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Otten, M. P. T.; Busscher, Hendrik; van der Mei, Henderina; Abbas, Frank; van Hoogmoed, Christianus

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Retention of Antimicrobial Activity in Plaque and Saliva following Mouthrinse Use in vivo

M.P.T. Otten[a] H.J. Busscher[a] H.C. van der Mei[a] F. Abbas[b]
C.G. van Hoogmoed[a]

[a]Department of Biomedical Engineering and [b]Center for Dentistry and Oral Hygiene, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Key Words
Mouthrinses · Oral antimicrobials · Plaque · Substantivity

Abstract
The aim of this study was to determine the contribution of plaque and saliva towards the prolonged activity, also called substantivity, of three antimicrobial mouthrinses (Listerine®, Meridol®, Crest Pro Health®), used in combination with a toothpaste (Prodent Coolmint®). Volunteers brushed for 4 weeks with a toothpaste without antimicrobial claims, while during the last 2 weeks half of the volunteers used an antimicrobial mouthrinse in addition to brushing. At the end of the experimental period, plaque and saliva samples were collected 6 h after oral hygiene, and bacterial concentrations and viabilities were determined. The contribution of plaque and saliva towards substantivity was assessed by combining plaque obtained after mechanical cleaning only with plaque and saliva obtained after additional use of an antimicrobial rinse. Subsequently, resulting viabilities of the combined plaques were determined. The viabilities of plaque samples after additional rinsing with mouthrinses were lower than of plaque obtained after mechanical cleaning only, regardless of the rinse involved. Moreover, plaque collected 6 h after rinsing with antimicrobial mouthrinses contained a surplus of antimicrobial activity. Only Listerine showed decreased viability in saliva, but none of the mouthrinses showed any residual antimicrobial activity in saliva. The findings indicate that plaque left behind after mechanical cleaning contributes to the prolonged substantivity of antimicrobial mouthrinses.

Mouthrinses may act as a valuable addendum to regular brushing, and therewith support adequate oral hygiene. Oral antimicrobials, present in mouthrinses, can chemically influence dental plaque formation either by preventing adhesion of bacteria to oral surfaces, affecting bacterial vitality or disrupting existing plaque [Baehni and Takeuchi, 2003]. The use of an antimicrobial mouthrinse in addition to habitual oral hygiene seems beneficial, since the bulk of the plaque is removed mechanically by brushing and interdental cleaning, while antimicrobial rinses act on the plaque left behind in e.g. fissures and interproximal spaces [Brecx, 1997].

An effective antimicrobial agent should not only have an immediate antimicrobial effect, but the effect should also persist in the oral cavity for periods of time longer than the application period. This is called substantivity [Addy, 1997; Brecx, 1997]. Substantive action is mostly ascribed to adsorption of antimicrobials to the abundant-

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Chris G. van Hoogmoed
University Medical Center Groningen and University of Groningen
A. Deusinglaan 1
NL–9713 AV Groningen (The Netherlands)
Tel. +31 50 3633 140, Fax +31 50 3633 159, E-Mail C.G.van.Hoogmoed@med.umcg.nl

Table 1. Toothpaste and antimicrobial mouthrinses included in this study, together with their main active components, manufacturer, version and rinsing volume as recommended by manufacturers

Product	Main active components	Manufacturer	Version	Rinse volume
Prodent Coolmint® (toothpaste)	sodium fluoride sodium lauryl sulfate	Sara Lee Household and Bodycare, Exton, USA	Sara Lee H&BC, The Netherlands	not applicable
Listerine® (mouthrinse)	alcohol phenols and essential oils	Pfizer Consumer Healthcare, USA	Coolmint, made in EU	20 ml
Meridol® (mouthrinse)	amine fluoride stannous fluoride	GABA Group, Basel, Switzerland	GABA Benelux	10 ml
Crest Pro Health® (mouthrinse)	cetylpyridinium chloride	Procter & Gamble, Cincinnati, USA	Refreshing Clean Mint	20 ml

ly available soft tissues and their subsequent slow release into saliva. However, since saliva is continuously refreshed, one may wonder whether there are also other mechanisms responsible for the prolonged action of oral antimicrobials. As a hypothesis, we forward that plaque left behind after mechanical cleaning may absorb oral antimicrobials and act as a reservoir enabling the prolonged presence of antimicrobials in the oral cavity.

