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ORIGINAL ARTICLE

Action of food preservatives on 14-days dental biofilm formation, biofilm vitality and biofilm-derived enamel demineralisation in situ

Nicole Birgit Arweiler · Lutz Netuschil · Daniel Beier · Sebastian Grunert · Christian Heumann · Markus Jörg Altenburger · Anton Sculean · Katalin Nagy · Ali Al-Ahmad · Thorsten Mathias Auschill

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Abstract
Aims The aims of this double-blind, controlled, crossover study were to assess the influence of food preservatives on in situ dental biofilm growth and vitality, and to evaluate their influence on the ability of dental biofilm to demineralize underlying enamel over a period of 14 days.
Materials and methods Twenty volunteers wore appliances with six specimens each of bovine enamel to build up intra-oral biofilms. During four test cycles of 14 days, the subjects had to place the appliance in one of the assigned controls or active solutions twice a day for a minute: negative control 0.9 % saline, 0.1 % benzoate (BA), 0.1 % sorbate (SA) and 0.2 % chlorhexidine (CHX positive control). After 14 days, the biofilms on two of the slabs were stained to visualize vital and dead bacteria to assess biofilm thickness (BT) and bacterial vitality (BV). Further, slabs were taken to determine mineral loss (ML), by quantitative light-induced laser fluorescence (QLF) and transversal microradiography (TMR), moreover the lesion depths (LD).
Results Nineteen subjects completed all test cycles. Use of SA, BA and CHX resulted in a significantly reduced BV compared to NaCl ($p<0.001$). Only CHX exerted a statistically significant retardation in BT as compared to saline. Differences between SA and BA were not significant ($p>0.05$) for both parameters. TMR analysis revealed the highest LD values in the NaCl group (43.6±44.2 μm) and the lowest with CHX (11.7±39.4 μm), while SA (22.9±45.2 μm) and BA (21.4±38.5 μm) lay in between. Similarly for ML, the highest mean values of 128.1± 207.3 vol% μm were assessed for NaCl, the lowest for CHX (−16.8±284.2 vol% μm), while SA and BA led to values of 83.2±150.9 and 98.4±191.2 vol% μm, respectively. With QLF for both controls, NaCl (−33.8±101.3 mm^2 %) and CHX (−16.9±69.9 mm^2 %), negative values were recorded reflecting a diminution of fluorescence, while positive values were found with SA (33.9±158.2 mm^2 %) and BA (24.8±118.0 mm^2 %) depicting a fluorescence gain. These differences were non-significant ($p>0.05$).
Conclusion The biofilm model permited the assessment of undisturbed oral biofilm formation influenced by antibacterial components under clinical conditions for a period of 14 days. An effect of BA and SA on the demineralization of enamel could be demonstrated by TMR and QLF, but these new findings have to be seen as a trend. As part of our daily diet, these preservatives exert an impact on the metabolism of the dental biofilm, and therefore may even influence demineralization processes of the underlying dental enamel in situ.

N. B. Arweiler (✉) · L. Netuschil
Department of Periodontology, Dental School, Philipps University, Georg-Voigt Straße 3, 35033 Marburg, Germany
e-mail: arweiler@med.uni-marburg.de

D. Beier · S. Grunert · M. J. Altenburger · A. Al-Ahmad
Department of Operative Dentistry and Periodontology, Dental School and Hospital, Albert-Ludwigs University, Freiburg, Germany

C. Heumann
Department of Statistics, LMU Munich, Munich, Germany

A. Sculean
Department of Periodontology, Dental School, University of Berne, Bern, Switzerland

K. Nagy
Department of Oral Surgery, Faculty of Dentistry, University of Szeged, Szeged, Hungary

T. M. Auschill
praxisHochschule, Neusser Str. 99, Cologne 50670, Germany

Keywords Dental biofilm · Biofilm in situ model · Food preservatives · Plaque vitality · Confocal laser scanning microscopy · Enamel lesions · TMR · Mineral content · QLF · Lesion depth

Introduction

The main factor contributing to the most common oral diseases including caries, endodontic infections and gingivitis/periodontitis/peri-implantitis is dental plaque, which currently is defined as a microbiological biofilm. Hence, the dental plaque shows the general characteristics of biofilms [1, 2]; moreover, it harbours a plethora of bacterial species and thus is extremely heterogeneous [2–5]. In contrast to planktonic cells, bacteria bound in the environment of biofilms exert a substantial resistance against antibiotics or other antibacterial compounds [6, 7]. The underlying mechanisms are complex and multifactorial [8].

