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Effect of Conventional Mouthrinses on Initial Bioadhesion to Enamel and Dentin in situ

C. Hannig[a] A. Gaeding[a] S. Basche[a] G. Richter[b] R. Helbig[c] M. Hannig[d]

Clinics of [a]Operative Dentistry and [b]Prosthetic Dentistry, Medical Faculty Carl Gustav Carus, TU Dresden, and [c]Leibniz Institute of Polymer Research, Research Division for Biofunctional Polymer Materials within the Max Bergmann Center of Biomaterials, Dresden; [d]Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital, Saarland University, Homburg/Saar, Germany

Key Words
Bacterial adherence · Bioadhesion · Chlorhexidine · Fluoride · Pellicle

Abstract
Aim: The study aimed to investigate the effect of a customary fluoride solution, containing sodium fluoride and amine fluoride, on initial biofilm formation on enamel and dentin in situ compared directly to chlorhexidine. ***Methods:*** Bovine enamel and dentin specimens were mounted on maxillary splints carried by 9 subjects. After 1 min of pellicle formation, rinses with tap water (control), chlorhexidine (meridol med CHX 0.2%, GABA) and a fluoride mouthrinse (elmex, GABA) were performed for 1 min. Subsequently, the slabs were carried for another 8 h. The adherent bacteria were determined by DAPI staining, live-dead staining and determination of colony-forming units after desorption; glucan formation was visualized with concanavalin A. Additionally, energy-dispersive X-ray spectroscopy (EDX) analysis of the in situ biofilm layers was conducted, and contact angle measurements were performed. Statistical evaluation was performed by means of the Kruskal-Wallis test followed by the Mann-Whitney U test ($p < 0.05$). ***Results:*** In the control group, significantly higher amounts of adherent bacteria were detected on dentin ($4.8 \times 10^6 \pm 5.4 \times 10^6$ bacteria/cm^2) than on enamel ($1.2 \times 10^6 \pm 1.5 \times 10^6$ bacteria/cm^2, DAPI). Chlorhexidine significantly reduced the amount of adherent bacteria (dentin: $2.8 \times 10^5 \pm 3.4 \times 10^5$ bacteria/cm^2; enamel: $4.2 \times 10^5 \pm 8.7 \times 10^5$ bacteria/cm^2). Rinses with the fluoride solution also significantly reduced bacterial adherence to dentin ($8.1 \times 10^5 \pm 1.5 \times 10^6$ bacteria/cm^2). Fluoride could not be detected by EDX analysis of the biofilms. Fluoride mouthrinsing did not influence the wettability of the pellicle-covered enamel surface. ***Conclusion:*** In addition to the reduction of demineralization and antibacterial effects, fluorides inhibit initial biofilm formation on dental hard tissues considerably, especially on dentin.

Introduction

Fluorides and chlorhexidine are the key components of oral health care preparations and chemotherapeutic mouthrinses [Shellis and Duckworth, 1994; Vitkov et al., 2005]. The decrease of caries in industrialized countries is, to a large extent, attributed to the adoption of fluorides [Brambilla, 2001], whereby the inhibition of demineralization and the promotion of remineralization at the

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Christian Hannig
Clinic of Operative Dentistry, Medical Faculty Carl Gustav Carus, TU Dresden
Fetscherstr. 74
DE–01307 Dresden (Germany)
E-Mail christian.hannig@uniklinikum-dresden.de

tooth surface are considered as main effects [Shellis and Duckworth, 1994; Duncan et al., 1999]. Furthermore, many studies indicate that fluorides have a noteworthy impact on the biological activity of *Streptococcus mutans* [Koo, 2008; Pandit et al., 2011]. This applies in the case of adherent bacteria and biofilms as well as for planktonic cells [Koo, 2008]. The effects of fluorides on *S. mutans* are the inhibition of enolases, proton-extruding ATPases and acidification of the cytoplasm [Van Loveren, 2001]. However, these effects have mainly been investigated in vitro; the inhibition of the glycolytic pathways in vivo by fluoride is still being researched [Van Loveren, 2001]. A recent study on the metabolic effects of fluoride on supragingival plaque in vivo yielded that lactate production is inhibited along with an increase in 3-phosphoglycerate and decrease in phosphoenolpyruvate [Takahashi and Washio, 2011]. This confirms previous in vitro data on inhibition of enolase. Fluoride-induced effects on plaque and biofilm composition have been described in vivo as well as in vitro, in humans and in animal models [Rolla and Melsen, 1975b; Hock and Tinanoff, 1979; Tinanoff and Weeks, 1979; Van Loveren, 2001]. Despite these findings, Van Loveren [2001] pointed out that the effect of fluorides on plaque (re)growth has to be investigated in vivo or in situ, respectively. The process of bioadhesion to the oral hard tissues starts with the formation of the pellicle layer, a physiological proteinaceous layer with protective and antibacterial properties [Lendenmann et al., 2000; Hannig and Joiner, 2006]. It is formed almost immediately on all surfaces exposed to the oral fluids and mediates the interactions of the saliva, the dental hard tissues, oral micro-organisms and oral-care preparations [Lendenmann et al., 2000; Hannig et al., 2009]. Bacterial biofilm formation in the oral cavity starts with an initial phase of microbial adhesion to the pellicle followed by coaggregation and bacterial cell cleavage as well as glucan synthesis [Hannig et al., 2009; Bowen and Koo, 2011]. Despite the well-known efficacy of fluorides, little is known about their interaction with the in vivo pellicle layer and the impact on initial bioadhesion as a starting point of oral biofilm formation; in situ models offer the opportunity to evaluate the relevant structures extraorally in the adherent state with elaborate methods such as enzyme assays and fluorescence and electron microscopy [Al-Ahmad et al., 2009; Jung et al., 2010]. The aim of this study was to investigate the effect of a customary fluoride solution containing sodium fluoride and amine fluoride on initial bacterial adhesion to enamel and dentin in situ, in direct comparison to chlorhexidine.

