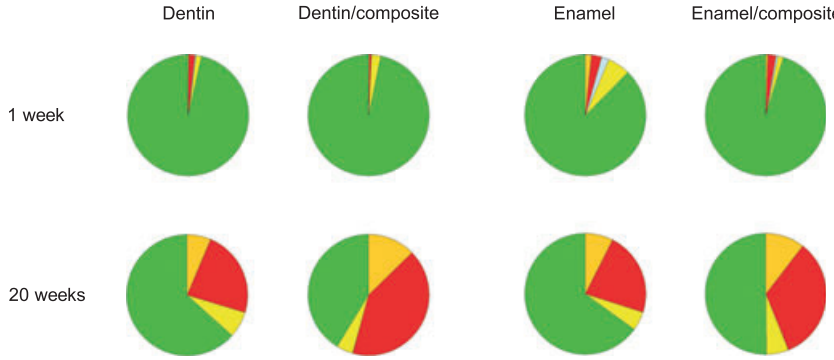

Fig. 2. Pie charts of caries-associated bacteria as a percentage of total viable counts cultured from dental plaque after 1 and 20 weeks of dentin and enamel, both restored and non-restored samples of eight subjects wearing *in situ* dentures. Subsequently mutans streptococci (orange), lactobacilli (red), candida (light blue), *Actinomyces odontolyticus* (yellow) and other bacteria (green) are represented.

caries-associated bacteria (Fig. 2). No significant difference in absolute numbers of total flora and specific bacteria was found between restored and non-restored samples (Table 1). Calculating the specific bacteria as a proportion of the total flora, more caries-associated bacteria (P = 0.04 for mutans streptococci and lactobacilli combined) were found on restored dentin and enamel samples (Fig. 2).

Red fluorescence of plaque was significantly higher after 20 weeks *in situ* than after 1 week (Table 1). Red fluorescence varied between subjects (Table 2). There was no relation between red fluorescence of the plaque and the number of CFU of orange fluorescent lactobacilli or red-fluorescent *A. odontolyticus*, either for separate samples or after pooling the data for all conditions in each subject (Fig. 3).

The lesion depth data in this *in situ* study have been reported before and have been included in Tables 1 and 2 (29). After pooling the data for all conditions in each subject, seven out of eight subjects harboured mutans streptococci. Lactobacilli and *A. odontolyticus* were found in all samples. *Candida* spp. were found in five out of the eight subjects. When the caries-associated bacteria were related to lesion progression positive associations of lesion depth with levels of lactobacilli in both dentin (R = 0.90) and enamel (R = 0.63) (exponential curve fit) and of *A. odontolyticus* in dentin (P = 0.0019, R = 0.59) (linear curve fit) were found, but for enamel a non-significant association (P = 0.27, R = 0.29 (linear curve fit) was found (Fig. 4B). No association was found with lesion depth and mutans streptococci, *Candida*, *A. odontolyticus* in enamel or total flora (Fig. 4A,C–E).

Discussion

Microbiological characteristics of *in-situ*-developed primary and secondary caries lesions were evaluated. Plaque was sampled 1 week after the sample holders were placed in the dentures of the edentulous subjects. This 1-week-old plaque was considered to contain the normal individual bacterial flora for sound tooth tissue. Plaque removal from the challenged surface is a requirement for quantification of lesion depth on the transversal T-WIM microradiographs, so every 4 weeks plaque had to be removed from the samples. Therefore the plaque sample taken after 20 weeks *in situ* was 4-week-old plaque

Table 2. Lesion depth (μm) for dentin and enamel at 16 weeks and bacterial composition at 20 weeks