Besides conventional mechanical oral hygiene measures, it is possible to affect the growth and metabolism of oral biofilms (bio)chemically. In dentistry, compounds which are able to reduce the amount or vitality of dental biofilms and/or their demineralizing potential, due to an immanent antibacterial action, are discussed as a valuable option in dental prophylaxis. They are used regularly and successfully in dental medicine as mouth rinses and toothpastes [9, 10]. Therefore, in spite of increased sugar consumption [11], the recent decline in the prevalence of caries in industrialized nations seems not only to be a consequence of optimized prophylactic measures. Possibly, in the context of changing dietary patterns, additional compounds may contribute to this reduction of caries prevalence. Weak organic acids, which are additives of food products and beverages, are acknowledged as potential substances with caries-protective properties [11–13]. They act as preservatives which delay the decay of food as caused by bacteria, candida species or yeasts, due to their action towards microbial enzymes or on microbial cell membranes. For instance, food preservatives such as sodium benzoate, sodium nitrite and sorbic acid are used to kill microorganisms or at least to control bacterial growth, thus prolonging the shelf life of food products [14].

The mentioned preservatives represent weak acids. Their nondissociated form is able to penetrate the bacterial cell membrane where they then dissociate in the neutral cytoplasm and produce protons. Due to this acidification of the cytoplasm, the growth of bacteria is blocked or hampered [15]. For example, potassium sorbate and sodium benzoate exerted an inhibitory effect on spoilage fungi on bakery products [16]. Their maximum antibacterial effect occurs at low pH values [17]. The restriction of bacterial growth occurs via an inhibition of key enzymes of glycolysis, such as enolase [18]. In particular, the effect of preservatives on growth and metabolism of dental plaque microorganisms was demonstrated in vitro and in vivo [13, 19]. In one study [13], 0.4 % sorbate or benzoate solutions, respectively, caused a marginal inhibition of bacterial acid production, while a significant restriction was registered at concentrations of 2 %.

Preservatives are foodstuff additives that are bound to official regulations. Within the European Union, they are assigned a number code (E200–E299) on the corresponding food packages. In recent decades, an increase in consumption of foods containing preservatives (i.e. soft drinks, meat products, ketchup and mayonnaise) was noted [20], while consumption of products without preservatives declined. In industrialized foods and beverages, the most widely used preservatives are benzoic acid and sorbic acid. Benzoic acid (E210), being a derivative of paraben, represents an aromatic carbonic acid. In spite of the fact that benzoic acid possesses a substantially stronger antimicrobial action, sodium benzoate (E211) is preferentially used due to its 200-fold better solubility. The latter has a sour taste and is used in acidic or acidified foods. Hence, it is part of soft and fruit drinks, pickled vegetables and sauces. Sorbic acid (E200) is a polyunsaturated carbonic acid that as a preservative is used as a free acid. However, due to its poor solubility in water, sorbates are preferred in most cases (sodium E201, potassium E202 and calcium E203). Foods containing sorbates include, but are not limited to, margarine, mayonnaise, lemonades, bakery products, dried fruits, yoghurt, cheeses and sausages.

Moreover, these mentioned substances are also added to house cleaning products, cosmetics and pharmaceuticals. Thus, it is evident that sorbate and benzoate are regularly in contact with the mouth, with intraoral surfaces and with the oral microbiota. As their antibacterial efficacy is proven in vitro and in vivo [13, 20], it seems possible that those compounds also exert an antimicrobial effect against the dental biofilm. Only few studies exist in the literature that discuss the influence of preservatives on the in situ dental biofilm. To the best of our knowledge, there are no studies examining in situ 14-day-old biofilms. Moreover, no investigations seem to exist regarding the influence of food preservatives on the quality of dental enamel in situ, underlying the dental biofilm. This may be due to increased efforts to establish the corresponding physicochemical data. The aims of this double-blind, randomized, four-cell crossover investigation were to evaluate the effects of two commercially widely used food preservatives, benzoic acid and sorbic acid, (a) on thickness (i.e. mass) and vitality of an in situ biofilm and (b) on the ability of the in situ biofilm to demineralize the underlying bovine enamel after a period of 2 weeks, regarding (1) we wanted to know whether the proven effect of preservatives on biofilm vitality [19] may still be prominent after 14 days. Our hypothesis was that biofilm development is still retarded under the influence of the preservatives, concerning (2) it was an open question whether this effect would even have an impact on enamel demineralization in this quite limited study period of only 2 weeks. Our expectation was that the preservatives would evoke a slight effect, if any.

