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# In vitro Effect of Chlorhexidine Mouth Rinses on Polyspecies Biofilms

Key words: chlorhexidine, biofilms, antimicrobial effect, mouth rinses

**Summary** The aim of this study was to use the Zurich polyspecies biofilm model to compare the antimicrobial effects of chlorhexidine mouth rinses available on the Swiss market. As positive and negative controls, aqueous 0.15% CHX solution and water were used, respectively. In addition, Listerine® without CHX was tested.

Biofilms in batch culture were grown in 24-well polystyrene tissue culture plates on hydroxyapatite discs in 70% mixed (1:1 diluted) unstimulated saliva and 30% complex culture medium. During the 64.5-hour culturing period, the biofilms were exposed to the test solutions for 1 minute twice a day on two subsequent days. Thereafter, the biofilms were dip-washed 3 times in physiological NaCl. Following the last exposure, the incubation of biofilms was continued for another 16 h. They were then harvested at 64.5 h. The dispersed

biofilms were plated on 2 agar media. After incubation, colonies (CFU) were counted.

All solutions containing CHX as well as Listerine® significantly reduced the number of microorganisms in biofilms. According to their efficacy, the mouth rinses were classified into 2 groups. The two Curasept ADS solutions, Parodontosan, and the Listerine® mouth rinse reduced the number of total CFU by 3 log<sub>10</sub> steps. This seems sufficient for a long-lasting prophylactic application. The two PlakOut® mouth rinses and the CHX control fell into the other group, where the number of CFU was reduced by 7 log<sub>10</sub> steps. These mouth rinses are predestined for short-term therapeutic use. However, reversible side effects must be taken into account. It has thus far not been possible to formulate CHX products with effective ADS (Anti Discoloration System) additives without reducing antimicrobial activity.

## Introduction

For over 40 years, chlorhexidine (CHX) has been known as an excellent compound for preventing dental plaque and gingival inflammation (LÖE & SCHIOTT 1970, FLOTRA ET AL. 1972). However, two pronounced side effects – superficial staining of the teeth and altered taste perception – were recognized almost immediately (SCHIOTT ET AL. 1970) and have hindered its unrestricted use in daily oral hygiene, although they are reversible after discontinuation of CHX treatment. These phenomena were observed in many studies, and resulted in two general

consequences. First: CHX rinses were limited to short-term applications in which the benefits clearly outweighed the mild but unpleasant side effects for patients. Second: Industry and research sought and still seek means and methods, for instance, adjusting the concentration and/or including additives in the formula, by which the side effects can be eliminated without reducing the antimicrobial effect (ADDY ET AL. 1989, 1991, 2005). This is an extremely difficult task because the cationic nature of the CHX molecule provides its substantivity and the associated sustained antimicrobial effect. In addition, the high reactivity of the molecule with anionic compounds

rapidly negates the antimicrobial activity, which makes antimicrobial active mixed formulas difficult if not impossible (JONES 1997).

The success of such efforts to consider all 4 critical parameters – plaque reduction, staining, altered taste sensation, and anti-inflammatory properties – can only be examined in clinical studies. Trials that only examine prevention of staining and altered taste perception *in vivo* (BERNARDI ET AL. 2004) or staining *in situ* (JONES 1997) are of limited value without the accompanying microbiological experiments.

Microbiological studies with CHX and products containing CHX or other antimicrobial substances are similarly limited if the minimum inhibitory concentrations are determined using planktonic bacterial suspensions (HOPE & WILSON 2004). A crucial breakthrough was achieved only when polyspecies biofilms were used to determine the effect of antimicrobial substances (KINNIMENT ET AL. 1996). Especially when using the Zurich biofilm model (GUGGENHEIM ET AL. 2001, SHAPIRO ET AL. 2002), a surprising degree of agreement was obtained between the optimal active-ingredient concentration for biofilms and the values found in clinical trials. Nevertheless, even biofilm models are not appropriate for determining 3 of the clinically measurable parameters mentioned above. They are, however, considerably less labor-intensive and enable biofilms to be exposed to test products in practice-relevant numbers, durations, and concentrations. Biofilm studies are therefore a tried and true selection procedure to test the effect and suitability of new antimicrobial products for therapeutic or prophylactic use in the oral cavity. Furthermore, they also allow cross-sectional comparison of the efficacy of products already on the market (SHAPIRO ET AL. 2002).

