The *in vivo* dynamics of *Streptococcus* spp., *Actinomyces naeslundii*, *Fusobacterium nucleatum* and *Veillonella* spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence *in situ* hybridization

Ali Al-Ahmad,[1] Axel Wunder,[1] Thorsten Mathias Auschill,[1] Marie Follo,[2] Gabriele Braun,[1] Elmar Hellwig[1] and Nicole Birgit Arweiler[1]

Correspondence
Ali Al-Ahmad
ali.al-ahmad@uniklinik-freiburg.de

[1]Department of Operative Dentistry and Periodontology, Albert-Ludwigs-University, Freiburg, Germany

[2]Department of Hematology and Oncology, Core Facility, Albert-Ludwigs-University, Freiburg, Germany

The formation and composition of dental plaque biofilm *in vivo* are important factors which influence the development of gingivitis, caries and periodontitis. Studying dental plaque biofilm in *in vitro* models can cause an oversimplification of the real conditions in the oral cavity. In this study, bovine enamel slabs were fixed in an individual acrylic appliance *in situ* to quantify dental plaque formation and composition using multiplex fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy. Each of the five oligonucleotide probes used for FISH was specific for either eubacteria or one of four frequently isolated bacterial constituents belonging to early and late colonizers of tooth surfaces. The thickness of formed biofilm increased from 14.9 ± 5.0 µm after 1 day to 49.3 ± 11.6 µm after 7 days. *Streptococcus* spp. were predominant in 1-day-old dental plaque and decreased significantly after 7 days ($P = 0.0061$). Compared to the first day, *Fusobacterium nucleatum* decreased after 2 days and increased significantly after 7 days ($P = 0.0006$). The decreases of *Actinomyces naeslundii* content on day 2 and day 7 were significant ($P = 0.0028$). Changes in *Veillonella* spp. were not significant during the study period ($P > 0.05$). The results showed that an *in vivo* observation period of 7 days was required to detect significant changes in *Streptococcus* spp. and *F. nucleatum*. The multiplex FISH used is suitable for analysing the dynamics of four important bacterial constituents in the oral biofilm in epidemiological studies.

INTRODUCTION

Dental plaque is the trigger for most dental diseases such as gingivitis, caries and periodontitis. Knowledge about the composition of dental plaque biofilm *in vivo* can be helpful in finding treatment and prevention strategies. Dental plaque consists of a multispecies biofilm of more than 500 species of bacteria and is the result of complex processes in the oral cavity (Foster & Kolenbrander, 2004; Kolenbrander *et al.*, 2002; Kroes *et al.*, 1999; Moore & Moore, 1994; Paster *et al.*, 2001). Coaggregation consisting of cell–cell recognition between genetically distinct bacteria and coadhesion due to the recognition between planktonic cells and surface-attached cells are two important processes in biofilm development in the oral cavity (Bos *et al.*, 1996;

Ciardi *et al.*, 1987; Gibbons & Nygaard, 1970; Kolenbrander *et al.*, 2002). The interactions between bacteria and salivary proteins as well as with the extracellular matrix are also major elements in the development of plaque biofilms. Additionally, one important factor for the initial colonization during the formation of the dental plaque biofilm is the salivary acquired pellicle, which is the basis for further bacterial adhesion and colonization of the tooth surface (Lendenmann *et al.*, 2000). The composition of the salivary pellicle formed *in vitro* has been found to be different from that formed *in situ* (Carlen *et al.*, 1998).

The spatial organization and development of oral biofilms have been studied intensively using different *in vitro* models (Guggenheim *et al.*, 2001; Foster & Kolenbrander, 2004; Kolenbrander *et al.*, 1999; Palmer & Calwell, 1995; Palmer *et al.*, 2001a). Though *in vitro* models cannot simulate all of the complex processes involved in biofilm

Abbreviations: CLSM, confocal laser scanning microscopy; FISH, fluorescence *in situ* hybridization.

development in the oral cavity, there have been very few *in vivo* studies of biofilm composition in the oral cavity. These involved the use of retrievable enamel chips or devices which did not disturb the already formed natural biofilms (Arweiler *et al.*, 2004; Foster *et al.*, 2003; Kolenbrander *et al.*, 2005; Palmer *et al.*, 2001b; Wecke *et al.*, 2000; Wood *et al.*, 2000, 2002). An oversimplification of the conditions in the oral cavity with regards to factors such as nutrient composition, flow of saliva and interaction with unculturable bacteria cannot be excluded in *in vitro* models using defined micro-organisms.