Materials and methods

Study population

Twenty healthy volunteers (9 female, 11 male, aged 23 to 36 years; mean age, 26.2 years) participated following the completion of a comprehensive dental anamnesis as described in Arweiler et al. [19]. Exclusion criteria were participation in other studies 30 days prior to start of study, pregnancy, the use of antibacterial mouthrinses or antibiotics during the last 6 months, as well as signs of destructive periodontitis or inflammatory symptoms. All volunteers were given written information about the study design and signed a consent form prior to their inclusion in the study. The design of the study was in accordance with the ICH note for guidance on Good Clinical Practice and the Declaration of Helsinki (1964). The study was not commenced until approval was obtained from the ethics committee of Freiburg University (No. 106/03).

Biofilm growth

The volunteers received individual acrylic appliances in which six plasma-sterilised [21] (Sterrad 100S, Advanced Sterilisation Products, USA) bovine enamel discs (diameter 3.4 mm, thickness 2 mm) were inserted for 14 days to generate intraoral biofilms (for further details, see [19, 22]). In order to standardize the oral hygiene conditions, all subjects received a professional toothcleaning, a standard toothpaste (Odol med3 Milchzahn, 500 ppm fluoride from sodium fluoride, GSK, Bühl, Germany) and an extra soft toothbrush (Lacalut med, Dr. Theiss Naturwaren, Homburg, Germany) prior to placing the intraoral appliance onto the maxillary teeth. Subjects maintained their normal diet during the four test cycles.

Appliances had to be worn for 14 days continuously except during eating and oral hygiene measures (twice daily for 2 min each using only the allocated toothpaste and toothbrush). During these short periods of time when the appliances were not worn, they were placed into a petri dish filled with tap water.

Test products and treatment

Solutions of benzoate (0.1 %; BA; natrium benzoate salt, Sigma, Munich, Germany) and sorbate (0.1 %; SA; potassium sorbate salt, Fluka, Buchs, Switzerland), respectively, in *aqua dest* were used as test solutions, while chlorhexidine (0.2 %; Chlorhexamed® forte; CHX; GlaxoSmithKline GmbH, Bühl, Germany) and saline (0.9 %; NaCl; B. Braun Melsungen AG, Melsungen, Germany) served as positive and negative controls, respectively. The pH values of the treatment solutions were physiol. NaCl 6.45, benzoate 6.75, sorbate 7.03 and CHX 7.85. Coded sterilized identical bottles (Schott Duran®, Duran Produktions GmbH & Co. KG, Mainz, Germany) containing BA, SA, CHX or NaCl were randomly distributed to the subjects in the individual test weeks following a Latin-square crossover design by a laboratory technician not otherwise involved in the study, so that neither investigator nor test subject could identify the corresponding product. The code was kept in a sealed envelope and was disclosed when all examinations were finished. Moreover, each subject received a petri dish (Cellstar® Tissue Culture dish, Greiner bio-one, Frickenhausen, Germany) for placement of the appliances in the corresponding test or control solutions twice daily for 1 min each time. After a washout period of 7 days, a new test cycle was started. Hence, the effects of the different solutions were tested 28 times during 14 days in a randomized order in a crossover design in four test cycles.

Bacterial staining, CLSM analysis and assessment of biofilm vitality

After 14 days of biofilm growth, the plaque-covered enamel specimens were removed from the splints, washed in saline at room temperature and then processed without any delay with vital fluorescence stain and subsequently evaluated by confocal laser scanning microscopy (CLSM) [23, 24]. In brief, the adhering biofilm was stained with 15 μl solution containing two fluorescent dyes, fluorescein diacetate (FDA) and ethidium bromide (EB) (both Sigma Chemie, Taufkirchen, Germany), in order to visualise the percentage of living (green) and dead (red) bacteria. Immediately after staining (2 min) and gentle washing (specimen were very carefully dipped in physiol. NaCl), a drop of saline buffer was placed onto a chambered coverslip (Lab-Tek II, Nalge Nunc International, USA). The specimens were then inverted onto the saline buffer drop in order to prevent disturbance and desiccation of the spatial structure of the biofilm and to allow imaging from below. Confocal images were obtained with the CLSM microscope (Leica TCS SP2 AOBS, Leica Microsystems, Heidelberg, Germany) equipped with an Argon laser [excitation 488 nm; emission (green) 497–541 nm, (red) 598–651 nm] using a ×63 water immersion objective [HCX PL APO (lbd-BL ×63/1.2 W Corr].

Biofilm thickness (BT in micrometre) and biofilm vitality (BV in per cent) were assessed according to Arweiler et al. [19] using two enamel discs of each panelist. To evaluate biofilm vitality, optical sections of approximately 1 μm were made at every second micrometre (to avoid overlaps) throughout the biofilm. The BV of each optical section was calculated in per cent using an automatic image analysis program (Axio Vision Rel. 4.5 Inc., Carl Zeiss Jena GmbH, Jena, Germany).