In the present study, products containing CHX currently commercially available in Switzerland were tested with respect

to the following questions: Are mouthwashes with anti-staining additives (Anti Discoloration System; ADS) suitable for both short-term therapeutic and long-term prophylactic use? Do such additives alter the antibacterial effect of CHX? Do differences exist in the efficacy of CHX mouthwashes containing ASD?

## Materials and Methods

### Mouth rinses and controls

The aqueous mouth rinses and the positive and negative controls used in this study are presented in Table I. Besides the rinses containing CHX, Listerine® was also included, because its manufacturer recommends it as a non-tooth-staining alternative to CHX mouthwashes. All products were purchased at a retail outlet.

### Growing the biofilms

The test methods employed have been published in detail elsewhere (GUGGENHEIM ET AL. 2001, SHAPIRO ET AL. 2002), so that a synopsis will suffice for understanding the current study.

Polyspecies biofilms containing the organisms *Actinomyces naeslundii* OMZ 745, *Veillonella dispar* OMZ 493, *Fusobacterium nucleatum* OMZ 598, *Streptococcus mutans* OMZ 918, *Streptococcus oralis* OMZ 607 and *Candida albicans* OMZ 110 were grown. To obtain a salivary pellicle on hydroxyapatite discs (HA discs; Ø 10.6 mm), each disk was placed in a well of a 24-well polystyrene cell culture plate and covered with 1.6 ml unstimulated mixed saliva. The medium, universal fluid medium (30% + 70% saliva) which was adjusted to pH 7.2 with 67 mmol Sørensen buffer, contained 0.3% carbohydrate. During the first 16.5 h of culturing, glucose was used; thereafter, from 16.5 h to 64.5 h, a 1:1 (w/w) mixture of glucose and sucrose was used. At time 0, the individual wells were inoculated with 200 µl

Tab. I Composition of the tested rinse solutions

Product, trade name	Manufacturer/sales	Active ingredient(s)	Concentration	Other additives
1. PlakOut®, rinse solution	KerrHawe SA, CH-6934 Bioggio***	CHX digluconate	0.1%	flavoring, dye: E127, ethanol 8% v/v, Exciapiens ad Solutionem
2. PlakOut®, Liquid	KerrHawe SA, CH-6934 Bioggio***	CHX digluconate	0.2%*	ethanol 45 vol.%, flavoring, Exciapiens ad Solutionem v**
3. Curasept ADS 212	Curaden Health-Care s.r.l. Saronno (VA), Italy	CHX digluconate	0.12%	Xylitol, propylene glycol, PEG 40, hyd. castor oil, ascorbic acid, Poloxamer 407, sodium metabisulfite sodium citrate, aroma Cl.42090
4. Curasept ADS 220	Curaden Health-Care s.r.l. Saronno (VA), Italy	CHX digluconate	0.2%	Xylitol, propylene glycol, PEG 40, hyd. castor oil, ascorbic acid, Poloxamer 407, sodium metabisulfite sodium citrate, aroma Cl.42090
5. Parodontosan rinse	Tentan AG, CH-4433, Ramlinsburg***	CHX digluconate	0.05%	Per ml: myrrh tincture 1.9 mg, sage oil 0.5 mg, peppermint oil 0.08 mg, ethanol 15 vol.%, xylitol and other adjuvants
6. Listerine®	Johnson & Johnson Maidenhead UK SL6 3UG	Thymol Menthol Eucalyptol	0.064% 0.042% 0.060%	Sorbitol, 1-propanol, ethanol 21%, methylsalicylate, Poloxamer 407, benzoic acid Cl I47005, sodium fluoride 100 ppm, and others
7. Chlorhexidine	Sigma-Aldrich Chemie GmbH D-Steinheim 88552*	CHX digluconate	0.15%	None (positive control)
8. Water		none		None (negative control)
* in the selected dilution	** in the undiluted original solution	*** sales		