Methods

Specimens and Subjects

Nine subjects (age: 24–42 years), all members of the laboratory staff, participated in the study. Visual oral examination was carried out by an experienced dentist; the subjects had a physiological salivary flow rate checked qualitatively and quantitatively during oral examination. The subjects showed plaque indices and gingivitis indices close to zero and no untreated carious lesions. The setup of the experiments was been approved by the medical ethic committee of the Medical Association of Saarland, Germany (52/05; Re. St./Gn 18/10). Subjects gave their informed written consent to participate in the study. Cylindrical dentin and enamel slabs (diameter 5 mm, surface area 1 9.63 mm^2 and height 1.5 mm) were obtained from the labial surfaces of bovine incisors of 2-year-old cattle. The animals were BSE-negative. For preparation of the dentin specimens, the enamel was removed and the outer dentin layer was used for the oral exposure experiments. The surfaces of all samples were polished by wet grinding with abrasive paper (400–4,000 grit, FEPA-P, Struers GmbH, Erkrath, Germany). Disinfection of the dentin samples was performed by ultrasonication in ethanol (70%) for 1 min, followed by air drying and 1-minute ultrasonication in EDTA (3%) in order to remove the smear layer [Jung et al., 2010]. The slabs were washed twice for 5 min in double-distilled water. The enamel samples were treated by ultrasonication for 1 min in 3% sodium hypochlorite to remove the superficial smear layer [Deimling et al., 2004, 2007]. Afterwards, 10-minute ultrasonication in 70% ethanol was performed for the purpose of disinfection, followed by 10-minute ultrasonication in double-distilled water. All specimens were incubated in distilled water for 24 h for the purpose of hydratization before exposure in the oral cavity [Hannig et al., 2007]. Different experimental setups were conducted to evaluate the effect of chlorhexidine and fluoride on initial bacterial adherence and on bioadhesion in situ (fig. 1).

Exposure of the Samples in the Oral Cavity

For in situ pellicle formation and bacterial colonization, individual upper jaw splints were vacuum-formed from 1.5-mm thick methacrylate foils. Cavities were prepared in the buccal aspects of the splints at the sites of the premolars and the 1st molar. The slabs were fixed on the splints using polyvinyl siloxane impression material (President Light Body, Coltène, Switzerland), exposing only the surfaces of the enamel or dentin slabs to the oral fluids. The splints were carried intraorally for 1 min, then rinses with the mouthrinses (8 ml for 1 min) were performed and the samples were exposed to the oral fluids for another 8 h. After intraoral exposure, the slabs were immediately dismounted from the splints and rinsed for 5 s under running water in order to remove non-adsorbed salivary remnants [Hannig et al., 2007].

Tested Solutions

A chlorhexidine-based mouthrinse (meridol med CHX, 0.2%, GABA, Lörrach, Germany) and a fluoride solution (elmex KARIESSCHUTZ, 100 ppm amine fluoride, 150 ppm sodium fluoride, GABA) were tested. Nonfluoridated tap water served as a reference.

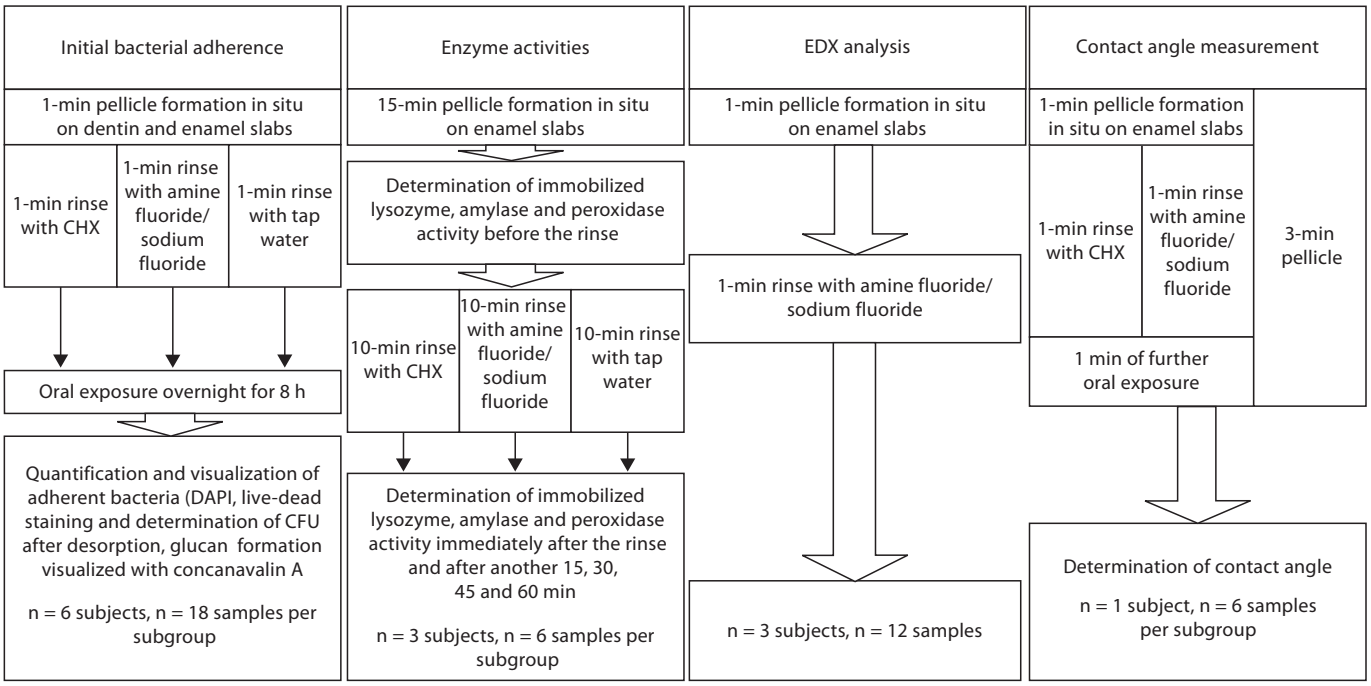


Fig. 1. Flow chart of the in situ experiments. CHX = Chlorhexidine.

Fluorescence Microscopic Assays

The epifluorescence microscopic analyses were performed at ×1000 magnification (Axioskop II, ZEISS, Oberkochen, Germany. The number of cells observed in 10 randomized microscopic ocular grid fields per sample was counted. The area of an ocular grid field (0.0156 mm^2) allowed the calculation of the number of cells per cm^2 [Hannig et al., 2007; Al-Ahmad et al., 2009; Jung et al., 2010].