Assessment of mineral content and lesion depth

The mineral loss (ML) of the four enamel slabs for every participant was assessed using quantitative light-induced laser fluorescence (QLF) [25, 26] and the ML by transversal microradiography (TMR, TMR 1.25e, Inspektor Research Systems B.V., Amsterdam, Netherlands) [27, 28]. Accordingly, the lesion depths (LD) in the enamel slabs were analysed by TMR.

TMR For TMR analysis, one half of the surface of the enamel samples was covered with a thin bonding layer (Heliobond, Ivoclar Vivadent GmbH, Ellwangen, Germany) before the samples were inserted into the splints, which served as a reference area of sound enamel [27, 28]. After the clinical phase, plane-parallel microsections of 120 to 130 μm thickness were produced and polished using sandpaper (4,000 grit). Subsequently, microradiograms were developed using a Cuα-source at 20 kV/20 mA (PW 1830/40, Philips Analytical X-Ray B.V., Almelo, Netherlands). The exposure time was set to 12 s on a holographic film (High-speed holographic film SO 253, Kodak AG, Stuttgart, Germany). Based on the transmission values (recorded as grey scale), graphs were drawn by a corresponding software (TMR 1.25e, Inspektor Research), which allowed ML as well as LD to be calculated.

QLF The change of fluorescence (delta *Q*) of the lesion area was monitored via light-induced laser fluorescence (QLF; QLF Device Inspektor Research Systems B.V.; Amsterdam, Netherlands) on two further slabs. Two measurements were conducted, namely baseline recordings at the start of the regime and endpoint recordings after 14 days of wearing according to Lennon et al. [29]. At the first appointment, the corresponding records of a CCD camera were processed by Software "QLF-Patient" (Inspektor Research Systems, Amsterdam, Netherlands), which produced a fluorescence image per sample. The samples obtained at the second appointment after 14 days were—as recommended by the manufacturer—gently cleaned and detached from the adhering biofilms using a soft toothbrush (meridol Paro, GABA GmbH, Lörrach, Germany) and aqua dest. Thereafter, another fluorescence image was recorded. The change of fluorescence (delta *Q*) was calculated with the aid of the analysis software "Software Substract 01" (QLF Image Subtraction Version 1.1, Inspektor Research Systems, Amsterdam, Netherlands). As the main parameter, the program calculates the change of the fluorescence levels across the lesion area (delta *Q*: integral of fluorescence loss in the lesion area: square millimetres×per cent) and depicts the data numerically and as false colour print.

With highly mineralised sound enamel, high levels of fluorescence are recorded with QLF, while demineralisation causes an increased scattering coefficient in the specimen and leads to a reduction of fluorescence. Thus, fluorescence loss indicates a mineral loss, while an increase in fluorescence resembles a mineral gain [25].

Statistical analysis

The statistical analysis was performed with SPSS 20. The data of BT and mean BV were averaged within each subject and then averaged across all subjects for each test cycle. The data series of BV in the different sections were subdivided into three equally prominent layers, i.e. bottom, middle and top layer according to Arweiler et al. [19]. The parameters BT and BV were analysed using linear mixed models with subject-specific random intercepts to adjust for the dependence of the measurements within one subject, followed by pairwise comparisons using a Bonferroni correction.

The data of TMR and QLF were analysed using linear mixed models with subject-specific random intercepts to adjust for the dependence of the measurements within one subject. Since not any significance could be determined, no further pairwise comparisons were conducted.

For all analyses, a difference was considered significant at the 95 % confidence level (α=0.05).

Results

Of the 20 volunteers recruited, one had to be excluded due to incompliance. Hence, 19 participants (9 female and 10 male) completed the study, and all their specimens could be analysed. Their mean age was 25.7±2.0 years.

Biofilm thickness (BT) and biofilm vitality (BV)

Mean values and standard deviations for BT and mean BV after 14 days of in situ biofilm growth during influence of either physiol. NaCl or the different test solutions SA, BA and CHX are presented in Table 1. With the negative control NaCl, a BT of 39.9±14.4 μm originated. Both food preservatives SA and BA influenced BT only slightly leading to mean BTs of 34.9±12.7 and 32.5±11.0 μm, respectively, without significant difference to the negative control. Only 0.2 % CHX reduced BT significantly (p<0.001) to a mean height of 15.8±7.8 μm. These data correspond to (relative) reduction rates of 12.5 % (n.s.) for SA, 18.5 % (n.s.) for BA and 60.4 % (p<0.001) for CHX compared to the negative control (Table 2).

