Clin Oral Invest (2008) 12:203–208
DOI 10.1007/s00784-008-0188-6

ORIGINAL ARTICLE

Effect of food preservatives on in situ biofilm formation

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Received: 8 October 2007 / Accepted: 21 January 2008 / Published online: 21 March 2008

Abstract The aim of this double-blind, controlled crossover study was to evaluate the influence of food preservatives on in situ dental biofilm growth. Twenty-four volunteers wore appliances with six specimens each of bovine enamel to build up intra-oral biofilms. During three test cycles, the subjects had to put one half of the appliance twice a day in one of the assigned active solutions (0.1% benzoate, BA; 0.1% sorbate, SA or 0.2% chlorhexidine, CHX) and the other into NaCl. After 5 days, the developed biofilms were stained with two fluorescent dyes to visualise vital (green) and dead bacteria (red). Biofilms were scanned by confocal laser scanning microscopy and biofilm thickness (BT) and bacterial vitality (BV%) were calculated. After a washout period of 7 days, a new test cycle was started. The use of SA, BA and CHX resulted in a significantly reduced BT and BV compared to NaCl ($p<0.001$). Differences between SA and BA were not significant ($p>0.05$) for both parameters, while CHX showed significantly lower values. Both preservatives showed antibacterial and plaque-inhibiting properties, but not to the extent of CHX. The biofilm model enabled the examination of undisturbed oral biofilm formation influenced by antibacterial components under clinical conditions.

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Keywords Biofilm · Plaque vitality · Confocal laser scanning microscopy

Introduction

Foods are mostly complex mixtures of macro- and micro-components. As these have prolonged contact with the oral cavity, diet can influence the oral microflora and can, for instance, constitute a caries risk even if adequate oral hygiene and the daily presence of fluorides have a greater effect [8].

The recent decline in the prevalence of caries has been primarily attributed to extensive exposure to fluoride. However, some authors indicate that during this same period, the consumption of food preservatives such as benzoates and sorbates has also increased substantially and could theoretically also have led to a decline in caries [9].

Food preservatives such as sodium benzoates, sodium nitrite and sorbic acid are used to kill microorganisms or at least to control bacterial growth [10, 22]. They are added to many food products and carbonated beverages (soft drinks) to prolong their use when opened and not to harm general health. However, these substances could also have an effect on oral bacteria, particularly with regard to their widespread use and the increase in consumption of these foods.

There is some data in the literature which suggests that these preservatives have an effect on cytoplasmic acidification which is not unique to fluoride [25] but common for a number of small weak acids. This leads to inhibition of enolase and other enzymes in the glycolytic pathway of carbohydrate metabolism [6].

An effect of food preservatives on the growth and metabolism of plaque bacteria has been shown to occur both in vitro and in vivo [14, 17]. However, neither of these studies took into account the presence of single bacteria or

the dental plaque flora as a biofilm. It has been shown that an established intra-oral splint design resulted in standardised in situ biofilm formation (mimicking supragingival plaque formation) irrespective of the position of the different specimens [1, 2, 4].

Therefore, it was the aim of this study to assess the effects of two food preservatives on in situ dental biofilm growth compared to chlorhexidine, the most efficient agent against dental plaque, and saline as a negative control.

Materials and methods

Study population

Twenty-four healthy volunteers (12 male, 12 female, aged from 23 to 36 years; mean age 26.4) were selected for this study. The caries risk of the subjects was classified according to decayed, missing and filled teeth (DMFT), salivary flow rate and lactic acid formation rate (Clinpro™ Cario L-Pop, 3M Espe AG, Seefeld, Germany; see also Table 3).

Exclusion criteria were participation in other studies 30 days before study start, 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 before 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 the approval of the ethics committee of Freiburg University had been obtained (#106/03).

Biofilm growth

All volunteers received an individual acrylic appliance for their upper jaw in which six sterilised bovine enamel discs (3.4 mm in diameter, 2 mm in height and negatively tested on bovine spongiform encephalopathy by a veterinarian) were inserted towards the interdental area between two adjacent teeth in such a way that biofilm growth was not disturbed by the tongue or the cheek. The positions of the enamel discs were chosen to mimic interproximal plaque (for details see Fig. 1).