DAPI Staining

DAPI (4′,6-diamidino-2-phenylindole; Merck, Darmstadt, Germany) forms fluorescent complexes with double-stranded DNA [Schwartz et al., 2003; Hannig et al., 2007; Jung et al., 2010]. This yields fluorescence specificity for adenine-thymidine (AT) clusters. DAPI is taken up into cellular DNA and the nuclei show pronounced fluorescence (maximum of fluorescence at λ = 461 nm). The staining was conducted as described previously [Hannig et al., 2007]. After the oral exposure, specimens were washed once in methanol. For staining, the slabs were covered with 1 ml of DAPI-methanol (working solution, 1 μg/ml) and incubated in a dark chamber for 15 min at room temperature. The solution was then poured off and the specimens were washed again with methanol and air-dried at room temperature. The slabs were fixed to a slide and analyzed by fluorescence microscopy. DAPI staining was also combined with concanavalin A (Invitrogen, Molecular probes, Darmstadt, Germany) for the visualization of glucan structures. The stock solution was 5mg/ml Alexa Fluor 594 conjugate in 0.1 M NaH_2PO_4-buffer, pH 8.3. The stock solution was stored at –20°C. The working solution was 10 μl of stock solution in 490 μl PBS (1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$).

BacLight Viability Assay

The LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen) adopts two nucleic acid stains – green-fluorescent SYTO® 9 stain and red-fluorescent propidium iodide stain [Hannig et al., 2010].

Experiments in situ: the BacLight assay was adopted for visualization of vital and avital bacteria on enamel and dentin slabs after oral exposure. Similar amounts of component A [Syto9 1.67 mM/propidium-iodide 1.67 mM and 300 μl dimethyl sulfoxide (DMSO)] and B (Syto 9 dye, 1.67 mM/propidium iodide, 18.3 mM and 300 μl DMSO) were mixed and 2 μl were pipetted to 1 ml of saline solution. The slabs were incubated in this solution for 10 min. The samples were then rinsed with saline solution and evaluated with fluorescence microscopy with the fluorescein-diacetate filter (FDA) and the ethidium-bromide filter.

Experiments in vitro: The assay was carried out according to the manufacturer's instructions and the fluorescence was measured in a 96-well plate reader. A suspension of *S. mutans* in saline solution was adopted after overnight cultivation; a share of the bacteria was inactivated by heating (at 95°C for 1 h). Dilutions of the mouthrinses were prepared with saline solution (0.9% sterile NaCl). The vital bacteria were mixed 1:1 with the diluted mouthrinses and incubated for 10 min. These suspensions were mixed with heat-inactivated bacteria (0:100; 10:90; 25:75; 50:50). A volume of 0.5 μl of BacLight staining solution (components A and B 1:1) was added to 250 μl of the mixtures. The staining was conducted over 10 min in a dark chamber. A volume of 100 μl from each sample was transferred into a microtiter plate and the fluorescence was measured. The excitation wavelength was 470 nm; emission was recorded at 530 nm for the vital bacteria and 620 nm

for the avital bacteria. The measurements were carried out in duplicate. For evaluation of the recorded data, the ratio of vital and dead/avital cells was calculated according to ratio = emission vital/emission dead bacteria. Experiments with saline solution served as a reference/negative control.

Colony-Forming Units

For determination of the colony-forming units (CFU), the specimens were rinsed in saline solution after intraoral exposure [Hannig et al., 2007]. The samples were then vortexed in sterile tubes with 1 ml 0.9% sodium chloride for 4 min in an ultrasonic bath on ice. In the following, this solution was serially diluted up to $1:10^3$ in saline solution and plated on Columbia blood agar (CBA, aerobic bacteria and facultative anaerobic bacteria) or on yeast-cysteine-blood agar, respectively (HCB, anaerobic bacteria). The HCB plates were incubated for 7 days in anaerobic jars (Merck) at 37°C using BBL GasPak Anaerobic System Envelopes (Becton Dickinson, N.J., USA), the CBA plates under aerobic conditions with 5% CO_2 for 2 days [Hannig et al., 2007].

Enzyme Assays

Additional experiments were carried out with 3 subjects to evaluate the effect of the mouthrinses on enzyme activities in the acquired pellicle. After 15 min of pellicle formation, the subjects rinsed for 10 min with the different mouthrinses. Each throughput of an individual was carried out with 12 enamel slabs fixed on buccal sites to the upper jaw splints. Before the rinse, immediately after the rinse and after another 15, 30, 45 and 60 min, 2 slabs were removed from the splint and transferred to the enzyme assays. Separate throughputs were conducted for the different enzymes. Due to the high inter- and intraindividual variability of the immobilized enzyme activities, the respective 15-minute pellicles served as a control. The activity of α-amylase was measured photometrically using 2-chloro-4-nitrophenyl-4-O-β-D-galactopyranosylmaltotriosid (GalG2CNP, Sorachim, Paris, France) as a substrate; peroxidase was determined with a fluorimetric assay based on the cleavage of 2',7'-dichlorofluorescin (Sigma, St. Louis, Mo., USA). The adopted lysozyme assay is based on the hydrolysis of cell walls from *Micrococcus lysodeicticus* linked to a fluorogenic substance (EnzCheck Lysozyme assay Kit; E-22013, Molecular Probes, Leiden, The Netherlands). All assays were carried out as described previously in detail [Hannig et al., 2004, 2008b, 2009].

Contact Angle Measurement

In order to analyze the influence of the mouthrinsing solutions on the wettability of the pellicle-covered enamel surface, one subject carried enamel samples over 3 min intaorally with intermediately applied rinses (fig. 1); 6 enamel samples were carried per subgroup.

The evaluation of the dynamic contact angle was processed out with a Goniometer OCA30 (Dataphysics, Filderstadt, Germany). For determination of the advancing contact angle, a volume of 1 μl water was dispensed to induce a liquid bridge between sample and pipette. Another 4 μl were dispensed (0.5 μl/s) and the progressing contact angle was recorded. The receding contact angle was then determined by the removal of 5 μl at the same speed.

Energy-Dispersive X-Ray Spectroscopy

Energy-dispersive X-ray spectroscopy (EDX) analysis of enamel samples after application of the fluoride rinse in situ was carried out with an XL 30 ESEM (Philips, Eindhoven, The Netherlands) with an EDX system (Phoenix, EDAX INC., Mahwah, N.J., USA) in order to detect fluoride immobilized, adsorbed in the biofilm layer or on the sample surface, respectively.

Statistics

Statistical evaluation was performed by means of the Kruskal-Wallis test and additional pair-wise comparison was carried out using the Mann-Whitney U test ($p < 0.05$). The software used was SPSS 16.0.

Results

Initial microbial colonization was visualized with DAPI and live/dead staining (fig. 2). On the enamel samples of the control group, monolayers of adherent bacteria were visible covering more than 50% of the surface. The respective dentin slabs showed a preferential bacterial colonization at the orifices of the dentin tubules (fig. 2). Both mouthrinses considerably reduced the bacterial adherence. Randomly adhering single bacteria as well as chains or small aggregates were detectable on the enamel slabs. A diminished microbial adherence was also recorded on the dentin slabs. Interestingly, there was nearly no bacterial accumulation at the orifices of the dentin tubules after the initial application of the tested mouthrinsing preparations.