The mean vitality (BV) was 67.2±15.9 in the NaCl control biofilms. Under influence of SA, BA and CHX, lower values were assessed, namely 36.7±10.9 %, 36.7±6.9 % and 32.5±11.4 %, respectively. These values were all significantly

Table 1 Effect of food preservatives and chlorhexidine on biofilm vitality (BV) and biofilm thickness (BT) after 14 days in situ development (n=19; mean±SD)

	BV (in %; n=19)	p value (compared to NaCl)	BT (in μm; n=19)	p value (compared to NaCl)
NaCl	67.2±15.9		39.9±14.4	
SA	36.7±10.9	<0.001	34.9±12.7	n.s.
BA	36.7±6.9	<0.001	32.5±11.0	n.s.
CHX	32.5±11.4	<0.001	15.8±7.8	<0.001 (also compared to SA and BA)

Statistical comparison by ANOVA and paired t test

n.s. non-significant

different from the negative control. Relative reduction rates of 45.6 %, 45.6 % and 51.6 %, respectively, could be calculated, thus reflecting an antibacterial effect on the in situ oral microbiota. There were no significant differences between the three active substances.

Table 3 depicts the distribution of BV (mean of all subjects) when subdivided in three layers. The negative control showed very similar BV data, with the top layer (69.7 %) being slightly more vital than the bottom layer (65.1 %). The test substances lowered the biofilm vitality to different extents. The CHX exerted the strongest effect especially on both outer layers (BV, 23.8–44.3 %). Both preservatives had somewhat weaker effects (BV, 31.6–44.1 %), especially in the middle layer(s).

TMR: lesion depth (LD) and mineral content (ML)

Regarding LD (Fig. 1), the highest values were assessed with NaCl (43.6±44.2 μm; mean±standard deviation), the lowest with CHX (11.7±39.4 μm), and SA and BA lying in between (22.9±45.2 and 21.4±38.5 μm, respectively). All comparisons were proven to be non-significant (p>0.05).

A similar data set was found for Delta ML (Fig. 2), where the highest mean values of 128.1±207.3 vol% μm were established for the control NaCl, the lowest for CHX (−16.8±-284.2 vol% μm), while SA and BA led to values of 83.2±150.9 and 98.4±191.2 vol% μm, respectively. Again, all comparisons were proven to be non-significant (p>0.05).

QLF: fluorescence change (delta Q)

With QLF (Fig. 3), for both NaCl and CHX controls, negative values (i.e. −33.8±101.3 and −16.9±69.9 mm^2 %, respectively) were recorded reflecting a fluorescence loss, while positive values were found with SA (33.9±158.2 mm^2 %) and BA (24.8±118.0 mm^2 %), depicting a fluorescence gain. These differences were also non-significant (p>0.05).

Discussion

Several in vitro and in vivo models of obtaining biofilms have been described [19, 22–24, 30–34]. In situ models with removable splints offer the opportunity to insert specimen of any material and to obtain several (six to eight) biofilm samples in one jaw [19]. However, studies examining biofilm growth over a period of more than 5 days are extremely rare or are non-existent. As in our previous investigation [19], the growing biofilms were dipped into the solutions instead of rinsing the mouth with them for the following reasons: (1) Although the concentration of the preservatives SA and BA had been within the maximum allowance for these products, rinsing with the solutions was avoided due to ethical reasons (direct contact with the mucosa may have the possibility of causing allergic reactions), as only the effect on biofilm formation was of interest; (2) rinsing with or dipping into the solutions seems not to differ strongly from one another.

Table 2 Comparison of the effect of the test products on biofilm vitality (BV) and biofilm thickness (BT) after 5 days [19] and 14 days [present study] (mean±SD)

Compound			NaCl	SA	BA	CHX
BV in %	Arweiler et al. [19]	5 days	57.7	42.9	44.5	21.7
	Relative reduction		–	25.6 %	29.0 %	71.8 %
	Present study	14 days	67.2	36.7	36.7	32.5
	Relative reduction		–	45.6 %	45.6 %	51.6 %
BT in μm	Arweiler et al. [19]	5 days	25.3	19.8	21.9	10.8
	Relative reduction		–	16.8 %	20.7 %	57.3 %
	Present study	14 days	39.9	34.9	32.5	15.8
	Relative reduction		–	12.5 %	18.5 %	60.4 %

Table 3 Comparison of BV (in per cent, ±SD) in different biofilm layers after 14 days of in situ biofilm growth

Compound	NaCl	SA	BA	CHX
Top layer	69.7±17.6	44.1±14.5	41.2±12.0	23.8±15.3
Middle layer	66.6±18.9	31.6±11.1	32.4±8.4	27.0±13.0
Bottom layer	65.1±20.8	33.0±14.2	33.0±12.1	44.3±12.9

Rinsing studies using not only the same splint design but also even the same controls showed very similar values for the controls CHX and NaCl, as well as similar reductions by CHX compared to NaCl [19, 35].

