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Effect of two antimicrobial agents on early in situ biofilm formation

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Abstract

Objectives: The aim of this observer-blind, controlled, three-cell cross-over study was to evaluate the influence of an amine fluoride/stannous fluoride (Meridol®, 250 ppm; ASF) and a chlorhexidine mouthrinse (CHX; Chlorhexamed forte®, 0.2%) compared with water on in situ biofilm growth.

Material and Methods: After a professional toothcleaning seven volunteers had to wear a special acrylic appliance, in which six specimens each were inserted to allow the build-up of intra-oral biofilms. The volunteers had to rinse twice daily for 1 min. with 10 ml of the allocated mouthrinse. After 48 h of wearing, the specimens with the adhering biofilms were removed from the splints and stained with two fluorescent dyes, which selectively stain vital bacteria green and dead bacteria red. Under the confocal laser scanning microscope biofilm thickness (BT) was evaluated. To examine bacterial vitality (BV%) the biofilms were scanned (1 µm sections) and digital images were made. An image analysis program was used to calculate the mean BV as well as the BV of the single sections. After a wash-out period of 14 days a new test cycle was started.

Results: The use of CHX and ASF resulted in a BT of 8.4 ± 4.4 µm and 15.7 ± 9.9 compared with 76.7 ± 29.4 µm using water. The mean vitality (in %) was reduced from 66.1 ± 20.4 to 23.3 ± 11.6 and 23.9 ± 12.4 using CHX and ASF, respectively. Both active solutions reduced BT and BV significantly compared with water ($p < 0.001$). Differences between the two active solutions were not significant ($p > 0.05$).

Conclusion: Both mouthrinses showed antibacterial and plaque-reducing properties against the in situ biofilm. The study design enables the examination of an undisturbed oral biofilm and for the first time shows the influence of antibacterial components applied under clinical conditions regarding biofilm formation.

Key words: amine fluoride/stannous fluoride; biofilm; chlorhexidine; CLSM; vital fluorescence technique

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The aim of controlling dental plaque is to prevent biofilm-associated diseases like caries and periodontitis. In this context, chemically active substances can be a valuable aid to mechanical plaque removal if manual measures are not performed long enough or on a regular daily basis.

Therefore, there is a big interest in using chemically active substances which can maintain, improve upon or even replace both preventive and therapeutic mechanical measures (ten Cate & Marsh 1994). While researchers are searching for active substances, which kill or at least inhibit the growth of causal organisms in classic infectious diseases, in oral biofilm-associated diseases active substances must not absolutely show bactericidal or bacteriostatic qualities as a primary characteristic. Some substances (e.g. furanones) have antipathogenic properties which are able to paralyse the command language [denoted quorum sensing (QS)] without affecting any vital function of the bacterium and thus will not interfere with its growth (Wu et al. 2004, Hentzer & Givskov 2004).

Based on the fact that the damaging microorganisms are present on the tooth in a relatively easily accessible biofilm, different active $x + y$substances as well as various methods can interfere in several ways with the mechanism of dental biofilm formation: (1) they can prevent the constitution of a biofilm; (2) they can destroy the existing biofilm; (3) they can prevent growth processes in the biofilm or (4) they can destroy individual microorganisms in the biofilm (Wilson & Pratten 1999). To date, most agents used intra-orally are antibacterial agents, which are aimed at inhibiting biofilm growth or the production of harmful bacterial metabolites although the first two approaches do not necessarily have such properties.

There are numerous studies about the effect of antimicrobial agents on oral microorganisms. However, in former times methods were used in which the examined bacteria were not available as a biofilm (Gjermo et al. 1970, Roberts

& Addy 1981). The formation of a biofilm produces a protected, encased community of cells in which environmental influences are vastly reduced (Anwar et al. 1992). Therefore, the biofilm may act as a barrier to later colonization by exogenous microorganisms (often also pathogens), as well as to chemical agents. A primary reason for the latter effect is suspected to be the changing of the cells at the surface of the biofilms or the inability of the inhibitor to penetrate the biofilm as well (ten Cate & Marsh 1994). However, there are indications that the diffusion of dissolved materials in a normally restrictive gel matrix is much less inhibited than has until now been assumed (Flemming 1994).