Subjects	Dentin μm (SE)[1]	Enamel μm (SE)[1]	Total flora $\log^{10}$ (SE)	Mutans strep. $\log^{10}$ (SE)	Lactobacilli $\log^{10}$ (SE)	Candida $\log^{10}$ (SE)	*A. odontolyticus* $\log^{10}$ (SE)	Red flu (%) (SE)
D	74.3 (11.4)	107.5 (43.2)	8.4 (0.04)	2.3 (0.16)	2.8 (0.14)	2.0 (0)	2.0 (0)	12.9 (0.2)
H	217.7 (73.5)	61.5 (12.2)	8.6 (0.06)	8.4 (0.11)	5.3 (0.25)	4.2 (0.7)	3.4 (0.8)	17.9 (2.0)
A	429.0 (145.9)[2]	89.8 (4.74)	6.8 (0.17)	2.0 (0)	6.2 (0.19)	2.0 (0)	5.9 (0.2)	25.5 (3.7)
G	433.5 (60.0)	197.3 (13.9)	8.6 (0.01)	6.9 (0.10)	7.7 (0.16)	2.0 (0)	4.1 (1.2)	10.4 (1.0)
B	600.8 (52.3)	181.0 (12.0)	8.3 (0.13)	2.0 (0)	7.7 (0.08)	4.1 (0.17)	6.0 (1.3)	14.7 (1.6)
C	751.3 (62.6)	361.5 (22.5)	8.2 (0.19)	5.4 (0.12)	8.0 (0.06)	2.0 (0)	4.4 (0.11)	12.1 (0.5)
F	787.7 (26.0)	357.8 (11.7)	9.3 (0.07)	8.1 (0.16)	8.4 (0.12)	6.7 (0.09)	6.7 (0.16)	18.1 (2.0)
E	902.3 (13.0)	280 (69.8)	7.1 (0.13)	4.1 (0.17)	6.9 (0.11)	2.0 (0)	6.1 (0.21)	30.0 (3.3)

[1]Data from Thomas et al. in press (29).
[2]No outer lesions were found in subject A. The lesion depth given is from wall lesions, measured perpendicular to the restoration zinterface.
Data shown are total CFU and specific bacteria as $\log_{10}$ (SE) per subject and red fluorescence of plaque pooled for the four conditions at 20 weeks (values are mean and SE). The table is ranked by increasing lesion depth in dentin.

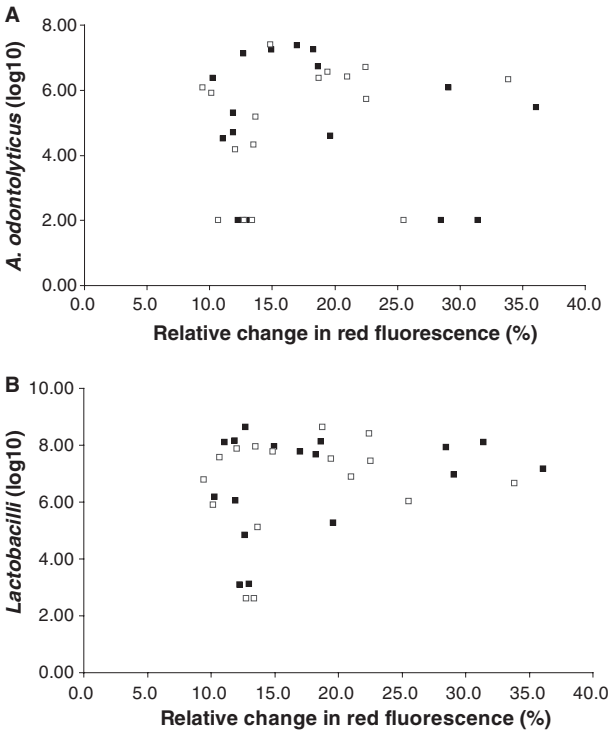

Fig. 3. Relation between relative change in red autofluorescence of plaque (%) and two red-fluorescent bacteria: *Actinomyces odontolyticus* (A) and lactobacilli (B). Each square symbol represents a sample of enamel (open) or dentin (closed).

that was removed from material that was demineralized for 20 weeks. Since plaque composition will not change significantly after 2–3 weeks (17, 27), based on plaque accumulation studies for experimental gingivitis, we assume that the 4-week-old plaque, accumulated from 16–20 weeks, resembles the plaque of a 20-week-old caries process. As in most studies on caries microbiology, we do not attempt to study the causative organisms involved in the caries process, because we cannot determine all of the possibly 800 species involved.

We hypothesized that caries-associated bacteria, such as lactobacilli and mutans streptococci, are more prevalent in the presence of composite material. Our theory is that plaque pH on restoration material stays low for a longer time. On or next to restorations, no or fewer dissolution products of enamel or dentin are available to neutralize acids; this may favour high proportions of aciduric microorganisms (4). The present study shows a higher proportion of lactobacilli and mutans streptococci on restored dentin and enamel ($P = 0.04$) (Fig. 2). Differences in proportion, but not in absolute numbers, may indicate that the secondary caries environment may not stimulate these specific species, but inhibits other species thereby disturbing the balanced ecosystem of the biofilm. Another reason can be that there are novel or unknown aciduric bacteria, which cannot be cultured on blood agar (2).