Additional experiments were carried out to visualize the glucan formation (fig. 2). Considerable glucan formation was observed on control samples, especially at the openings of the dentinal tubules. Generally, the glucans were detected mainly around the adherent bacteria. Both mouthrinses distinctly reduced the glucan formation over a period of 8 h, notably at the dentinal tubules.

The bacteria were quantified in the adherent state with DAPI and with live/dead staining (fig. 3). Furthermore, the amount of CFU units was determined after desorption (fig. 4). In the control group (tap water), significantly higher amounts of adherent bacteria were detected on the dentin ($4.8 \times 10^6 \pm 5.4 \times 10^6/cm^2$) than on the enamel ($1.2 \times 10^6 \pm 1.5 \times 10^6/cm^2$) as recorded with DAPI (table 1). With all methods, a significant impact of the mouthrinses was recorded. On the dentin samples in particular, significantly fewer bacteria were detected after the adoption of fluoride and chlorhexidine rinsing solution (demonstrated with all adopted methods) (table 1). Slightly different observations were made in the case of the enamel slabs. All methods indicated a significant effect of chlorhexidine; a significant effect of fluoride solution on enamel was, however, only observed for the viable bacteria (BacLight, fig. 3, table 1).

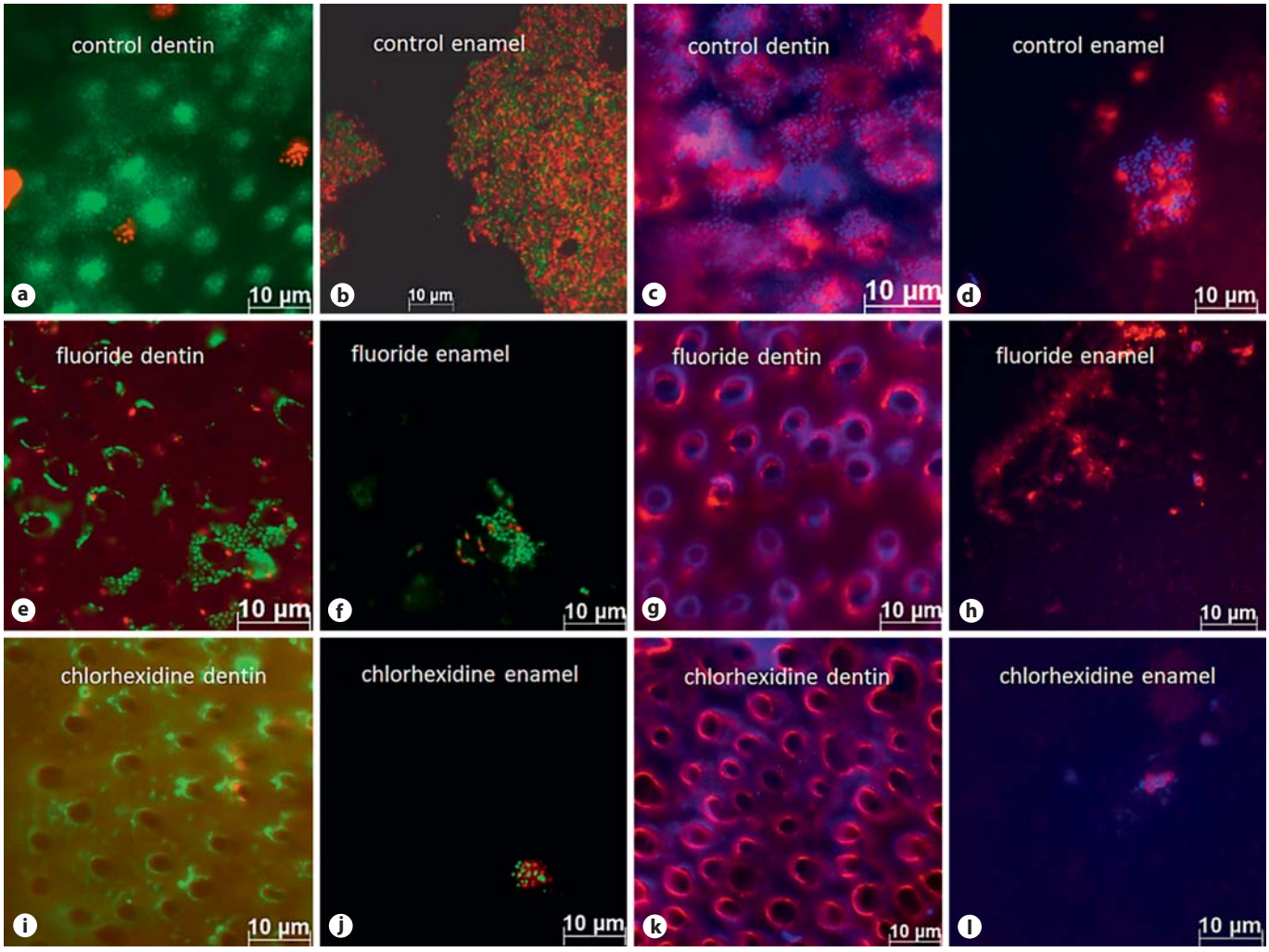


Fig. 2. Bacterial adherence to dentin and enamel, BacLight™ staining (**a**, **b**, **e**, **f**, **i**, **j**), and DAPI (blue) combined with concanavalin A for visualization of glucans (red) (**c**, **d**, **g**, **h**, **k**, **l**) representative examples. With the viability assay, clear differentiation of viable (green) and dead (red) bacteria was possible. Vast areas of the control samples were coated by bacterial aggregates or monolayers, and dentin tubules were almost occluded by bacterial agglomerates. Furthermore, monolayers of bacteria were observed on parts of the surface. Initial rinses with fluoride solution or chlorhexidine reduced the bacterial adhesion considerably. Remarkably, the colonization of the tubules was diminished nearly completely. Following chlorhexidine application, vast areas of the samples were completely free of bacteria. On control samples, considerable glucan formation was observed. It is noteworthy that glucan formation occurred mainly together with bacterial adherence. Initial rinses with fluoride as well as with chlorhexidine reduced not only bacterial adhesion but also glucan formation.