The bovine enamel samples were sterilised by ultrasonication for 2 min in 2% sodium hypochlorite followed by ultrasonication in 70% ethanol for another 2 min. After that, the samples were washed twice in sterile distilled water (see also [1]).

Before inserting the intraoral appliance, all subjects received a professional tooth cleaning, a standard toothpaste

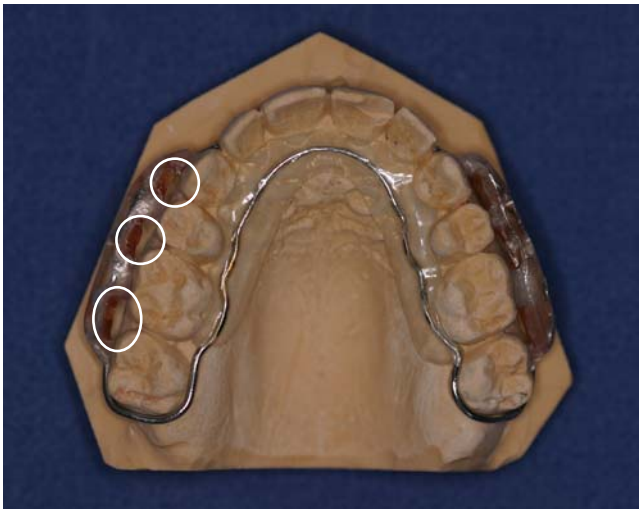

Fig. 1 Locations of the specimens (fixed with sticky wax) in the intra-oral splint (*left side* highlighted with *white circles*)

(Odol med3 Milchzahn, 500 ppm fluoride, GSK, Bühl, Germany) and a toothbrush (elmex® interX, GABA, Lörrach, Germany) to standardise the conditions. During the three test cycles, the subjects maintained their normal diet.

Appliances had to be worn for 5 days continuously except during eating, oral hygiene measures (twice daily for 2 min each using only the allocated toothpaste and toothbrush) and twice daily treatment of the specimens.

Test products and treatment

Solutions of benzoic acid (0.1%; BA) and sorbic acid (0.1%; SA) 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. BA, SA and CHX were tested over a course of three cycles, while NaCl always served as a control for biofilm growth.

During the 5 days of wearing, subjects had to dip twice daily for 1 min one arm of the splint into either a test (BA, SA) or CHX solution and the other arm into NaCl. To avoid confusion about which arm was to be put in which solution, the splint material of one arm was pink, while the other one was fabricated from clear acrylic. Concurrently, the bottles of the active solutions had a pink labelling, but were otherwise identical (labelled only with a code number). The coded bottles (BA, SA, CHX) together with the control (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.

After a washout period of 7 days, a new test cycle was started.

Bacterial staining and confocal laser scanning microscopy analysis

After 5 days of biofilm growth (120 h), the plaque-covered enamel specimens were carefully removed from the splints, gently washed in saline (room temperature) and then processed without delay for the vital fluorescence staining as described by [9] and the evaluation by confocal laser scanning microscopy (CLSM). In brief, the adhering biofilm was stained with two fluorescent dyes, fluorescein diacetate and ethidium bromide to visualise the percentage of living (green) and dead (red) bacteria. Immediately after the staining procedure (3 min), 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 drops 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) using a 63× water immersion objective. The highest point of the biofilm surface was searched for by focusing on the substratum and moving until the outermost biofilm cells were in focus. Then, optical sections of approximately 1 μm were made at every second micrometer (to avoid overlaps) throughout the biofilm. The area of each section was transformed into a digital image containing 512×512 pixels. The size of each pixel represented 0.625×0.625 μm in the specimen. This procedure was repeated twice on each biofilm resulting in three series of sections throughout the biofilm. Thus, biofilm thickness (BT) of each test product arose from three measuring points and three specimens.

Assessing biofilm vitality and the vitality of each biofilm section

To assess mean biofilm vitality as well as biofilm vitality per layer from the digitised data, an automatic image analysis program (KS 300 3.0, Carl Zeiss Vision GmbH, Hallbergmoos, Germany) was used. This program calculated the percentage of vital (green-stained) bacteria as compared to vital and dead (red-stained) bacteria in each section as well as the vitality values of the entire biofilm (BV %).