The shift from flora with lower caries activity (1 week) to a dominance of caries-associated bacteria with high caries activity (20 weeks *in situ*) is expected and in agreement with the literature (23) (Fig. 2). Lesion progression data varied considerably between the subjects and this was mainly attributed to natural variation in caries susceptibility. Seven out of eight subjects (except for subject A, forming wall lesions) harboured mutans streptococci irrespective of the caries lesion depth. This result confirms a recent theory that mutans streptococci may be good markers of the disease but are not necessarily the aetiological agents (2). The positive association between lactobacilli and lesion depth confirms previous work (33). The prevalence of *Candida* spp. agrees with a reported isolation frequency of 63% (19). The positive association of *A. odontolyticus* with lesion progression in dentin is a new finding. Despite the fact that very early stages of demineralization were associated with increases in *A. odontolyticus* (19), an association of *A. odontolyticus* with lesion progression has not been reported before. The mechanism behind *Actinomyces* species and root caries is unknown, it has been suggested that *Actinomyces* species may bind selectively to collagen (18).

Red fluorescence of plaque was significantly higher after 20 weeks *in situ* than after 1 week (Table 1). Increase of red fluorescence, combined with a less complex flora, containing a few dominant caries-associated bacteria, confirms the hypothesis that red-fluorescent plaque is mature plaque (3, 31). Interestingly, no correlation was found between red fluorescence of plaque and the number of CFU of orange–red-fluorescent lactobacilli species (15) or of intense-red-fluorescent *A. odontolyticus* species (14), indicating that red fluorescence is probably not caused by a single species of lactobacilli and/or *A. odontolyticus*. This result supports a previous finding of our group, where it was concluded that the intrinsic characteristics of the biofilm are probably more responsible for the autofluorescence than the characteristics of the single species (31). These results could be influenced by the use of the red fluorescence measurements on *in situ* tooth material, while the method has been developed for *in vivo* studies. Furthermore, it must be realized that in the microbial analysis we did not analyse the complete composition but focused on the known red-fluorescent bacteria.

Özer and Thylstrup (24) concluded that 'secondary caries is not a universal attack along the entire interface between tooth and restoration, but rather a new lesion or a re-beginning of caries on the surface due to local conditions for plaque formation with cariogenic potential'. We found no difference between primary and secondary outer lesion progression (29), while this was expected based on the levels of mutans streptococci and lactobacilli. We should however realize that a higher proportion of aciduric bacteria, such as the mutans streptococci and lactobacilli, on restorations might be the results of a lower environmental pH because of the absence of enamel buffer. This buffer capacity is present on non-restored sites. We suggest that when the enamel is decalcifying, the plaque pH rises, thereby inhibiting the growth of aciduric organisms. On the contrary, a higher concentration of aciduric bacteria, resulting in a lower localized pH on composite, may lead to a higher chance of demineralization of the tooth tissue next to the composite. Based on our present results it is unclear which of these opposing mechanisms plays the major role. The restorations in our *in situ* model are made