of a mixed microorganism suspension in physiological NaCl solution, which consisted of equal volumes of each species (OD  $1.0 \pm 0.05$ ). The biofilm cultures were anaerobically incubated at 37 °C. The medium was changed at 16.5 and 40.5 h, after an exposure to the test solutions.

**Determining the antimicrobial effect of the test solutions**

The biofilm-covered HA discs were taken out of culture, placed in another culture plate, immersed in the test solution for 1 min. and shaken lightly, and finally washed by dipping 3 times in 2 ml of physiological saline solution. The biofilms were exposed to test solutions after 16.5, 24.5, 40.5, and 48.5 h. Following the final treatment, incubation of the biofilms was continued; after 64.5 h, they were harvested by vortexing vigorously in 1 ml of saline for 2 min. The harvested, suspended biofilms were treated with ultrasound for 5 seconds, pre-diluted, and using a spiral plater were plated onto (a) Columbia Blood Agar Base (Difco Laboratories, Inc., Detroit, MI, USA) with 5% (v/v) human blood and (b) Mitis-Salivarius Agar (Difco Laboratories, Inc., Detroit, MI, USA). After 72 h of anaerobic incubation, the colony forming units (CFU) were counted under a stereomicroscope. The total CFU were determined on Columbia blood agar, and the *S. mutans* and *S. oralis* CFU were counted on Mitis-Salivarius agar. All experiments were repeated 3 times in triplicate (N=9).

**Statistical analysis**

The log<sub>10</sub>-transformed CFU values obtained on the two nutrient media were statistically analyzed to determine the effect

of the different test solutions on the biofilm microbes. The distribution of the values (total CFU, *S. mutans* CFU, *S. oralis* CFU) is depicted as box-plots (CHATFIELD 1983). The differences in the antimicrobial effect of the test products Curasept ADS 212 and 220, Parodontosan, and Listerine® were examined for statistical significance using analysis of variance (ANOVA) and Scheffe tests in the StatView II software program (Abacus Concepts, Inc., Berkley, Calif., USA).

**Results**

Figure 1 depicts a summary of the results. The graph shows that the tested solutions can be assigned to three distinct groups according to their antimicrobial effect. Group 1 contains just the negative water control; group 2 includes the Curasept ADS 212, Curasept ADS 220, Parodontosan, and the Listerine® mouth rinses; group 3 contains the PlakOut® rinse, PlakOut® liquid, and the positive CHX control. As clearly shown by the box-plots (even without statistical analysis), great differences exist between the 3 groups.

Group 3 shows remarkable results: with only 2 daily one-minute exposures to CHX solutions at concentrations of 0.1 to 0.2%, biofilm formation was reduced by 7 log<sub>10</sub> steps in two days, which even exceeds the microbe reduction demanded by sterilization procedures. However, compared to the water control, the mouth rinses in group 2 also demonstrated a marked reduction in total microbes of approximately 3 log<sub>10</sub> steps. The differences between the mouthwashes within group 2 were not as pronounced. The ANOVA showed no significant differences between the mouth rinses in terms of total CFU of the micro-