Statistical analysis

The statistical analysis was performed with SPSS 11.0. The data of BT and mean BV were averaged across the three series within each subject and then averaged across all subjects for each test cycle. Then, the parameter BT and BV were analysed using single factor analysis of variance (ANOVA). The significance of any differences was determined using the paired *t* test. The data series of BV in the different sections were subdivided into three equally thick layers, layer 1: bottom layer, layer 2: middle layer, layer 3: top layer (Fig. 2), so that the effect of the different solutions could be compared by layer. Differences between the three layers were tested by ANOVA.

Results

All 24 volunteers finished the study and all specimens could be analysed. Both food preservatives were able to significantly reduce biofilm thickness and vitality compared to the NaCl solution. As previously shown [1, 2, 4], the statistical analysis detected again no differences between the three specimens of one appliance (with the same treatment) in any parameter, which speaks for the quality rating and reproducibility of the biofilm model used in the study.

Mean values as well as standard deviations for BT and mean BV are presented in Table 1.

The use of SA, BA and CHX resulted in BT of 19.8, 21.9 and 10.8 μm, respectively, which corresponds to a reduction of 17% ($p=0.032$), 21% ($p>0.001$) and 57% ($p>0.001$) in comparison with the negative control.

The mean vitality (in %) under the influence of SA, BA and CHX was 42.9%, 44.5% and 21.7% and reduced by 26%. 29% and 62% (all $p>0.001$) when compared to the negative control.

As the control data (BT and BV) of the three test cycles did not differ significantly, the three active solutions were compared directly. Differences between SA and BA were not significant ($p>0.05$), while both preservatives were significantly different from CHX ($p<0.001$).

The distribution of biofilm vitality of all subjects divided into three layers is presented in Table 2. Besides CHX, both test products as well as the negative control showed a very similar vitality pattern with lower values in the bottom and

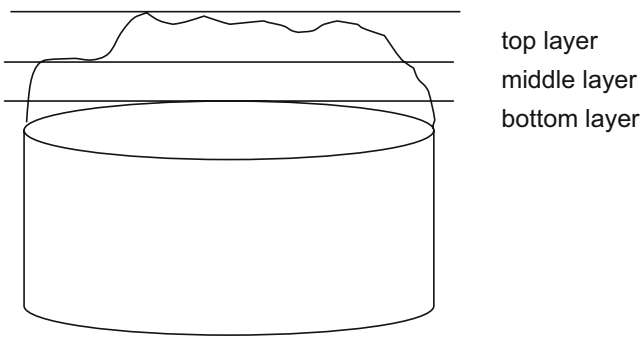

Fig. 2 Definition of the different biofilm layers

Table 1 Biofilm thickness (BT, in μm) and mean proportion of vital bacteria (BV; vitality values in %) during the different test cycles

	SA cycle		BA cycle		CHX cycle	
	SA	SA control (saline)	BA	BA control (saline)	CHX	CHX control (saline)
BT (in μm)	19.76b	23.76a	21.91b	27.63a	10.78c	25.33a
	±6.92	±7.59	±5.88	±6.78	±3.74	±11.97
BV (in %)	42.93e	57.66d	44.47e	62.59d	21.69f	56.79d
	±12.23	±13.44	±15.33	±14.43	±11.1	±13.74

Different letters (a–f) indicate statistical significance ($p<0.05$).

higher values in the top layers. No statistically significant differences between the different layers (top, middle and bottom) of the dental plaque biofilms analysed in this study were found.

Additionally, the BT of the subjects (during the SA control cycle, in ascending order) was set in relation to the assessed caries risk parameter (Table 3). Neither DMFT, nor salivary flow rate, nor lactic acid formation rate showed any correlation with the BT of the negative control.

Discussion

Many in vitro and some in vivo models of obtaining biofilms have been described [1, 2, 4, 20, 24, 26, 28, 29]. While in vitro models (also known as laboratory models) with single species or multispecies biofilms only capture a small part of the oral flora, in vivo or in situ models reflect the natural intra-oral situation. In situ models with removable splints offer the opportunity to insert mounting specimens of any material and to obtain several (six to eight) biofilm samples in one jaw. In contrast, specimens fixed directly at the tooth [20] are often difficult to remove without disturbing the adhering biofilm. Moreover, the acceptance of our splint model by the subjects is very good, as oral hygiene measurements are possible without disturbing biofilm formation. While the present model tries to imitate interproximal plaque, the aim of another in vivo splint model was to imitate fissure plaque [28, 29].