The laboratory examination of antibacterial substances by means of biofilm-based models (in which one can only imitate the intra-oral situation), is an important step in selecting the agent which should be used in clinical studies. In such studies the chemicals chlorhexidine (CHX), amine fluoride/stannous fluoride, triclosan and phenolic agents were shown to be effective. They inhibited the development of the biofilm with respect to its maturation as well as to the metabolism of the exposed bacteria (Millward & Wilson 1989, Pratten et al. 1998, Shapiro et al. 2002).

The clinical examination of in situ biofilms and their reaction to antibacterial substances is expensive, but indeed anti-plaque activity can only be examined in such studies. For example, it is believed that the turnover rate of saliva itself or the proteins present in saliva reduces the activity of different substances (Hjelford et al. 1973, Addy & Wright 1978, Roberts & Addy 1981). The first studies examining the influence of antibacterial substances on undamaged biofilms through the use of intra-oral devices were enhanced by the application of confocal laser scanning microscopy (CLSM) combined with a particular staining technique (vital-fluorescence technology) (Auschill et al. 2001, 2002, 2004, Arweiler et al. 2004, Zaura-Arite et al. 2001).

The current observer-blind study with a cross-over design used the above-mentioned techniques to examine the *in vivo* effects of an amine fluoride/stannous fluoride-containing mouthwash (Meridol®, GABA International, Basel, Switzerland), as well as a 0.2% CHX solution (Chlorhexamed Forte®, GlaxoSmithKline, Bühl, Germany) on the thickness of developing intra-oral biofilms and on the vitality of the bacteria therein. An in situ splint system was used to build in vivo biofilms imitating appoximal plaque.

Material and Methods

Surveys

Seven volunteers from 25 to 29 years of age (average age 26.9) were selected. Each of them signed an informed consent form in order to participate in this study. The general anamnesis for each subject was unremarkable. They were in healthy dental conditions. Exclusion criteria were signs of destructive periodontitis or inflammatory symptoms, as well as the use of antibacterial mouthwashes and antibiotics in the last half-year, because of the possible disturbing effect on plaque formation.

The presence of *Streptococcus mutans* and *Lactobacillus* was determined with the help of a bacterial test (CRT bacteria, Vivadent, Ellwangen, Germany). Additionally, the Decayed, Missing, Filled Surfaces (DMFS) value of the subjects was recorded. Three subjects showed low caries prevalence (*S. mutans* value 0; DMFS 2.7 ± 1) and four subjects high caries prevalence (*S. mutans* value 1–3; DMFS 11.5 ± 4.8).

Study design

After a professional toothcleaning, each participant had to wear an individual, ready-made, special acrylic splint on the upper jaw (Auschill et al. – in situ device 2004). As carry plates, six sterile glass slabs (float glass, Menzel, Braunschweig, Germany) were inserted into depressions with sticky wax towards the natural teeth ($n = 6$; localizations A–F; Fig. 1). The specimens had a diameter of 3 mm and were each already industrially polished in the same manner (4000 grid).

The surface to be examined was turned towards the tooth, but was not in direct contact with it, so that the approximal spaces of the teeth remained free. This allowed the biofilm to be washed with saliva and nutrients but prevented the possibility of disturbing the biofilm through contact with the tongue or cheek. The goal of this experiment was to imitate an approximal plaque.

The appliances were carried by the subjects day and night for 48 h continuously, and were allowed to be taken out only during meals or for cleaning of the teeth.

They were stored in a physiological sterile saline solution. The only additional mouth hygiene aids allowed were brushing of teeth only with water and the use of fluoride-free dental floss.

Test products

The antibacterial solutions used were either a non-alcoholic, amine fluoride/stannous fluoride -containing solution (ASF; Meridol®; GABA International) with the effective components 0.0125%