The acceptance of our splint model by the subjects was very good. Although in situ model systems standardize biofilm growth at the different positions, the distinct models of the research groups vary in kind of biofilm growth (e.g. fissural, interproximal or smooth surface plaque) and treatment. While the present model tries to imitate interproximal plaque, the aim of another in vivo splint model using "dentinal grooves" was to imitate fissure plaque [31, 36].

Biofilm thickness (BT) and biofilm vitality (BV)

The findings of the present 14-day biofilm growth study can be compared best directly with the study of Arweiler et al. [19], who used exactly the same study design and the same solutions, however, on 5-day biofilm growth. As obvious from Table 2, a BT of 25 μm had emerged after 5 days of growth, while 40 μm were measured after 14 days of biofilm development. Related to the fact that the study time increased nearly threefold, the gain in biofilm thickness was less than expected. However, if native dental plaque is left undisturbed, 50- to 100-μm-thick plaque biofilms may develop after 2 to 3 weeks [37]. Possible phenomena like erosion and sloughing of biofilm shreds are responsible for relatively thinner biofilms after 14 days.

In accordance with the literature, the 0.2 % CHX positive control significantly retarded biofilm growth [9, 35]. In both the 5-day and the 14-day investigations, CHX reduced BT in the same magnitude. As well, in the two studies, both preservatives reduced BT to a similar extent. Also in line with the literature [38, 39] with respect to the negative control, the mean BV increased with increasing plaque age from 57.7 % in the 5-day biofilms to 67.2 % in the 14-day specimen. This increase was not as pronounced as anticipated. Moreover, the BV of the 14-day biofilms increased from the bottom layers to the outer top layers (Table 3). However, this effect was less pronounced than in the 5-day biofilms [19] and in other CLSM vital fluorescence studies [23, 24]. The preservatives SA and BA showed less effect on the 5-day microbiota (mean BV reduced to 42.9 % or 44.5 %, i.e. reduction rates 25.9 % or 29.0 %, respectively) than on the more mature 14-day biofilm (mean BV, 36.7 %; relative reduction, 45.6 % for both substances). CHX as well as the acidic food preservatives had different effects on the various layers of the biofilms (Table 3).

CHX reduced the mean percentage of the vital microbiota the most (Table 2), more so in the 5-day-old biofilms (mean BV, 21.7 %; relative reduction, 71.8 %) than in the 14-day-old biofilms (mean BV, 32.5 %; relative reduction, 51.6 %). Table 4 compares the antibacterial effect of 0.2 % CHX as

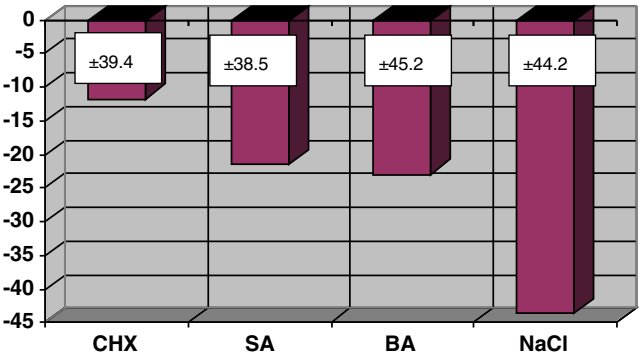

Fig. 1 Lesion depth (in micrometre; ±standard deviation) assessed by TMR

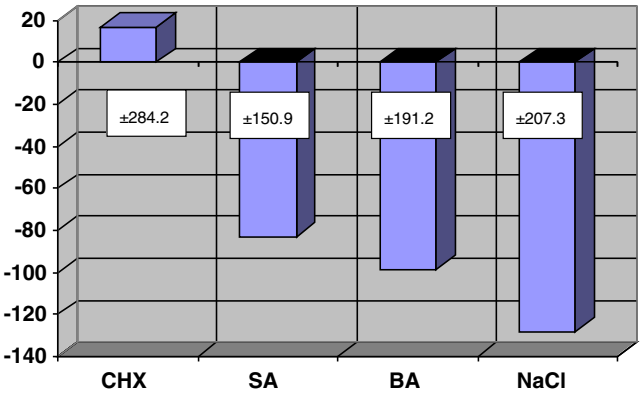

Fig. 2 Mineral loss (in volume per cent×micrometre; ±standard deviation) assessed by TMR