Viable plate count and culture-dependent techniques select for certain bacteria present in the dental plaque, which can lead to false results with regards to the actual bacterial structure. Fluorescence *in situ* hybridization (FISH) is a useful method for detection of bacteria without disruption of their natural environment (Amann *et al.*, 1995; Moter & Göbel, 2000). The combination of FISH with confocal laser scanning microscopy (CLSM) has been used to obtain images of three-dimensional reconstructions of natural microbiological environments (Wagner *et al.*, 1994; Sunde *et al.*, 2003).

In this study, a multiplex FISH assay was first established on pure bacterial cultures and then used in combination with CLSM to study the levels of important bacterial members of *in vivo* dental plaque biofilm formed after different periods of time (1, 2, 3, 5 and 7 days). In addition to a probe which allowed detection of all eubacteria, specific probes for *Streptococcus* spp., *Fusobacterium nucleatum*, *Actinomyces naeslundii* and *Veillonella* spp. were simultaneously used to detect the different bacteria present in dental plaque biofilm.

METHODS

Plaque growth *in situ*. A healthy 27-year-old volunteer was selected who did not use antibacterial mouthrinses or antibiotics for 6 months prior to the start of the investigation. The volunteer wore an individual acrylic appliance in the upper jaw over periods of 1, 2, 3, 5 and 7 days. Six sterilized and BSE-free bovine enamel discs (each 3 mm in diameter and 2 mm in height; Fig. 1A–F) were inserted facing the interdental area between two adjacent teeth in such a way that biofilm growth was not disturbed by the tongue or the cheek. The enamel discs were held in place by red wax. The bovine enamel samples were sterilized by ultrasonication for 2 min in 2 % sodium hypochlorite followed by ultrasonication in 70 % ethanol for another 2 min. Following this, the samples were washed twice in sterile distilled water. The subject maintained his regular diet and wore the appliance (intra-orally) throughout all test periods, except during his daily mechanical oral hygiene protocol. After each of the five test periods, all six enamel chips were removed from the splint and processed without delay.

FISH and confocal microscopy. FISH was conducted according to Amann (1995). In brief, biofilms grown on enamel chips were fixed in 4 % paraformaldehyde in PBS (1.7 mM KH_2PO_4, 5 mM Na_2HPO_4 with 0.15 M sodium chloride, pH 7.2) for 12 h at 4 °C. After fixation all specimens were washed with PBS and fixed again in solution containing ethanol (50 %, v/v, in PBS) for 12 h. The probes were then washed twice with PBS, followed by incubation in a solution
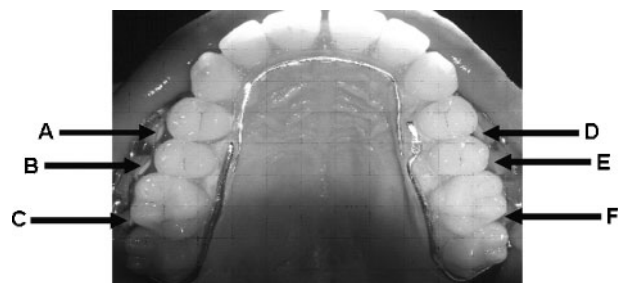

Fig. 1. Intra-oral micrograph of the acrylic appliance *in situ* in the upper jaw with the specimens in place at the different locations (A–F). The exposed surfaces are fixed towards the tooth enamel. The enamel discs were held in place by wax.