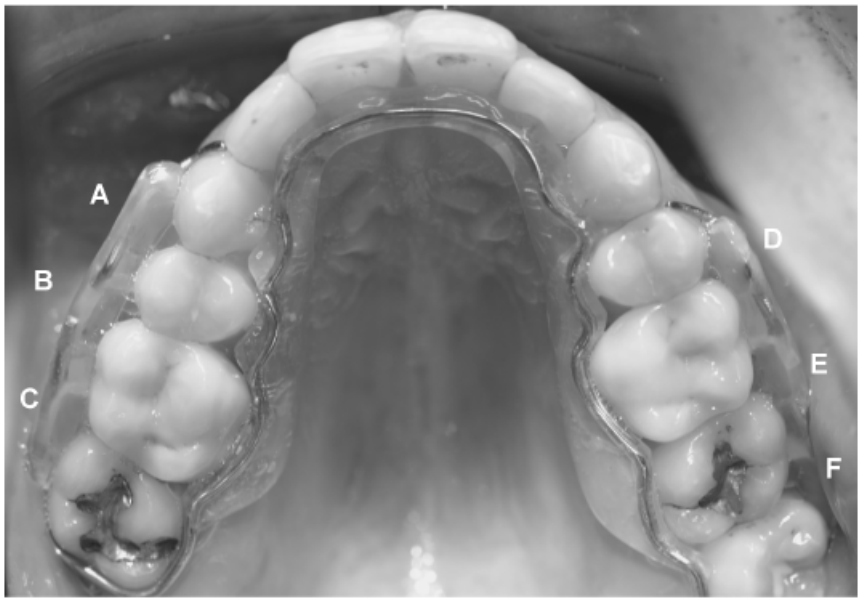

Fig. 1. Appliance in situ (locations A–F).

amine fluoride and 0.0125% stannous fluoride (= 250 p.p.m. fluorides), or a 0.2% chlorhexidinedigluconate solution with 7.0 vol.% ethanol (CHX; Chlorhexamed forte®, GlaxoSmithKline). Water served as a negative control. The three solutions were assigned for the respective test weeks by randomization (balances cross-over design). The subjects had to rinse twice daily – once in the morning and once in the evening – for 1 min. each with 10 ml of the allocated mouthwash while wearing the splint. Therefore, during the 48 h that the splint was worn, the subjects rinsed for a total of four times. After a washout period of 14 days, the next test was carried out, so that each subject had applied the three test solutions in a random sequence.

Vital fluorescent staining

After being worn for 48 h, the plaque-covered specimens were removed from the splint and carefully washed in physiological sterile saline at room temperature. The bacteria were then directly stained with a fluorescent vital stain (as described in Arweiler et al. 2004). The adhering biofilm was stained with fluorescein diacetate (FDA) and ethidiumbromide (EB) by applying a solution containing the two fluorescent dyes, which selectively stain the metabolism-active bacteria (green) and the dead bacteria (red) visibly. FDA does not fluoresce on its own. It is however, membrane-permeable and is enzymatically reduced in living cells to the green fluorescent fluorescein, which is not able to leave intact cells. Since the formation of fluorescein is tied to the active metabolism of the cell, only the living cells stain green. EB serves as a counterstaining. It can only enter bacteria where the cell membrane is no longer intact, where it then fluoresces red upon binding their nucleic acids. In this way we are able to distinguish at the single cell level between dead cells and those with an active metabolism.

CLSM

After a 3 min staining, the specimens were put into a special object-chamber (Lab-Tek II Chambered Coverglass, Nalge Nunc International, Naperville, IL, USA) and immediately examined under the CLSM, (LSM 410, Carl Zeiss, Jena, Germany), that was equipped with an argon laser which excited the sample at 488 nm, and with a helium–neon laser which excited the sample at 543 nm. Fluorescence emission was measured at 510–525 nm (for fluorescein) and 590–610 nm (for EB). Confocal images were generated with the help of a water-immersion objective (C-Apochromat × 40/1,2 WS, Carl Zeiss). The upper and lower boundaries of the biofilm probe were assumed to be where no more fluorescence was detectable. The biofilm was examined at several locations in order to find the thickest point of the biofilm. At this position, a series of images was taken through the biofilm. In order to avoid bacterial overlaps, slices, which were optically 1 µm thick were taken at intervals of 2 µm. The maximum biofilm thickness (BT) in microns could therefore be measured by doubling the number of optical slices.

Evaluation of biofilm vitality

The digitized serial pictures were examined by means of an automated image analysis program (KS300, Carl Zeiss, Göttingen, Germany), which examined the per cent vitality of whole biofilm as well as of the single-biofilm sections (BV%). The program appraises green and red pixels (metabolically active and dead bacteria, respectively) and is capable of assigning a bacterial vitality (BV) (in %) to each biofilm in that the green areas are determined as the percent-share of all stained bacteria.

Statistical analysis

The statistical analysis took place by means of SPSS 11.0. The calculation average of the plaque thickness was calculated for each mouthrinse from all subjects, resulting from the six locations.