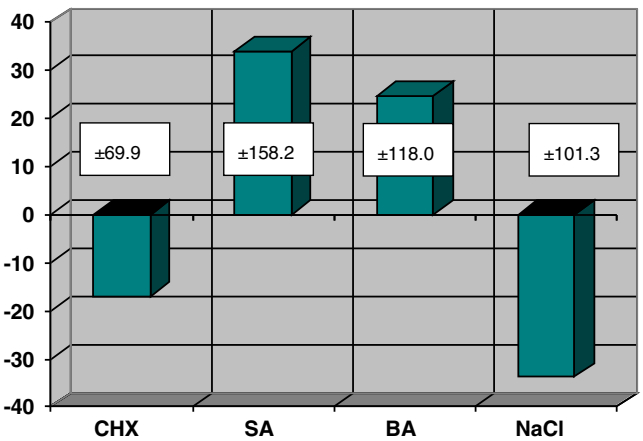

Fig. 3 Delta *Q* (in per cent×square millimetre; ±standard deviation) assessed by QLF

Table 4 Decrease of biofilm vitality caused by 0.2 % chlorhexidine

Authors	Days[a]	*N*	Top[b]	Middle[b]	Bottom[b]
Zaura-Arite et al. [31]	2 days	6	−18.7 %	−11.1 %	−6.7 %
Arweiler et al. [19]	5 days	24	−35.0 %	−46.8 %	−27.2 %
This study	14 days	19	−45.9 %	−39.6 %	−20.8 %

Absolute decrease of biofilm vitality as measured by FDA/EB vital fluorescence (VF%) as related to control specimen

[a] Of biofilm development in situ

[b] Layer of biofilm

assessed by FDA/EB vital fluorescence in different studies and documented in distinct layers of the corresponding biofilms. In the present 14-day in situ biofilms, the top and the middle layers were affected the most by CHX and the bottom layers the least. This is in accordance with findings in even younger, 2-day-old biofilms [31]. Conversely, the top layers in the 5-day biofilms had been less affected, whyever, and the middle layer the most affected [19].

The latter finding could be interpreted as a diffusion phenomenon. CHX as a dicationic substance binds to bacterial cells [40, 41] and precipitates outer-mural and inner-mural proteins, thus leading to a concentration-dependent bacteriostatic or bactericidal action. In that way, CHX may be bound and "consumed" preferentially in the outer parts of oral biofilms. This effect became obvious in the more mature 14-day-old biofilms as well as in less mature bacterial films after 1 and 2 days of development [31]. In contrast, both BA and SA are small molecules compared to CHX. They seem not to bind to components of the bacterial biofilm cells. They contain hydrophobic (aliphatic or cyclic) as well as hydrophilic elements and act as weak acids (cf. "Introduction" section) and weak detergents. In both the 5-day biofilm study and in the 14-day investigation, they exerted a stronger action on the inner and the middle layer of the biofilms than on the outer top layer due to their penetration characteristics [30, 32]. While the medicament(oid) CHX as well as the common food preservatives BA and SA exert antibacterial effects on in situ developing oral biofilms, their modes of biofilm penetration and of extracellular and intracellular antibacterial action seem to differ.

TMR and QLF

The methods described for mineral quantification are mainly used to examine the strong and quite easy assessable impact of fluoride on the remineralisation of dental enamel in vitro and in vivo [25–29, 42]. However, in our study, BA and SA could only indirectly influence remineralisation. The impact of fluoride on bacterial metabolism and glycolysis is considered as a "weak-acid effect" [43]. Thus, fluoride and weak acids (like BA and SA) act against bacteria in a similar manner, especially in an acidified environment. Benzoate has been shown to affect the metabolism of oral microorganisms (in a similar way to that of fluoride) by reducing the acid tolerance of the oral flora causing cell death [28, 37, 43]. Thereby, weak acids exert a similar [44] or a lower efficacy than fluoride [45]. In an animal model, fluoride and sodium benzoate were able to reduce caries activity [46]. Overall, the preservatives, which act indirectly via the bacterial biofilm, seem to have a lower potential to reduce enamel demineralisation than fluoride. This is not surprising, since fluoride is known for its direct effect by straight interaction with dental surfaces. Moreover, other studies used more influencing conditions, for example, sugar rinses were conducted to provoke demineralization in situ [30], or the specimen were demineralised to generate obvious lesions before re-remineralisation [28, 29, 42]. Compared to those striking conditions, the "natural" acid-effect was weaker, thus resembling even a new challenge for the employment of TMR and QLF.