The ratio of viable and dead bacteria amounted to 1.27 for enamel and to 2.42 for dentin in the control group. It was decreased by the application of chlorhexidine (enamel: 0.84; dentin: 0.57) and fluoride (enamel: 0.49; dentin: 0.58) i.e. there was a higher proportion of dead bacteria.

Additional in vitro experiments with *S. mutans* indicated an antibacterial effect of both tested preparations in a dose-dependent manner (fig. 5).

EDX analysis of the enamel samples was carried out directly after the application of fluoride mouthrinse in situ. Interestingly, no accumulation of fluoride was traceable (data not shown).

Additional experiments indicated that neither of the mouthrinses affected the immobilized activities of amylase, lysozyme and peroxidase in the in situ pellicle layer (table 2).

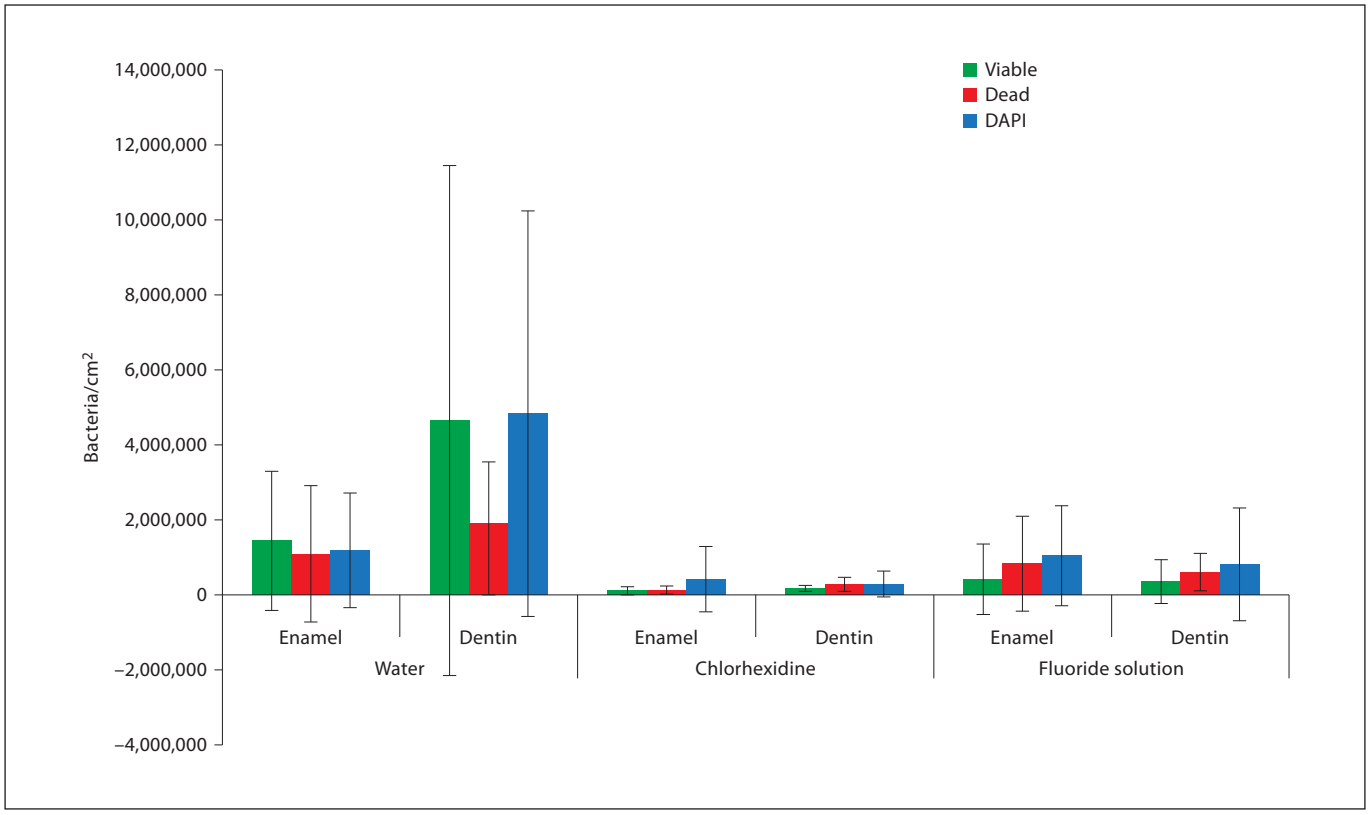


Fig. 3. BacLight staining for detection of adherent viable and dead bacteria, and DAPI staining for detection of adherent bacteria after rinses with water or different mouthrinses. Exposure of enamel slabs at buccal sites of the upper 1st and 2nd premolar and 1st molar for 8 h, mean ± SD, n = 18 samples per subgroup (n = 9 subjects, 2 samples per subject, and mouthrinse).

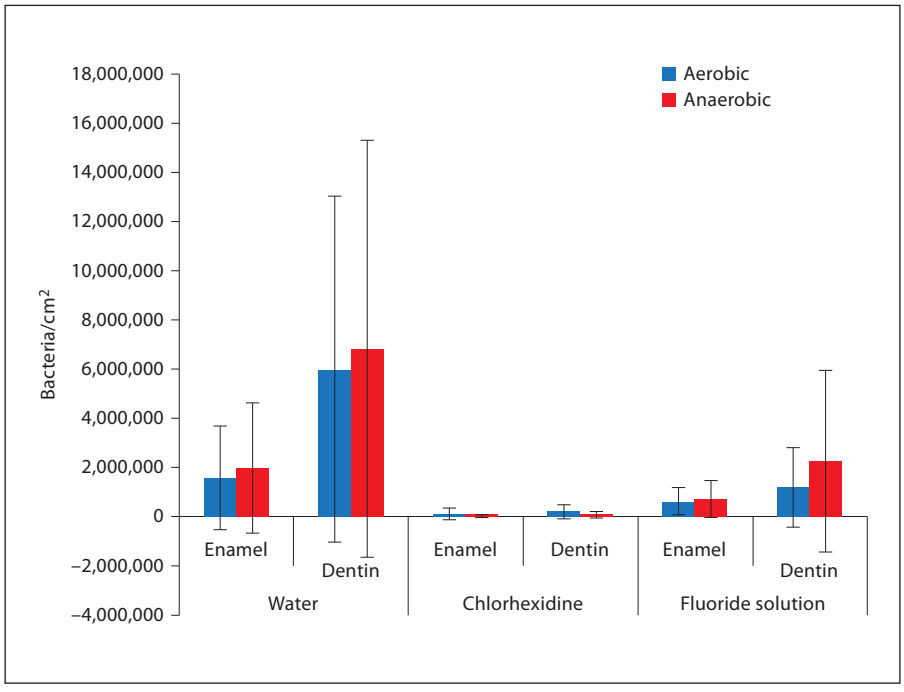
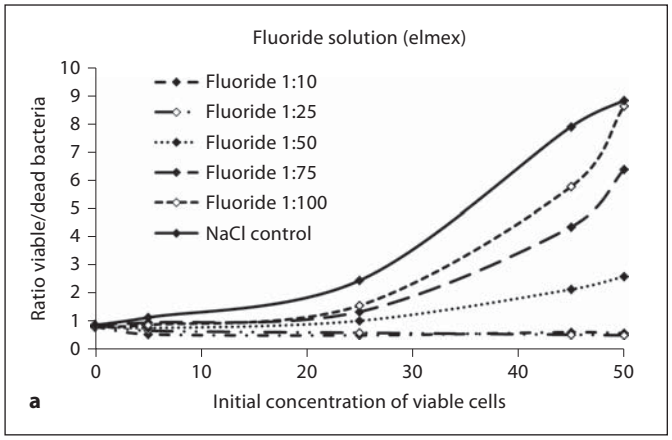