containing 7 mg lysozyme per ml 0.1 M Tris/HCl, 5 mM EDTA (pH 7.2) for 10 min at 37 °C in order to permeabilize cells in the plaque biofilm. The biofilms were then dehydrated with a series of ethanol washes containing 50, 80 and 100 % ethanol for 3 min each. Specimens were then incubated with the oligonucleotide probes at a concentration of 50 ng each per 20 μl hybridization buffer [0.9 M NaCl, 20 mM Tris/HCl (pH 7.2), 25 % (v/v) formamide and 0.01 % (w/v) sodium dodecyl sulphate]. Hybridization was conducted in 96-well plates (Greiner bio-one) at 46 °C for 2 h. Following probe hybridization, specimens were incubated for 15 min at 48 °C in wash buffer containing 20 mM Tris/HCl (pH 7.5), 5 mM EDTA, 159 mM NaCl and 0.01 % (w/v) sodium dodecyl sulphate. After washing, the labelled biofilms were analysed in a chambered coverglass (Lab-Tek II, Nalge Nunc International) by CLSM (Leica TCS SP2 AOBS) using a ×63 water immersion objective (HCX PL APO/bd.BL 63.0 × 1.2 W; Leica).

All HPLC-purified oligonucleotide probes used in this study were synthesized commercially and 5′-end-labelled with different fluorochromes (Thermo Electron). The 5′-modification was chosen after testing the different fluorochromes in a multiplex FISH assay using the bacterial strains which were taken to test the specificity of the different probes. EUB 338 was used to visualize the entire bacterial population within the plaque specimen. The sequence specificities of the oligonucleotide probes were tested using the following strains: *A. naeslundii* DSM 17233[T], *A. naeslundii* clinical isolate, *Actinomyces viscosus* clinical isolate, *Candida albicans* ATCC 90028, *Enterococcus faecalis* clinical isolate, *F. nucleatum* subsp. *nucleatum* ATCC 25586, *F. nucleatum* clinical isolate, *Fusobacterium necrophorum* ATCC 27852, *Lactobacillus brevis* DSM 20054, *Lactobacillus salivarius* DSM 20555, *Peptostreptococcus micros* ATCC 23195, *Prevotella nigrescens* NCTC 9336, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* DSM 1798, *Streptococcus mutans* ATCC 25175, *Streptococcus oralis* ATCC 35037, *Streptococcus sanguis* DSM 20068, *Streptococcus salivarius* DSM 20067, *Streptococcus sobrinus* DSM 20381, *Veillonella parvula* DSM 2008[T] and *V. parvula* clinical isolate. All clinical isolates were kindly provided by the Institute of Medical Microbiology and Hygiene of the Albert-Ludwigs-University of Freibug.

The sequence, target species, reference and 5′-modifications are listed in Table 1.

Excitation of the FISH probes was carried out at the following wavelengths: Pacific blue, 405 nm; fluorescein, 488 nm; Cy3, 546 nm; Texas red, 594 nm; Cy5, 633 nm. Fluorescence emission of the probes was measured at the following wavelengths: Pacific blue, 406–473 nm; fluorescein, 495–565 nm; Cy3, 493–538 nm; Texas red, 599–670 nm; Cy5, 550–592 nm. In order to minimize spectral overlap between the

Table 1. Sequence, target species, reference and 5′-modifications of used oligonucleotide probes

Probe	Sequence (5′–3′)	5′-modification	Target	Reference
EUB 338	GCTGCCTCCCGTAGGAGT	Fluorescein	Eubacteria	Amann *et al.* (1990)
E 79	AATCCCCTCCTTCAGTGA	Texas red	*Veillonella* spp.	Paster *et al.* (1998)
FUS 664	CTTGTAGTTCCGC(C/T)TACCTC	Cy5	*F. nucleatum*	Thurnheer *et al.* (2004)
IF 201	GCTACCGTCAACCCACCC	Pacific Blue	*A. naeslundii*	Foster & Kolenbrander (2004)
STR 405	TAGCCGTCCCTTTCTGGT	Cy3	*Streptococcus* spp.	Paster *et al.* (1998)

probes, confocal scanning was carried out sequentially for each image. The curve of fluorescence response is principally sigmoid. However, this is dependent on the concentration of fluorescent probes attached to the ribosomes. Since FISH detects cells with different numbers of ribosomes, the overall detected fluorescence response is sigmoid, but it could also be linear for individual cells.