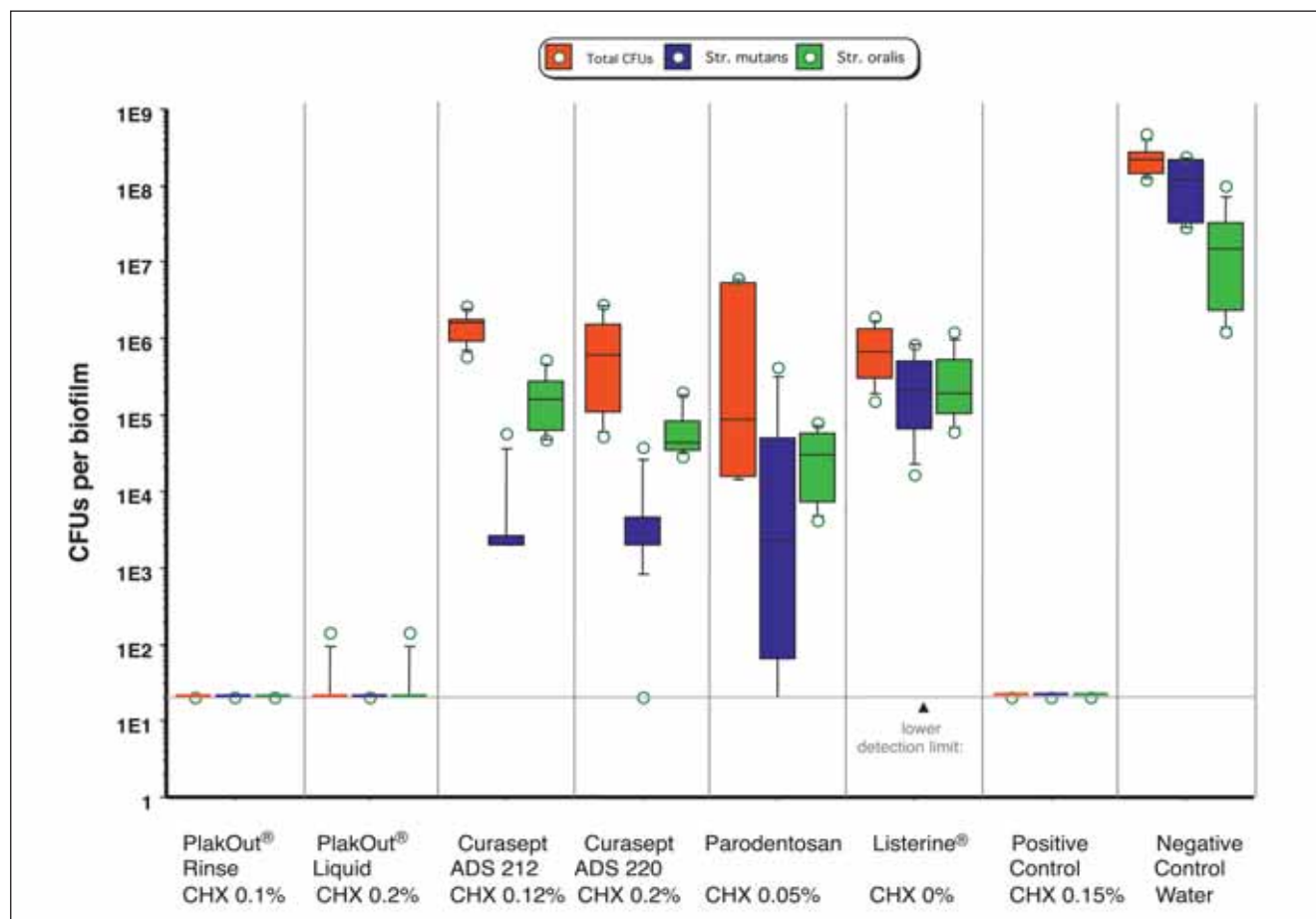


Fig. 1 Box-plot depiction of the inhibition of microbiota using various mouthwashes in the biofilm model (N=9).

organisms, although regarding *S. mutans*, these differences were statistically significant (ANOVA  $P < 0.001$ , Scheffe test  $P < 0.05$  to  $P < 0.01$ ). In addition, significant differences were found in the CFU of *S. oralis* using Listerine® vs. Curasept ADS 220 or Parodontosan (Scheffe test:  $P < 0.05$  and  $P < 0.001$ , resp.). Furthermore, a significant difference was observed between Curasept ADS 212 and Parodontosan (Scheffe test:  $P < 0.01$ ).