Nevertheless, the differences between the different controls and test substances as documented in our study were obvious. Taking the lesion depth of 43.6 μm, as assessed with the negative control NaCl as a 100 % niveau, this value was retarded by BA and SA by approximately 50 % and with CHX by 73 %. Concerning ML assessments, mineral loss with NaCl, SA and BA was even reversed to mineral gain in the case of CHX. However, in comparison to fluoride data from the literature [27–29, 36, 42], our 14-day investigation had quite high standard deviations for all of the parameters assessed (LD, ML and QLF) as well as for all of the test and control substances. Therefore, the evident differences could not be proven as statistically significant. Moreover, the small lesion size could also be a reason for large standard deviations and thus the lack of significance. TMR may not be sensitive enough to lead to reproducible measurements for superficial lesions.

Since mineral loss and lesion depth are presented, a statement about changes in surface layer is warranted. In the case that both mineral loss and lesion depth decrease in a similar manner, it can be concluded that the remineralizing effect occurs within the body of the lesion. However, in the present investigation, CHX lesion depth (Fig. 1) decreased only slightly compared to mineral loss (Fig. 2) which showed a distinct effect even towards positive values. Thus, it can be concluded that under the use of CHX, mineral change happens rather in the outer layers of the enamel and seems to have a superficial effect.

In general, results as established with both different optophysical methods (TMR and QLF) should be comparable, so far, however, only tested for directly re- or demineralizing substances or saline [47, 48]. In the present study, this holds true for the negative control, NaCl, which "created" the most prominent lesion depth and the highest mineral loss as measured with TMR and QLF. Both preservatives BA and SA had

very similar effects in retarding LD as well as ML (TMR); in case of delta *Q* (QLF), they even showed a mineral gain. It should however be kept in mind that preservatives represent antibacterial substances which only indirectly influence hard tissue surfaces and were so far not tested by TMR and QLF.

Furthermore, the positive control CHX resulted in contrasting outcomes with TMR and with QLF. Due to its strong antibacterial efficacy [40, 41, 49], CHX not only exerts an anti-plaque and anti-gingivitis action but also has an anti-caries impact [50]. As a consequence, CHX became the gold standard as an antibacterial mouthwash and can also retard enamel demineralization. This is shown in our study using TMR, which also aligns with other literature [36, 42]. These authors assessed the effect of CHX only by TMR and on previously demineralized hard tissue specimens. To our knowledge, no studies currently exist with respect to the (re)mineralizing effect of CHX on QLF. QLF has so far only been used to measure tooth stain caused by CHX [51]. However, these authors had to develop an algorithm to detect stain on QLF images of teeth captured in vivo.

Therefore, it can be speculated that the mere adhesion of CHX on the enamel surface and its well-documented effect of surface staining as a consequence [52, 53] may severely hamper the quantification of mineral loss via QLF by changing surface characteristics and its fluorescence. In our study, the test slabs for QLF analyses were cleaned to remove only the bacterial biofilms. Care was taken not to alter the enamel surface characteristics. During the time span of the study, the 0.2 % CHX solution was used 28 times, which may have led to pertinacious surface precipitation, even when not visible. Possibly, this may have been also deteriorating for QLF analysis. Analogous recent modern opto-physical methods for caries detection [54, 55] rely on their accuracy in measuring specific fluorescence patterns (or deposits) in order to differentiate between enamel and dental calculus [54] or to differentiate white from light brown/dark brown spots [55]. As a consequence, the clinician is advised to clean the enamel surface thoroughly when applying these diagnostic devices.

Conclusion

Based on our results, benzoic and sorbic acid retard biofilm thickness and vitality in 14-day-old biofilms. However, these substances were not as effective as chlorhexidine. The latter reduced BV statistically significantly by more than 50 % compared to the negative control. Additionally, the BT was reduced even more by relatively 60 % in comparison to the control. The food preservatives were also able to retard the BV by approximately 45 %, which is very similar to the effect of CHX. In the case of SA and BA, this retardation did not lead to a significant negative change of BT, with only 12.5 % and 18.5 %, respectively. Such a phenomenon was already described and discussed in earlier studies with mouthrinse solutions containing essential oils or with amine/stannous fluoride compounds that had been compared to CHX as positive control [49, 56, 57]. It seems that CHX, due to its high substantivity, is even able to retard biofilm thickness in an in situ growing biofilm, while the tested food preservatives are "only" able to reduce bacterial vitality of the biofilm.

After 2 weeks of biofilm development, the preservatives benzoate and sorbate had a clear diminishing effect on demineralization of the enamel. This was demonstrated by TMR as well as by QLF for the very first time. However, unexpectedly high standard deviations hampered the statistical analysis. Thus, these new findings have to be seen as a trend. As part of our daily diet, these preservatives, and possibly numerous others, exert not only a direct impact on the vitality and metabolism of the dental biofilm but also on this way, they even influence demineralization processes of the underlying dental enamel in situ.

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