Finally, differing treatments for each arm of the splint are possible as performed in the present investigation.

Growing biofilms were dipped into the solutions instead of rinsed with them for the following reasons:

1. Although the concentration of the preservatives SA and BA lie within the maximum allowance for products, rinsing with the solutions was avoided due to ethical reasons (direct contact with the mucosa, possibility of allergic reactions), as only the effect on biofilm formation was of interest.
2. To prove or control the reproducibility of the biofilm formation during the three test cycles, it was necessary and important to always run a control, as maintaining standardised conditions (diet, oral hygiene measures) for the subjects is often difficult to fulfil. Therefore, rinsing the whole mouth was excluded.

In addition, rinsing with or dipping into the solutions did not seem to differ greatly from one another, as control data of CHX and NaCl and reductions of CHX compared to NaCl were very similar to those obtained in a rinsing study (same CHX product and water as negative control) using the same splint design [5].

Rinsing would have both positive and negative influences on the efficacy of the substances. In the real situation, it must be assumed that the products are diluted by saliva or greatly increase transient flow rate, thus, reducing the exposure period to preservatives. Conversely, adhesive foods may extend the persistence of such material in the mouth, and contact more than twice a day due to increased consumption must also be assumed. Data from the USA show that individuals are consuming as much as 662 mg sodium benzoate from two cans of a carbonated beverage daily (660 ml) [21]. This corresponds exactly to the concentration of benzoate used in the present study (1,000 mg in 1 l), presuming a volume of 330 ml per can.

Table 2 Biofilm vitality (BV in %) and standard deviation (±SD) of all subjects ($n=24$) in the different layers

Test cycle	BV (%) bottom layer	BV (%) middle layer	BV (%) top layer
SA control	49.32 (SD 16.78)	60.76 (SD 12.63)	63.41 (SD 14.4)
SA	37.28 (SD 17.46)	43.78 (SD 12.84)	49.08 (SD 15.23)
BA control	56.57 (SD 18.96)	64.95 (SD 12.9)	67.02 (SD 14.38)
BA	39.76 (SD 17.96)	43.65 (SD 15.47)	49.37 (SD 16.01)
CHX control	45.97 (SD 20.05)	61.53 (SD 12.37)	63.93 (SD 13.5)
CHX	18.74 (SD 12.85)	14.69 (SD 9.27)	28.89 (SD 16.59)

Table 3 Caries risk data (DMFT, salivary flow rate, lactate rate) in relation to biofilm thickness (in ascending order of BT in the control cycle of SA)

Subject no.	BT (μm; of SA control)	DMFT	Salivary flow rate (ml/min)	lactic acid formation rate (1–9)
03	3.90	10	2.0	3
18	7.44	14	1.3	4
14	11.58	7	3.5	4
21	16.52	8	1.8	4
04	21.88	7	1.3	2
09	22.29	7	1.2	3
01	22.70	13	1.8	3
12	23.00	8	2.0	7
11	23.13	12	2.3	9
24	23.57	2	2.3	3
19	23.63	10	2.0	8
23	25.19	2	1.4	7
06	25.75	0	2.0	4
15	25.77	3	1.5	3
20	25.77	0	1.5	2
07	26.46	6	2.3	3
13	26.92	14	2.0	5
02	27.33	6	2.5	1
22	27.37	4	1.0	3
10	28.01	6	2.0	2
05	29.52	4	1.3	7
17	31.01	12	1.3	1
08	34.16	20	3.5	6
16	37.37	0	1.6	1

When considering the importance of plaque thickness in metabolic processes involved in dental diseases, it has to be pointed out that there are only a very limited number of investigations which have evaluated intra-oral plaque thickness. Many in vitro biofilm models (constant depth film fermentor) or in situ models with dentinal grooves only assess biofilms of a defined thickness. These models are ideally suited to building up (established) biofilms and then studying diffusion phenomena, but assessing the influence of antibacterial substances on biofilm development and thickness is not possible with such models.