## Discussion

The present results allow definite answers to the questions posed at the outset. Although all mouth rinses containing CHX reduced the biofilm population – even with exposure limited to 4 one-minute applications –, they did so to greatly varying extents. All CHX mouth rinses and Listerine® as well (without CHX) that were formulated to reduce or prevent tooth staining demonstrated a highly significantly lower antimicrobial activity than the two PlakOut® rinses and the CHX control. This clearly defines the application areas of these two product groups. All rinses in group 2 seem to meet the requirement for use as long-term prophylactic mouth rinses, as they also prevent tooth staining. For the numerous clinical situations in which extant, bacterially caused diseases of the dental hard or soft tissues are the primary problem, the mouth rinses in group 3 are much better suited for short-term therapeutic use. Under such conditions, the well-known side effects (staining, altered taste sensation) must simply be accepted.

The different effective concentrations of the various CHX rinses are noteworthy. For the solutions in group 3, the present biofilm model does not detect differences beyond a concentration of 0.1%, since the maximum efficacy is already attained at that concentration. In group 2, the total CFU did not differ significantly between the 0.12 and 0.2% Curasept ADS rinses. This can only be explained by the limited compatibility of the stain-inhibiting additives with the antimicrobial effect of CHX. Many earlier studies also observed this phenomenon (ADDY ET AL. 1991 2005, SHAPIRO ET AL. 2002, SLOTS 2002, ETC.). The manufacturer of Parodontosan has developed an interesting strategy for preventing staining. The minimal amounts of additives (etheric oils, ethanol, xylitol, etc.) do not seem to interfere with the very low CHX concentration (0.05%), and Parodontosan can still be assigned to group 2. The wide scatter can be explained as follows: After the first exposure to CHX, the number of surviving microorganisms in the biofilm was more widely scattered due to the low concentration of CHX. This scatter was further amplified in the subsequent CHX treatments.

Polyspecies biofilm models have been used successfully for over 10 years to test and compare the effect of antimicrobial substances for use in the oral cavity (Review: TEN CATE 2006). The determination of the minimum inhibitory concentration using planktonic cultures has become obsolete, because the values found in that way differ by a factor of up to 1000 from clinically effective concentrations. The reasons are simple. Biofilms can be exposed to test substances for short periods of time and at short intervals which correspond to daily hygiene habits. Their diffusion properties are very similar to those of dental plaque and they are more resistant to antimicrobial substances (GILBERT ET AL. 1997), which is explained by different gene expression and therefore the presence of various phenotypes in the biofilms, in contrast to the situation with planktonic microorganisms. Two models have primarily been used successfully to date: the Zurich model used here (GUGGENHEIM ET AL. 2001, SHAPIRO ET AL. 2002) and the constant-depth film

fermenter described elsewhere (KINNIMENT ET AL. 1996, PRATTEN ET AL. 1998). In the latter, biofilms are grown on plugs located in sample pans which are inserted in a rotating turntable (laboratory fermenter) submerged in nutrient solution. A constant biofilm thickness is achieved by two rotating scrapers or knives set at the desired distance. The biofilm fermenter is inoculated with a continual influx of mixed culture from a second fermenter, which supplies the biofilms growing on the plugs with medium (variable flow rate). Through a periodic and temporally limited influx of test solutions at the desired concentration, their antimicrobial effect can be tested. However, this method has considerable disadvantages: it is very labor-intensive and can only be used for one test substance and one concentration at a time. The complete elimination from the medium requires time, so that a clinically relevant, very short exposure to the test solution is impossible. The advantage of this method is that by adjusting the flow rate, constant, selectable shear forces can be brought to bear on the biofilms. The Zurich biofilm model does not have these disadvantages: it is not labor-intensive, and per experiment, 8 procedures with 3 repetitions each can be tested. Admittedly, constant shear forces are absent. The biofilms are intermittently exposed to high shear forces during the frequently repeated dip-washing, which involves moving between gas and liquid phases.