The data-series of the BV on each of the specimens was subdivided into three equally thick layers, layer 1: inner layer, layer 2: middle layer, layer 3: outer layer, so that the vitality data of the different products could be compared by layers.

The data rows for BT and BV were examined with the Kolmogorow–Smirnow Test on normal distribution and by means of ANOVA for significant differences. Because the distribution was normal and significant differences with respect to the test-products were apparent ($p < 0.05$), the differences between the individual groups were compared by means of a paired *t*-test for significance.

Results

All seven subjects went through each of the three test sequences of the study. No specimen got lost so every probe could be evaluated in the study.

After 48 h of application, the thickness (BT) as well as the vitality of the plaque (BV) was significantly reduced with the two antibacterial mouthrinses when compared with the negative control ($p < 0.001$).

With regards to BT, the ASF-solution achieved a reduction of 80% and CHX-solution a reduction of 89% as compared with water (Table 1). The difference between the two active solutions was not significant ($p > 0.05$).

The mean BV (in %) was reduced from 66.1 ± 20.4 to 23.3 ± 11.6 and 23.9 ± 12.4 using CHX and ASF, respectively. This corresponds to a 64% reduction of both solutions compared with the negative control. The vitality distribution in the three layers (1: inner layer, 2: middle layer, 3: outer layer) is represented in Table 2. Comparing the different layers within each test product no significant differences were found ($p > 0.05$ by ANOVA).

The caries prevalence of the individual subjects had no influence on either BT or BV ($p > 0.05$).

Table 1. Mean biofilm thickness (BT in µm)

Volunteer	CHX	ASF	Water
High caries prevalence			
1	6.0	12.4	88.0
2	14.4	20.0	24.4
3	4.0	7.7	74.3
4	15.0	6.4	120.4
Low caries prevalence			
5	7.0	13.3	80.3
6	6.0	14.3	88.7
7	6.5	35.7	61.0
Mean BT ± SD	8.4 ± 4.4	15.7 ± 9.9	76.7 ± 29.4

CHX, chlorhexidine; ASF, amine fluoride/stannous fluoride; SD, standard deviation.

Table 2. Mean biofilm vitality (BV in %; ± standard deviation) in the different layers, 1: inner, 2: middle, 3: outer layer

Slice	CHX	ASF	Water
Layer 1	25.31 ± 25.49	24.53 ± 23.06	68.96 ± 27.46
Layer 2	23.64 ± 22.90	27.42 ± 23.76	71.16 ± 21.90
Layer 3	29.29 ± 27.12	30.71 ± 22.56	66.95 ± 21.71

CHX, Chlorhexamed forte; ASF, amine fluoride/stannous fluoride.

In Fig. 2, serial pictures of the two representative biofilms show the influence of water and CHX.

Discussion

The effect of ASF– and CHX mouthrinses on oral microorganisms and plaque formation has already been demonstrated in numerous other studies (Addy 1986, Netuschil et al. 1995, Arweiler et al. 2001a, Shapiro et al. 2002). However, these other clinical studies either observed the clinical effect on gingiva or plaque indices or the plaque was scraped off the teeth for further examination. There are laboratory studies which consider the presence of test organisms in the form of a biofilm (Pratten et al. 1998, Wilson & Pratten 1999). Other studies used a microcosm of plaque which is more similar to the clinical situation than those studies in which only single species were used (Sissons et al. 1996). In these experiments the interactions between bacteria of the same type and other species, which play such a big role in the formation and ripening of dental plaque were studied (Pratten et al. 1998). Such in vitro attempts are a more practical and affordable method with which one can assess potentially active substances. However, they are in many respects insufficient in imitating the clinical situation. Influences in the oral cavity such as, for example the turnover rate of saliva (Goodson 1989), or the ability of antibacterial substances to adhere to the pellicle of the tooth or to the surface of soft tissues in order to achieve their effects (Cummins & Creeth 1992), cannot be modelled in such experiments. In order to better understand the metabolic process or the clinical effects of agents that take place within the biofilm it is necessary to choose an examination method in which the biofilms grow directly in the oral cavity and in which their three-dimensional structure is not manipulated with the examination. Through the use of CLSM, biofilms and their structure can not only be analysed in vitro but, with the help of the intra-oral splint, in situ as well (Auschill et al. 2001, 2002, 2004, Zaura-Arite et al. 2001, Arweiler et al. 2004). Additionally, the staining of the bacteria with specific dyes enabled statements about bacterial viability in these studies.