Intra-oral plaque indices give only a rough impression of the amount of plaque. There are methods to measure plaque thickness by electronic probes [15] or by a laser scanning probe [27]. The examination of plaque thickness microscopically by means of CLSM is one alternative and has been used in many investigations [3–5, 16]. In this instance, biofilm thickness is defined to be the distance between the substratum and the peaks of the highest cell clusters [19].

The caries risk did not seem to have any influence on the data. These findings were also confirmed by two recent studies in which no differences in undisturbed 48-h biofilm between heavy and light plaque formers were observed [2, 29].

In contrast to a general expectation when looking at approximal plaque accumulation after 5 days, all thicknesses seem to be very small, but—irrespective of the explanation by the present individual study population—it should be mentioned that (1) the study model only tried to mimic approximal plaque and that (2) there are no in vivo data of approximal plaque thickness after 5 days to compare with. Interestingly, Thurnheer et al. [24] found similar thicknesses (30 μm after 64.5 h) in an in vitro biofilm model.

Additionally, as seen in this study, retarded diffusion phenomena are also possible in thin biofilms. However, it should not be forgotten that the test agents were continuously applied on the developing biofilms (nine times over a course of 5 days) and not on established ones where penetration plays a more important role. Moreover, SA and BA, with their very low molecular weights, should penetrate biofilm better than the substances examined in [24]. CLSM pictures recomposed of the scans showed frond-like structures and similar vitality patterns in all layers of the biofilms, which could be an indication for continuous and equal penetration.

Substances like sorbic acids and benzoates are not only used in foods and carbonated beverages. Benzoate for example occurs naturally in cranberries, prunes, cinnamon and ripe olives [7]. Recently, it was shown that cranberry juice presents multiple inhibitory activities, e.g. inhibition of the development of *Streptococcus mutans* biofilm in vitro, especially the glucan-mediated processes [13], which could be caused in part by the benzoic acid content.

Benzoic and sorbic acids have demonstrated a reduction of intracellular pH and inhibition of growth of *Escherichia coli* [22]. Benzoate has already been shown to affect oral microorganisms in a similar way to that of fluoride by reducing the acid tolerance of the oral flora causing cell death [11, 14, 17]. Especially when combined with fluoride, benzoate can inhibit growth in vitro and cause acid killing of bacteria such as *S. mutans* [6] as well as *S. sobrinus* in rodents and rats [9].

The effect of benzoate and sorbate on in situ oral biofilm was not as pronounced as the effect of the positive control CHX, the gold standard treatment against dental plaque and gingivitis [12], which, in addition to a lower antibacterial effect, could also be due to differences in biomass penetration [24]. While CHX is known to have surfactant properties, SA and BA were diluted in water. However, both preservatives showed significant reductions between 17% and 21% in BT and between 26% and 29% in BV compared to the negative control. It is known that external stress such as absence of nutrients or exposure to toxic substance can better be compensated when organisms are embedded in a biofilm in comparison with free-floating (planktonic) bacteria [18, 23].

Regarding the different layers of the biofilms, SA, BA and NaCl showed an identical vitality distribution with the

highest values at the top of the biofilm, which is in line with the knowledge that bacteria in deeper biofilm layers are rather metabolically inactive (dormant zones) [16, 23]. A constant reduction in all layers compared to NaCl points to a uniform effect of the regularly applied preservatives during biofilm growth. It is also an indication that the effect of any substance will be best when applied on thin developing biofilms and when no penetration through thick, established ones is necessary, which is still a considerable problem.

The splint design used in the study together with CLSM represents an excellent tool to study dental biofilm growth, which could—for the first time—prove an antibacterial effect of preservatives on in situ biofilm formation.

Based on our results, benzoic and sorbic acid significantly inhibited biofilm thickness and vitality, but were not as effective as chlorhexidine. Irregardless of the effects of CHX, the increased usage of preservatives and their availability in foods could be contributing to the decline in prevalence of dental caries.

Acknowledgements This investigation was financially supported by a grant from the Deutsche Forschungsgemeinschaft (DFG; Ar 341/3-1). The authors thank Marie Follo, Ph.D., Department of Hematology and Oncology, Core facility, University of Freiburg for her help in the image analysis and preparing the manuscript.

Declaration of Interests There are no conflicts of interest for any author.

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