Image and statistical analysis. The measured areas were from three separate and representative locations on the bovine enamel slabs covered with dental plaque biofilm. The biofilm was examined at several locations. Within each area the thickest point was measured by determination of the upper and lower boundaries of the biofilm. This procedure was repeated twice so that a mean thickness of the biofilm was then determined from the three measurements. Biofilms were scanned from these three starting points, generating sections of a thickness of approximately 0.5 µm each at 2 µm intervals throughout the biofilm layers (to avoid overlaps). Standard images were made with a zoom setting of 1.7 corresponding to physical dimensions of 140×140 µm for each image. The area of each section was transformed into a digital image containing 1024×1024 pixels.

In order to quantify the biomass of the different targets within the oral biofilm, total fluorescent staining of the confocal micrographs was analysed using the image analysis program MetaMorph 6.3r7 (Molecular Devices Corporation). The EUB 338 corresponding fluorescent volume was set as 100 % of bacterial biomass in the biofilm. All other targets were calculated as percentage of the biomass calculated by EUB 338. The program was used to calculate the biofilm composition from stacks of five-channel images by measuring voxel intensities. Fluorescence intensity thresholds were manually set for each of the fluorescent colours. Eighteen biofilm points, three on each of six bovine enamel slabs, were analysed for each experimental period (each biofilm age). The resulting biofilm contents were analysed for statistical significance.

One-way ANOVA was used to calculate significant differences in biofilm thickness over the plaque growth period. Three-way ANOVA (PROC GLM, SAS 8.2) and the Tukey test were used to determine whether significant differences existed for the different biofilm targets over the different biofilm-forming periods (1–7 days). In addition to calculating the global significance values (*P* values), the days were pairwise analysed using the Tukey test to deliver detailed significant differences between the different ages of plaque. Bonferroni's corrections as a post test are not necessary.

RESULTS AND DISCUSSION

In Table 2, the means and standard deviations of biofilm thickness after different growth periods are presented. After 1 day, a mean biofilm plaque thickness of 14.9 ± 5.0 µm was measured. After 2 days, the thickness of the oral biofilm increased significantly to 33.6 ± 7.4 µm ($P \leqslant 0.001$, by ANOVA). A 3-day-old oral biofilm showed a mean thickness of 34.3 ± 10.2 µm, which was not significantly

different compared to the 2-day-old biofilm ($P = 0.804$). A significant increase in biofilm thickness to 44.9 ± 6.2 µm ($P = 0.001$) was detected at day 5. The increase in biofilm thickness to 49.3 ± 11.6 µm after 7 days was not significant ($P = 0.165$) compared to day 5.

In Fig. 2, confocal micrographs of dental plaque biofilm hybridized with five different specific probes as presented in Table 1 are shown. The quantitative results of bacteria detected are presented in Fig. 3(a–d). The percentages presented below are all median values. One day after biofilm formation, *Streptococcus* spp. made up 40.8 % of the grown biofilm, whereas the percentages of *F. nucleatum*, *A. naeslundii* and *Veillonella* spp. were detected to be 9.9, 7.7 and 1.4 %, respectively. After 2 days of biofilm formation *in situ*, *Streptococcus* spp. were still the majority of detected bacteria and made up 26.7 %, followed by *F. nucleatum* with 4.6 %, *Veillonella* spp. with 4.0 % and *A. naeslundii* with only 0.2 %. The content of *Streptococcus* spp. was 25.7 % in 3-day-old oral biofilm and 33.9 % and 13.1 % in 5-day- and 7-day-old biofilm, respectively. For *F. nucleatum*, the following percentage values were detected in 3-, 5- and 7-day-old biofilms: 10.3, 9.5 and 22.1 %, respectively. The *A. naeslundii* content was 6.1 % in 3-day-old biofilm, 4.4 % in 5-day- and 0.2 % in 7-day-old biofilm. *Veillonella* spp. were found at 5.1, 2.5 and 3.2 % after 3, 5 and 7 days of biofilm cultivation, respectively.