The major advantage of this model is its excellent reproducibility. For instance, the antibacterial effect obtained 9 years ago with Listerine® (SHAPIRO ET AL. 2002) was almost identical to that found in the present trials under clinically relevant conditions.

How important are the current results for the clinical application of CHX mouth rinses? The extraordinarily strong clinical effect of both well-formulated and pure CHX solutions at concentrations from 0.1% to 0.2% without anti-discoloration systems (ADS) is known and was confirmed by these biofilm experiments. Reversible tooth discoloration and altered taste sensations must simply be accepted, and they are the reason that these mouth rinses are only suitable for short-term therapeutic use. Attempts to prevent discoloration by ADS additives or reducing the CHX concentration but still maintain the antimicrobial effect must be considered as having failed, at least in regard to bacterial reduction in biofilms. As it could be expected, there was good agreement between the results of the present biofilm experiments and a clinical plaque study by ARWEILER ET AL. 2006.

As the results of group 2 show, the antimicrobial effect of CHX products compared to group 3 was 10,000 times ( $4 \log^{10}$  steps) weaker, but this certainly suffices for long-term prophylactic use. However, Listerine® – which is CHX-free – is equally effective as the products containing CHX, but it should be pointed out that the latter are particularly effective against *S. mutans* (Fig. 1), which may be advantageous in caries prophylaxis.

Finally, it must again be mentioned that *in vitro* biofilm trials allow very exact estimates of the *in vivo* antimicrobial effect. Other parameters (staining, taste alterations, inflammation inhibition, acceptance, etc.) that are decisive for a product's suitability in practice can still only be examined in clinical studies.

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## Résumé

Le but de cette étude était de comparer les effets antimicrobiens de bains de bouche à base de chlorhexidine (CHX) disponibles sur le marché suisse en utilisant le modèle zurichois du biofilm polymicrobien. Une solution aqueuse contenant 0,15% de CHX a été utilisée comme contrôle positif et de l'eau constituait le contrôle négatif. De plus, la Listerine® sans CHX a été testée.

Les biofilms ont été mis en culture sur des plaques à 24 puits sur des disques d'hydroxylapatite. Pour la culture 70% de salive non stimulée mixte +30% d'un medium complexe ont été utilisées. Durant la période de culture de 64,5 h, les biofilms ont été exposés aux solutions tests pendant une minute, deux fois par jour, durant deux jours consécutifs. Ensuite, les biofilms ont été rincés trois fois avec une solution saline. Suite à la dernière exposition, l'incubation s'est poursuivie pendant 16 h. Le prélèvement s'est effectué après 64,5 h. Les biofilms disper-

sés ont été ensemencés sur deux plaques d'agar. Puis le nombre de colonies formées (CFU) a été comptabilisé.

Toutes les solutions de chlorhexidine, de même que la Listerine®, ont significativement réduit le nombre de micro-organismes. En relation avec leur efficacité, les bains de bouches peuvent être répartis en deux groupes. L'ADS Curasept, Parodontosan et la Listerine® ont réduit le CFU d'une magnitude de trois sur l'échelle logarithmique. Cet effet semble suffisant pour une application prophylactique de longue durée. Le rinçage au PlakOut® et le contrôle à base de CHX ont réduit le CFU de sept sur l'échelle logarithmique. Ces solutions de rinçage sont prédestinées à un usage thérapeutique à court terme. Les effets secondaires réversibles doivent cependant être pris en considération.

La fabrication de produits contenant de la CHX avec des additifs ADS efficaces en conservant les propriétés antimicrobiennes semble être vouée à l'échec.

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