Fig. 4. Quantification of CFUs after desorption for determination of adherent aerobic and anaerobic bacteria. Exposition of enamel slabs at buccal sites of the upper 1st and 2nd premolar and 1st molar for 8 h, mean ± SD, n = 18 samples per subgroup (n = 9 subjects, 2 samples per subject, and mouthrinse).



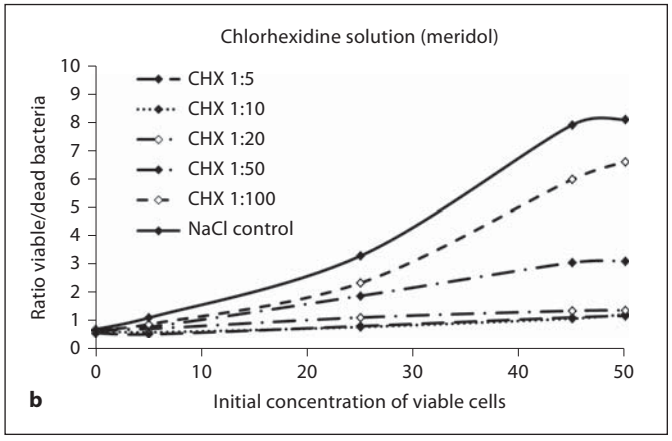


Fig. 5. Effect of fluoride (**a**), chlorhexidine (CHX) (**b**) and saline solution (control) on the viability of *S. mutans* in vitro. A suspension of *S. mutans* was incubated with fluoride- and chlorhexidine-containing solutions in different concentrations. After incubation, the samples were admixed to proportions of the basic suspension, the amount of vital and dead bacteria was determined using the BacLight bacterial viability assay, the measurements were carried out in duplicates and the ratio was calculated, emission 530 nm/emission 620 nm representing emission vital/emission dead bacteria, respectively.

Table 1. Statistical evaluation of the in situ experiments with the different rinsing solutions; Kruskal-Wallis test and additional pairwise comparison with Mann-Whitney U test ($p < 0.05$)

Kruskal-Wallis test: $p < 0.001$	Enamel		Dentin		
	fluoride solution	chlorhexidine	water	fluoride solution	chlorhexidine
DAPI					
Enamel					
Water	n.s.	0.002	0.001	n.s.	0.001
Fluoride solution		0.003	0.000	n.s.	0.004
Chlorhexidine			0.000	0.037	n.s.
Dentin					
Water				0.000	0.000
Fluoride solution					0.034
BacLight viable bacteria					
Enamel					
Water	0.033	<0.001	n.s.	0.006	<0.001
Fluoride solution		0.01	<0.001	n.s.	n.s.
Chlorhexidine			<0.001	0.005	0.017
Dentin					
Water				<0.001	<0.001
Fluoride solution					n.s.
BacLight dead bacteria					
Enamel					
Water	n.s.	<0.001	0.005	n.s.	0.013
Fluoride solution		0.003	0.001	n.s.	n.s.
Chlorhexidine			<0.001	<0.001	0.012
Dentin					
Water				<0.001	<0.001
Fluoride solution					0.003
CFU: aerobic					
Enamel					
Water	n.s.	<0.001	0.005	n.s.	<0.001
Fluoride solution		<0.001	<0.001	n.s.	0.001
Chlorhexidine			<0.001	<0.001	n.s.
Dentin					
Water				0.001	<0.001
Fluoride solution					0.001
CFU: anaerobic					
Enamel					
Water	n.s.	<0.001	0.026	n.s.	<0.001
Fluoride solution		<0.001	<0.001	n.s.	<0.001
Chlorhexidine			<0.001	<0.001	n.s.
Dentin					
Water				0.009	<0.001
Fluoride solution					<0.001

Furthermore, a water contact angle measurement was conducted (table 3). Bare enamel (control) showed a very high hydrophilicity and the drop of water spread nearly immediately on the surface. Oral exposure led to an increase of the contact angle, indicating a more hydrophobic surface. The mouthrinse with fluoride yielded similar data to the 3-minute pellicle. In contrast, the application of chlorhexidine resulted in a slightly more hydrophilic surface.

The contact angle measurements on specimens carried in situ showed a high variability which might be a result of the physiological pellicle structure which is a somewhat heterogeneous proteinaceous network. Furthermore, epithelial cells, quite often detected on the enamel slabs during the fluorescence microscopic evaluation, are expected to influence the contact angle mea-

Table 2. Impact of mouthrinses on enzyme activities in the in situ pellicle in 3 subjects with 2 throughputs per subject, enzyme and beverage

		Baseline (15 min-pellicle)	At 0 min	After 15 min	After 30 min	After 45 min	After 60 min
Peroxidase, mU/cm^2	chlorhexidine	11.76 ± 9.10	9.86 ± 7.13	9.95 ± 7.53	15.54 ± 13.87	15.18 ± 17.47	13.19 ± 11.85
	fluoride	6.50 ± 6.30	2.95 ± 3.63	5.16 ± 5.85	6.47 ± 6.29	4.75 ± 4.73	9.86 ± 15.57
Lysozyme, U/cm^2	chlorhexidine	16.53 ± 12.45	15.27 ± 5.14	18.34 ± 12.28	12.58 ± 5.06	15.48 ± 6.95	9.02 ± 4.67
	fluoride	14.14 ± 10.57	8.95 ± 2.12	13.58 ± 6.89	8.58 ± 4.63	13.64 ± 7.81	16.12 ± 10.02
Amylase, mU/cm^2	chlorhexidine	0.60 ± 0.60	0.34 ± 0.27	0.53 ± 0.53	0.50 ± 0.48	0.37 ± 0.52	0.64 ± 0.68
	fluoride	0.47 ± 0.37	0.24 ± 0.21	0.33 ± 0.15	0.28 ± 0.27	0.27 ± 0.16	0.34 ± 0.26

Twelve slabs were carried out on buccal sites. After 15 min of pellicle formation, the subjects rinsed for 10 min with the different mouthrinses. At the different time points, 2 slabs were removed from the splint and transferred to the enzyme assays. Mean ± SD.