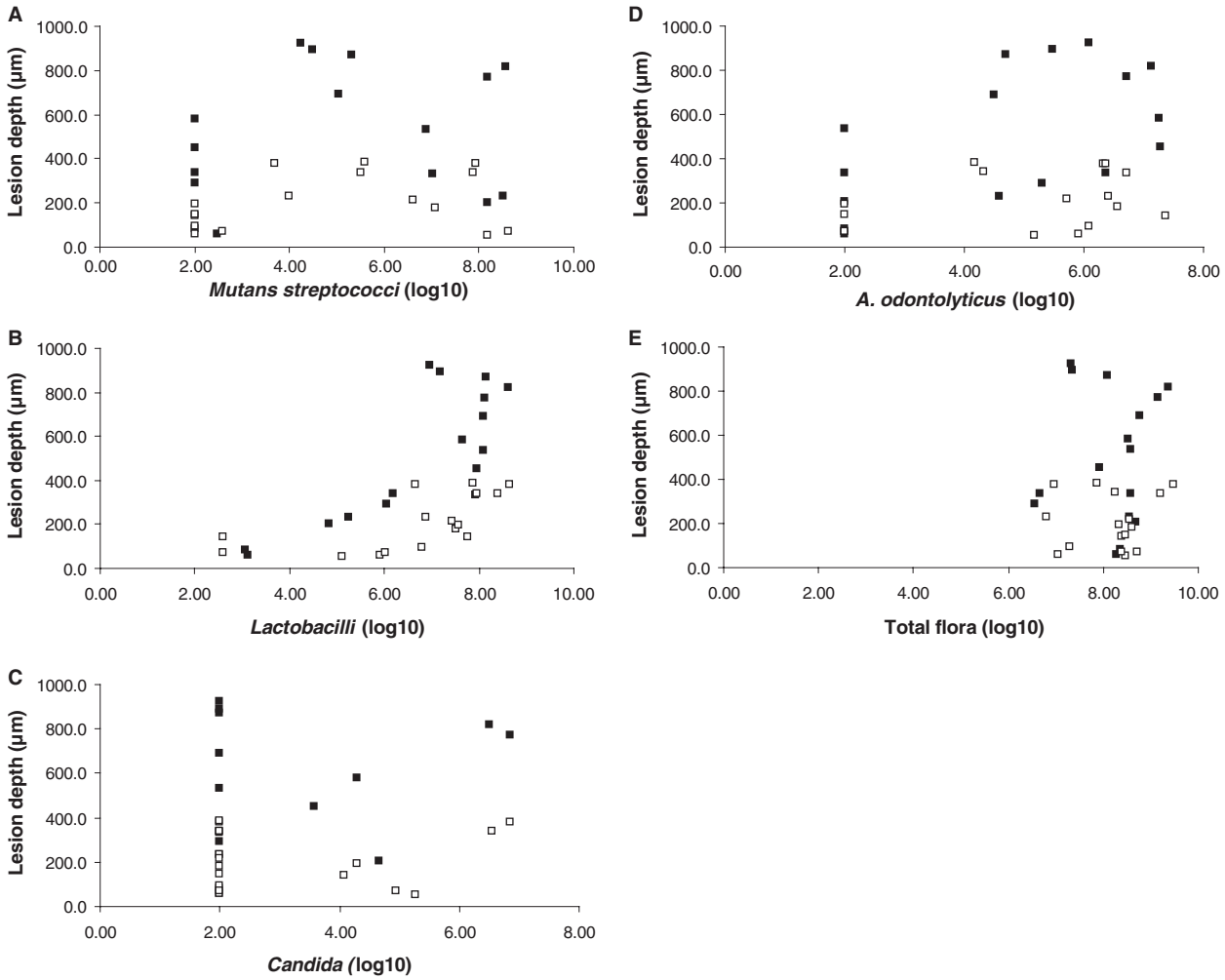

Fig. 4. Association between lesion depth (of both dentin and enamel) and caries-associated bacteria: mutans streptococci (A), lactobacilli (B), candida (C), *Actinomyces odontolyticus* (D), and total viable flora (E). Each symbol represents a sample of enamel (open squares) or dentin (closed squares).

under ideal circumstances and highly polished; the tooth restoration interface will therefore probably not function as a plaque stagnation site. After making an ideal restoration, the only difference between primary and secondary caries lesion initiation and progression from a microbiological point of view is the adherent substrate (restoration).

Although Kidd et al. (11) found no differences between the microflora in the carious dentin of primary and secondary lesions next to amalgam, our results indicate that the ecology on the surface of the primary lesions differs from that on the surface of lesions next to composite, and that secondary caries next to composite may differ from the primary process. A higher proportion of caries-associated microorganisms on restored surfaces may explain the clinical experience of higher secondary caries rates for composite restorations. Further evaluation of microbiological composition and progression of secondary caries lesions is recommended.

Acknowledgments

The work in this study was financially supported by University Medical Centre Groningen. The authors wish to thank the subjects who participated in the study and the dentists who provided the extracted teeth. R.Z.T. is indebted to Jan Ruben, Rolf de Ruijter, Janine Oosterhof and Pascal Lokin for their help during and the continuance of the study protocol during her maternity leave.

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