The antibacterially active substance CHX is the most representative of the chemoprophylactic agents, and is widely considered to be the most effective agent against plaque and gingivitis (Rölla & Melsen 1975, Addy 1986, Mandel 1988, Jones 1997). All ''new'' antibacterial products will therefore have to be compared with the ''gold standard'' CHX (Jones 1997). In the present study, the bactericidal- and plaque-inhibiting effect of CHX could be confirmed. In a previously published study a biofilm, consisting of five bacterial species, was exposed to a 0.2% CHX solution for 5 min. and only a minimal effect could be observed on the biofilm (Pratten et al. 1998). Clear reductions in bacterial viability were reached only after a contact time of 60 min. in that experiment, while significant effects were observed in the present study after a rinsing time of only 1 min. These results clarify the effectiveness of intra-oral application of antimicrobial products and show why intra-oral tests are of such importance. While Dibdin (1984) and Melsen et al. (1983), describe the biofilm as a diffusion barrier, today it is believed that, although the diffusion of dissolved materials are inhibited in the gel–matrix of biofilms as compared with in free water, they are inhibited to a far lesser extent than has been until now assumed. For smaller molecules like fluorescein the diffusion–coefficient in the biofilm hardly lies below that of free water (Flemming 1994), so that antibacterially active substances can work in principle not only on planktonic but also on bacteria in the biofilm. However, it should also be noted that the antibacterial substances used in this study were already used at an early phase of biofilm formation.

In a very similar study design Zaura-Arite et al. (2001) examined the effect of clorhexidine on 24 and 48 h old, in situ-grown biofilms. As a carrier substance they used dentin slices, which were equipped with grooves. A CHX solution (0.2%) was applied extra-orally for 1 min., so that these data are only marginally comparable with the present study in which the mouthwashes were applied in a clinical situation twice daily.

Over the last years, in clinical short-term studies (Netuschil et al. 1995, Arweiler et al. 2001a, b), as well as in long-term studies (Banoczy et al. 1989, Brecx et al. 1990, 1993), the combination of the organic amine fluoride (''Olaflur'', GABA International, Basel, Switzerland) with the anorganic stannous fluoride was examined as an antibacterial and consequently plaque inhibiting substance. The results were, without exception, positive. The antibacterial effects derive from the individual components, since the complex is stable in water but at the place of action, stannous ions with antimicrobial effects are set free. Additionally, it was shown that amine fluoride (Dolan et al. 1974, Schneider & Mühlemann 1974) as well as the combination ASF, inhibited both the metabolic activity of different oral bacteria as well as their acid production (Bley & Gülzow 1991, Gehring 1991). Furthermore, it could be shown that a mouthwash with this combination of active substances had a high substantivity as well as sufficient antibacterial activity in the oral cavity (Netuschil et al. 1997, Arweiler et al. 2001a, b).

In the present study, the determined effectiveness of ASF and CHX with respect to BV and plaque formation agrees with the data of numerous other studies (Brecx et al. 1990, Netuschil et al. 1995, Arweiler et al. 2001b, Shapiro et al. 2002) whereby in these studies the biofilms were either grown in a laboratory model or were obtained through scraping of the tooth surface, so that the study design is only conditionally comparable. Somewhat lower vitality values were obtained in the present study, but because these were lower for the placebo as well as for the anti-bacterial solutions, the relationship of the mouthwashes to each other is very similar. In the studies to date, the plaque quantity could only be compared through the use of the plaque index. The combination of the vital fluorescence technique together