Statistical analysis showed that decreases of *Streptococcus* spp. content were significant only after 7 days ($P = 0.0061$). Compared to the first day, the content of *F. nucleatum* decreased after 2 days and then increased significantly after 7 days ($P = 0.0006$). The decreases in *A. naeslundii* content on day 2 and day 7 were significant ($P = 0.0028$). All other changes were not significant in comparison to day 1 ($P > 0.05$). Changes in *Veillonella* spp. were not significant throughout the entire plaque growth period ($P > 0.05$). The percentage results presented in our study were also

Table 2. Thickness of analysed dental plaque biofilm

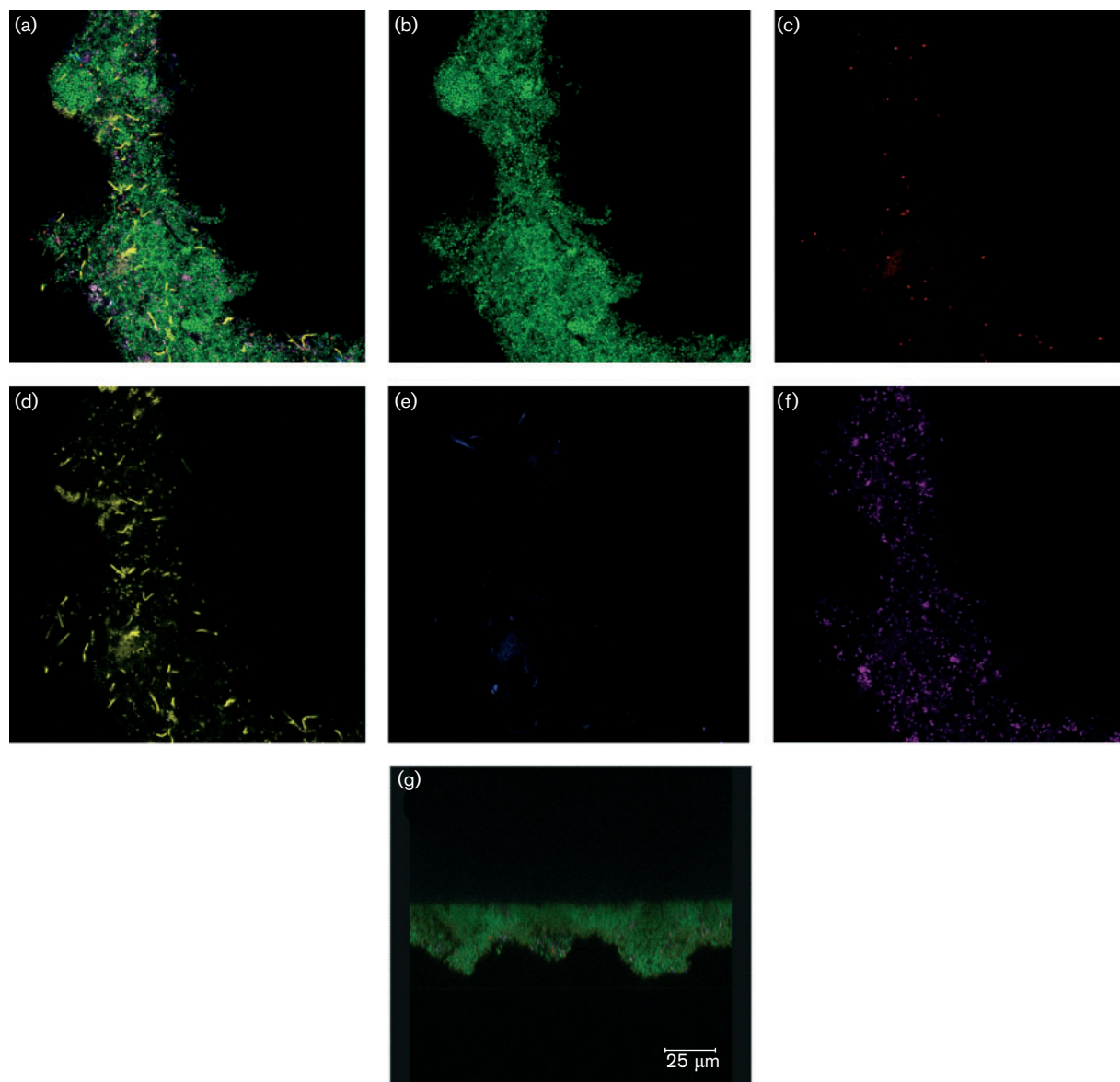| Age of dental plaque (days)	Mean thickness ± SD (µm)
1	14.9 ± 5.0
2	33.6 ± 7.4
3	34.3 ± 10.2
5	45.0 ± 6.1
7	49.3 ± 11.6