Table 3. Determination of the advancing contact angle on enamel slabs (6 specimens per subgroup)

	Mean ± SD	Min./max.
Bare enamel	<10°	<10°
3-minute pellicle	39 ± 10°	22–51°
Fluoride: 1 min of pellicle formation followed by a 1-minute rinse with fluoride solution and 1 min of additional oral exposure	40 ± 11°	25–51°
Chlorhexidine: 1 min of pellicle formation followed by a 1-minute rinse with chlorhexidine solution and 1 min of additional oral exposure	27 ± 3°	25–34°

surements. Dewetting was not observed with any of the specimens. Accordingly, the receding contact angle was given as <10°.

Discussion

The antibiofilm and antibacterial efficacy of chlorhexidine as well as amine fluoride/stannous fluoride preparations has been demonstrated in several previously published studies [Addy et al., 1983; Netuschil et al., 1989; Addy et al., 1990; Brecx et al., 1990; Rundegren et al., 1992; Brecx et al., 1993; Lindhe et al., 1993; Jenkins et al., 1994; Netuschil et al., 1995; Newcombe et al., 1995; Riep et al., 1999; Otten et al., 2010]. However, to the best knowledge of the authors, in this study, the effect of a mouthwash based on amine fluoride and sodium fluoride on initial bioadhesion in situ was tested for the first time. Whenever amine fluoride/stannous fluoride preparations were directly compared to chlorhexidine, the chlorhexidine-containing mouthrinsing solution showed a superior effect with regard to the plaque-reducing properties [Netuschil et al., 1989; Brecx et al., 1990, 1993; Netuschil et al., 1995; Riep et al., 1999]. Their observation agrees with our findings, indicating that the amine fluoride/sodium fluoride rinsing caused a less extensive reduction in bacterial adherence than chlorhexidine. Interestingly, only a few studies have been published focusing on the antiplaque effect of amine-fluoride- and sodium-fluoride-containing agents without the addition of stannous fluoride [Balmelli et al., 1974; Baehni and Takeuchi, 2003]. Research on these fluorides mainly focused on remineralization or on antibacterial effects [Tinanoff and Weeks, 1979; Shellis and Duckworth, 1994]. This study has clearly outlined and confirmed that amine fluoride and sodium fluoride also have a considerable impact on the process of initial bacterial adhesion and bacterial growth, not only on enamel but also on dentin.

The initial biofilm formation was carried out in situ on bovine enamel and dentin samples, an approach adopted in several previous studies on pellicle formation and initial bioadhesion [Deimling et al., 2004; Hannig et al., 2008a; Jung et al., 2010]. The prepared slabs allow the investigation of bioadhesion in a standardized manner and very like the situation in the oral cavity, although minor microscopic irregularities and residual organic material on the native tooth surface could modulate the interaction of the fluoride or chlorhexidine preparations with the enamel and dentin surface in vivo. For the visualiza-

tion and quantification of the adherent bacteria, well-established fluorescence microscopic techniques were adopted appropriate for the evaluation of dentin and enamel carried in situ [Hannig et al., 2007, 2010; Jung et al., 2010]. The application of fluoride was deliberately performed with a mouthrinse, to ensure direct comparability with the chlorhexidine rinse and to avoid the mechanical effects possibly induced by a toothbrush. Chlorhexidine was included in our study as a gold standard and positive control [Ferretti et al., 1990; Ribeiro et al., 2007]. Rinses with tap water served as negative controls. It was apparent that both chlorhexidine and amine fluoride/sodium fluoride modify the surface properties of dental hard tissues or interact with planktonic salivary bacteria, thereby hampering bacterial adherence significantly over the first 8 h of intraoral bioadhesion. In particular, bacterial colonization of the rugged dentin surface was significantly reduced. This is a remarkable finding, as bovine dentin tubules have a greater diameter than those in human dentin, and there are more tubules/cm^2. Usually, the openings of the tubules offer excellent niches for initial bacterial adherence [Jung et al., 2010]. When interpreting the present data, the high clearance in the oral cavity has to be kept in mind. Direct antibacterial effects at the surface cannot explain the observed effects in full, underlined by the presence of adherent vital bacteria. In vitro models indicated that the concentrations of amine fluoride and chlorhexidine required to kill *Streptococcus sobrinus* in the adherent state are about 100 times higher than those required to affect bacteria in the planktonic phase [Shani et al., 2000]. Antibacterial effects on suspensions of *S. mutans* were confirmed in our experiments. Accordingly, surface interactions also seemed to be relevant for the observed effects [Hannig et al., 2009]. It is well documented in the literature that chlorhexidine is of high substantivity, with a high affinity to the tooth surface modifying the pellicle and reducing bacterial adhesion [Rolla and Melsen, 1975a; Ferretti et al., 1990; Vitkov et al., 2005; Ribeiro et al., 2007]. Analogous mechanisms might occur, if fluorides are applied. It has been assumed that the pellicle layer serves as a reservoir for fluorides and facilitates the retention of fluorides at the tooth surface [Arends and Schuthof, 1981; Grobler, 1981]. Amine fluoride, in particular, is assumed to have a high affinity to the proteinaceous pellicle layer. However, the potential accumulation of fluorides at the enamel and dentin surface was not confirmed by EDX analysis. There was perhaps actually no fluoride bound to the surface after the rinse. Fluoride retained in dental plaque on natural tooth surfaces and on the oral mucosa could have affected the process of bacterial growth and bioadhesion; plaque left behind after tooth-brushing also retains antimicrobials and serves as a slow-release reservoir [Otten et al., 2010]. An effect of adherent amine groups in the pellicle layer can also not be excluded. This applies to antiglycolytic, antibacterial and antiadherent effects [Breitenmoser, 1975; Van Loveren, 2001].