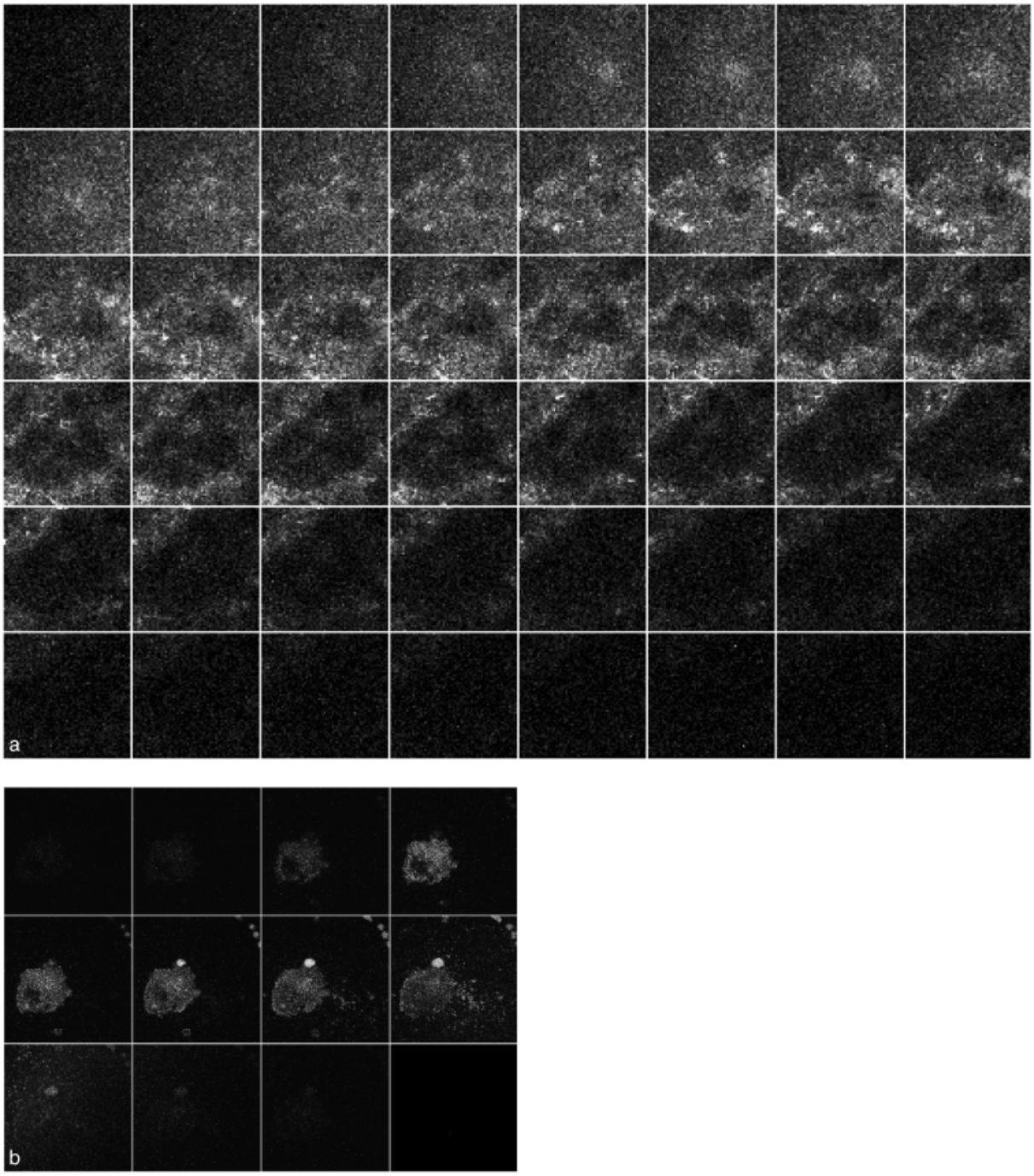

Fig. 2. Gallery of the sectional images through the biofilm under the influence of (a) water and (b) Chlorhexamed forte® (from the left above to the right below: slices through the biofilm from top of the biofilm to the bottom).

with CLSM technology allows the drawing of conclusions about the effect of mouthwashes on plaque thickness. Although our data can only be conditionally compared with the reduction of plaque index through antibacterial mouthwashes, they lie within a similar range of magnitude (Moran et al. 1992, 1994). Interestingly, our data confirmed the results of earlier studies, in which the ASF solution seems to influence the vitality of plaque just as strongly as CHX, while the effect on plaque thickness is somewhat less pronounced compared with that of CHX (Brecx et al. 1990, Arweiler et al. 2001b). Additionally, the data show a constant effect on the different biofilm layers when used during biofilm formation.

One can conclude from the results of the study that both mouthwashes show a significant antibacterial- and plaque-reducing effect on biofilms in situ. The present study design made possible for the first time the examination of biofilms grown directly in the oral cavity under the influence of antibacterial mouthwash, which was applied under real clinical conditions.

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