Fig. 2. Confocal micrographs of 3-day-old dental plaque biofilm hybridized with five different specific probes as presented in Table 1. (a) Single optical section from five channel image stack. (b–f) All channels of the stack. (b) Green, eubacteria-specific probe; (c) red, *Veillonella* spp.-specific probe; (d) yellow, *F. nucleatum*-specific probe; (e) blue, *A. naeslundii*-specific probe; (f) magenta, *Streptococcus* spp.-specific probe. (g) Orthogonal slice of the plaque biofilm.

statistically compared in three levels of the biofilms (top, middle and bottom). This revealed no statistically significant differences, which means that these results were not influenced by the penetration of the probes used.

The four detected bacterial targets exemplify a wide range of metabolic and physiological characteristics which represent a diverse array of oral bacteria (Foster & Kolenbrander, 2004). The advantages of using FISH to identify micro-organisms in oral biofilms include the possibility of the detection of uncultured bacteria, the development of new probes is more rapid than production

and characterization of antibodies and that the correlation shown between FISH and the results after staining with diamino-2-phenylindole dihydrochloride was good (Foster *et al.*, 2003).

There have been several studies using FISH to detect either multiple Gram-negative or Gram-positive bacterial species within biofilms and in activated sludge (Amann *et al.*, 1996; Schmid *et al.*, 2000). Recently, Thurnheer *et al.* (2004) showed that FISH using triple hybridizations to detect both Gram-positive and Gram-negative bacteria in *in vitro* biofilm consisting of six-species is possible.