However, the formation of a calcium fluoride layer is a well-established model for the main effect of fluoridated toothpastes and mouthrinses, and the adsorption of amine fluoride to enamel was shown previously in vitro with considerable effects on the surface-free energy [Busscher et al., 1988]. They postulated that such fluoride layers will be rapidly covered by pellicle structures masking the physicochemical properties of the adsorbed amine fluoride, and confirmed this with additional in vivo experiments. Nevertheless, an adsorption of fluoride occurring at the nano-scale potentially adds different surface characteristics to dentin and enamel modulating the tenacity of initial unspecific bacterial adherence to the pellicle layer. These physicochemical properties act through the pellicle layer.

Water contact angle measurements were carried out as well. The results differed from the observations described in the literature; several studies measured water contact angles between 40 and 50° on bare enamel [de Jong et al., 1982; van Dijk et al., 1987; Perdok et al., 1990]. However, different grinding procedures and especially varying pretreatments of the enamel with different buffer solutions such as SDS or sodium hypochlorite certainly affect the wettability of the surface, as does ultrasonication [Hannig and Hannig, 2009; Muller et al., 2010a]. In a recent study, bare bovine enamel was shown to have a low water contact angle after pretreatment with SDS and water ($22.3 \pm 15.5°$), and a very high hydrophilicity [Muller et al., 2010a]. Furthermore, in contrast to many other studies, in our experiments, the enamel samples were stored for over 24 h in distilled water, for the purpose of hydratization before exposure in the oral cavity [Hannig et al., 2007]. This offers an explanation for the spreading of the drop of water due to the high hydrophilicity. In this context, it is noteworthy that natural enamel is not a homogenous structure but a nanostructured surface which might be an explanation for the high standard deviation [Hannig and Hannig, 2010; Muller et al., 2010a]. As expected, the pellicle formation led to a more hydrophobic character of the enamel surface [Hannig and Hannig, 2009].

However, the water contact angle measurements performed in our study indicated no differences between

pellicle samples with and without fluoride application, whereas after chlorhexidine application the specimens yielded distinctly lower contact angles.

Previous in vitro experiments indicated that fluoride solutions inhibit adsorption of bacteria and bioadhesion to hydroxylapatite [Rolla and Melsen, 1975b]. Preadsorbed amine fluoride reduced the number of adherent bacteria *(S. sobrinus)* on hydroxylapatite in vitro [Shani et al., 2000]. In another more elaborate biofilm model based on a monospecies biofilm, bacterial adhesion to pellicles over 16 h with and without application of amine fluoride was tested, and the amount of adherent bacteria was also determined with the BacLight method [van der Mei et al., 2008]. The adhesion of *S. mutans* strains to the pellicle was distinctly reduced after the application of amine fluoride. Irrespective of the strain tested, the proportion of dead bacteria was considerably higher in the fluoride groups than in the controls [van der Mei et al., 2008]. This observation was confirmed in our study. Due to their cationic properties, amine fluorides are assumed to interact with the pellicle through electrostatic interactions [Shani et al., 1996a, b; van der Mei et al., 2008]. Adsorption of amine fluorides has been shown to have considerable effects on the hydrophobicity and surface charge of the experimental pellicles [de Jong et al., 1984; Shani et al., 1998]. However, the respective effects occurring in vivo or in situ have not been investigated much [van der Mei et al., 2008]. Water contact angles of the in vitro pellicle are decreased by application of amine fluoride, making the surface more hydrophobic than that of untreated pellicles [van der Mei et al., 2008]. Interestingly, these data correspond to the water contact angles measured in vivo directly after brushing with toothpaste containing amine fluoride. These observations support the theory that fluorides alter the physicochemical properties of the tooth surface and thereby the process of bacterial adherence. Van der Mei et al. [2008] already pointed out that the effect of fluorides on plaque reduction can be attributed to effects on initial bacterial adhesion; this is in line with our study. A distinctly reduced number of adherent bacteria was observed on enamel specimens carried in situ over 48 h following the adoption of rinses containing stannous fluoride [Tinanoff and Weeks, 1979]. The respective preparation was adopted once a day and the adherent bacteria were visualized with scanning electron microscopy. So it should be kept in mind that the stannous ion itself has strong antibacterial properties.

The masking of specific receptors in the pellicle due to fluoride or chlorhexidine cannot be excluded, but it is not certain. The activity of the pellicle's key enzymes mirrors the biological activity of this proteinaceous layer covering all known surfaces and modifying all structures exposed to the oral fluids [Hannig et al., 2005]. Lysozyme, amylase and peroxidase are the main nonbacterial enzymes in the acquired pellicle and represent its biological function [Hannig et al., 2005]. Interestingly, these enzymes in the in situ pellicle were not inhibited by rinses with chlorhexidine or fluoride, as was shown in separate experiments. The findings indicate that the tested solutions do not hamper the physiological function of the pellicle layer, despite the observed effects on bacterial adhesion. However, the relatively small number of experiments carried out for the determination of enzyme activities has to be considered as well as the high standard deviation (albeit typical for enzymes) in the acquired pellicle [Hannig et al., 2004, 2008b]. Bacterial glucosyltransferases (GTFs) are linking bacterial pioneer enzymes during initial biofilm formation and microbial adhesion [Bowen and Koo, 2011]. Inhibition of immobilized GTF on hydroxylapatite surfaces in an experimental biofilm model required about 100 times more amine fluoride than necessary for the free enzyme [Shani et al., 2000]. Despite the limited impact of fluorides on immobilized GTF in vitro described in the literature, a considerable reduction of glucan formation was observed in our study, especially on the dentin slabs. In this context, it is noteworthy that we observed dense glucan formation mainly around the adherent bacteria, though the glucan-forming GTFs are distributed randomly in the pellicle layer in an active conformation before bacterial adhesion [Hannig et al., 2008a]. This applied for the controls as well as for fluoride and chlorhexidine. GTFs seem to adhere not only to the pellicle but also to the bacteria, transforming them to glucan-forming bacteria [Bowen and Koo, 2011]. In conclusion, our data confirm the key role of fluorides in preventive dentistry, and indicate that surface interactions seem to be an explanation for the unsurpassed efficacy of this substance in dental prophylaxis. Furthermore, the findings underline that the effect of fluorides is a topic and not a systemic effect; the adoption of fluoride pills should not be regarded as the therapeutic approach of first choice. Despite these considerations, there are still many unanswered questions on the mode of action of fluorides, particularly on the nanoscopic level [Muller et al., 2010b].

Disclosure Statement

The authors have no conflicts of interest to declare.

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