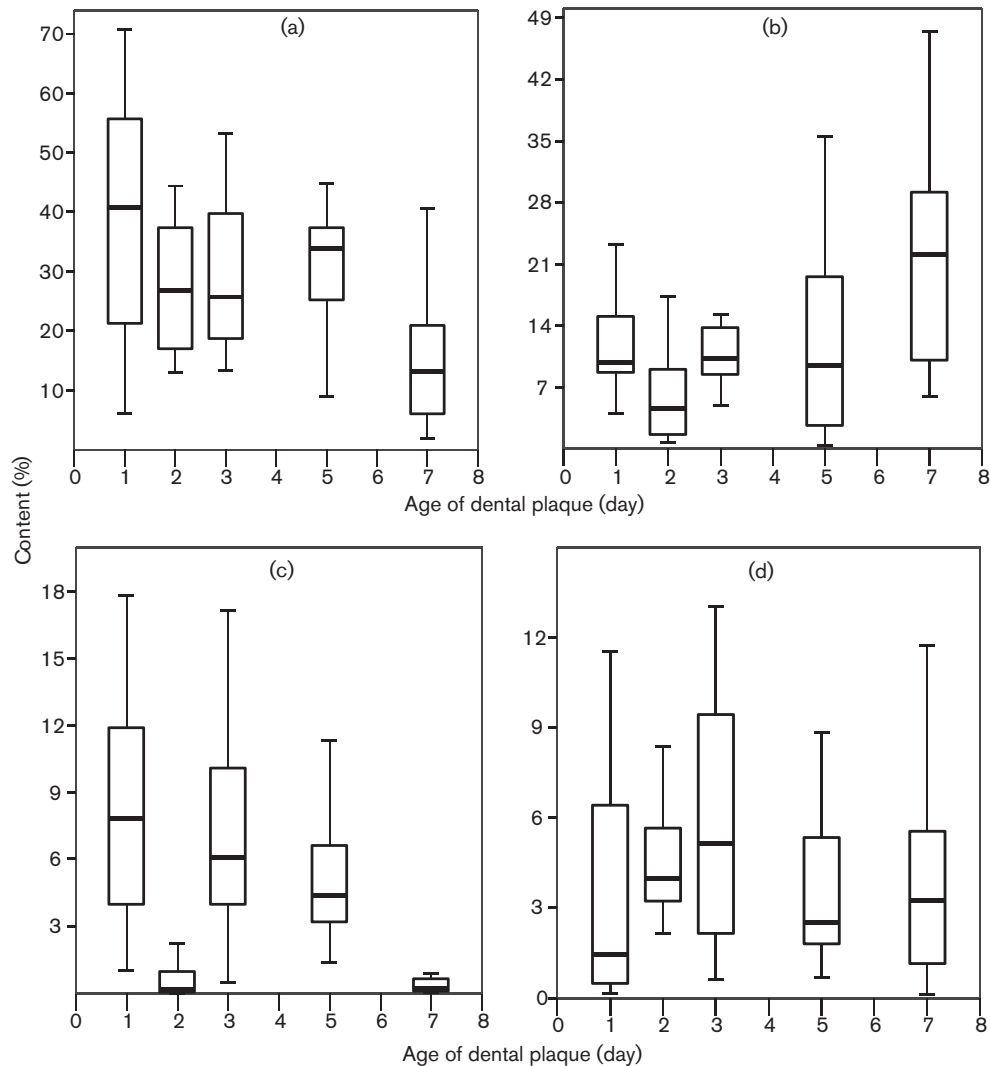
Fig. 3. Boxplots depicting percentages of the different bacterial members as detected by multiplex FISH. Each box indicates the lower and upper quartiles; the central line is the median; whiskers indicate minimum and maximum. (a) *Streptococcus* spp.; (b) *F. nucleatum*; (c) *A. naeslundii*; (d) *Veillonella* spp.

However, all recent FISH studies were conducted on plaque specimens which were not older than 72 h. A longer time interval in order to investigate coaggregation of oral bacteria may reveal the advantage of interaction of different bacterial species, i.e. streptococci and *A. naeslundii*, as suggested by Palmer *et al.* (2003).

Our results showed that all of the investigated bacterial targets were detected in each dental plaque over the course of 7 days. The specifically detected bacterial targets made up 35.5–59.8 % of the total amount of bacteria in dental plaque. In each of the oral biofilms, coaggregations of streptococci, *A. naeslundii*, *Veillonella* spp. and *F. nucleatum* were detected. Our earlier studies showed that up to 77 % of bacteria in 2-day-old oral biofilm from different volunteers were determined to be viable as detected using the vital fluorescence technique (Arweiler *et al.*, 2004). This

indicates that it is likely that the bacterial members detected in this study using FISH made up the major part of viable bacteria in the formed dental plaque, but the detection of cells that died just prior to fixation cannot be excluded. However, the relative abundance of ribosomes in the bacterial population should represent a reasonable measurement of relative physiological activity (Amann *et al.*, 1995).

In the study of Foster & Kolenbrander (2004), the composition of 1- and 14-h-old biofilm in a flow cell containing four species as an *in vitro* model was investigated. The *Streptococcus gordonii* content was between 57 and 73 % of the four-species biofilm. For *A. naeslundii*, a value of 21 % for coaggregate-inoculated biofilm was found. *Veillonella atypica* made up 5–12 % of the biofilm and *F. nucleatum* 3–11 % (*F. nucleatum* needs

an obligately anaerobic environment in the oral biofilm). In contrast, the content of *A. naeslundii* in the *in vivo* dental plaque investigated in this study was much lower, which is evidence of differences between the *in vitro* and *in vivo* results with regards to the role of *A. naeslundii* as an early colonizer. The role of *F. nucleatum* was reported to be that of a late colonizer that is only prominent in dental plaque after early colonizers have colonized the tooth surface (Socransky *et al.*, 1998). The authors scraped dental plaque from the tooth surface before analysis. Our results showed that after just 1 day, *F. nucleatum* was present in dental plaque at a higher level, in fact as high as that seen for *A. naeslundii* and *Veillonella* spp., yet much lower than that of the streptococci. We suggest that the disturbance of dental plaque may lead to microbial results other than those revealed *in situ*. In the five-species *in vitro* oral biofilm model reported by Guggenheim *et al.* (2001), *F. nucleatum* dominated the biofim by levels higher than 50 % after 62 h of cultivation. This high content was suggested to be caused by a decrease in oxygen. In our study, *F. nucleatum* reached a content of 20 % after 7 days of cultivation. Bradshaw *et al.* (1996) reported that *F. nucleatum* dominated their *in vitro* model of a multispecies biofilm only after a period of 7 days. This agrees with our results. A significant increase in *F. nucleatum*, which dominated the detected bacterial members after 7 days, was observed. Again the question arises about the interpretation of the differences between *in vivo* and *in vitro* models.

Thurnheer *et al.* (2001) detected total streptococci in different dental plaque samples using automated FISH. The authors found that total streptococci comprised 8.2 % of total bacteria detected. This difference from our results may have been caused by pre-treatment of the samples, which, in their study, were strongly vortexed, sonicated and diluted five times before fixation. This pre-treatment could cause a lower number of ribosomes corresponding to a lower metabolic activity, which results in variable fluorescence intensity (Oda *et al.*, 2000). In our study, such pre-treatment was not conducted before fixation.

After initial colonization of human enamel surfaces *in vivo*, oral streptococci have been found to compose 78–88 % of the total number of isolates as detected using culture-dependent methods (Nyvad & Kilian, 1987, 1990). This higher detected level of streptococci could be caused by the culturing methods. Furthermore, the authors found that *A. naeslundii* comprised only approximately 1 % of all bacteria that were isolated from 24 h plaque. In the study by Liljemark *et al.* (1993) using culture-dependent methods, *A. naeslundii* comprised 0.2 % and its proportion in plaque did not change throughout the time period of 2 through 72 h in periodontally healthy adults. The results presented in this study confirm the role of oral streptococci, particularly in early formed plaque (1 day), as well as the increased presence of *F. nucleatum* in older and thicker dental plaque biofilm. Furthermore, these results for FISH-based microbial analysis showed that culture-dependent methods overestimate some bacterial members, i.e. oral streptococci, in dental plaque and underestimate other bacteria, i.e. *A. naeslundii*.

Since no statistically significant differences between the different levels (top, middle and bottom) of the dental plaque biofilms analysed in this study were found for any of the probes studied, we suggest that the penetration of the oligonucleotide probes used did not influence our results. This is in agreement with the suggestions of Wood *et al.* (2002) and the results of Zhu *et al.* (2001).

Only a combination of methods allows a realistic representation of microbial distribution and the dynamics of dental plaque bacteria and can examine the role of distinct species as a coadhesive microbial agent in dental plaque. This aspect should be confirmed by the determination of supragingival plaque from many different volunteers.

To our knowledge, this is the first time that five important bacterial constituents of supragingival plaque belonging to both Gram-negative and Gram-positive bacteria have been detected simultaneously using FISH in combination with the quantitative CLSM technique. Possible structural changes of dental plaque biofilms caused by fixation and processing for FISH should be taken into consideration in future studies. This multiplex FISH can be used to visualize the distribution of different bacteria in supragingival dental plaque simultaneously as well as to study the spatial composition of dental plaque in order to reveal bacterial prevalence in persons with different states of health.

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