The aim of this study is to determine the contribution of plaque left behind after mechanical cleaning and saliva towards the substantive action of oral antimicrobials, after the use of mouthrinses.

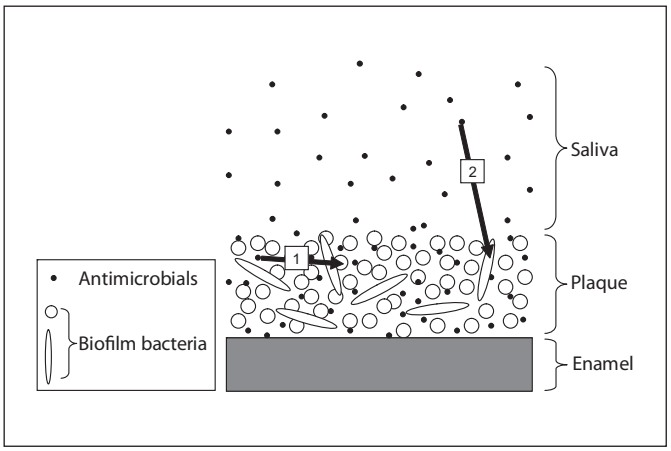

Fig. 1. Schematic presentation of the potential contributions of plaque (route 1) and saliva (route 2) towards substantive efficacy of antimicrobials. Route 1 involves antimicrobials absorbed in plaque. Route 2 involves antimicrobials in saliva.

Subjects and Methods

Human Volunteers and Oral Hygiene Products

Volunteers included in this study are healthy dental and oral hygiene students (in total 10 males and 14 females, aged 19–32 years; see 'Experimental Protocol' for further explanation). The study was performed according to the guidelines of the Medical Ethics Committee of the University Medical Center Groningen, Groningen, The Netherlands, including the informed consent by the volunteers and the tenets of the Declaration of Helsinki. For this study, three antimicrobial mouthrinses and one toothpaste without antibacterial claims were selected, as listed in table 1, together with their main active components and manufacturers. All products were commercially purchased.

Experimental Protocol

Every volunteer brushed for 2 weeks with a toothpaste only (control group), subsequently followed by 2 weeks of both brushing with a toothpaste and additional use of a mouthrinse (experimental group). Mechanical cleaning, consisting of brushing and interdental cleaning, was done twice a day according to the habitual routine of the volunteers. Rinsing was done for 30 s with the appropriate volume of the mouthrinse as recommended by the manufacturer (table 1), immediately after every mechanical cleaning. Every experiment comprised 2 volunteers, 1 from the control and 1 from the experimental group. Plaque and saliva samples of these 2 volunteers were collected 6 h after the morning cleaning of the oral cavity (see 'Collection of Plaque and Saliva'). In total 4 samples were obtained in one experiment: a control plaque (Pc) and saliva sample (Sc), obtained after mechanical cleaning only, and an experimental plaque (Pe) and saliva sample (Se), obtained after mechanical cleaning followed by the additional use of a mouthrinse. All samples were studied with respect to the number of bacteria and their viability (see 'Analysis of Plaque and Saliva Samples'). For each mouthrinse evaluated, experiments were performed in fivefold.

If plaque left behind after mechanical cleaning has absorbed effective amounts of antimicrobials and the absorbed antimicrobials have the ability to desorb, they should be able to kill bacteria in untreated plaque, in order to contribute to the substantivity of the antimicrobial (fig. 1). To evaluate the residual antimicrobial activity in a Pe sample, a 1:1 mixture of the Pc and Pe samples was made, denoted PcPe, of which the bacterial viability was assessed 2 h after combining. Similarly, the contribution of saliva toward substantivity (fig. 1) was determined in an Se sample. To this end,

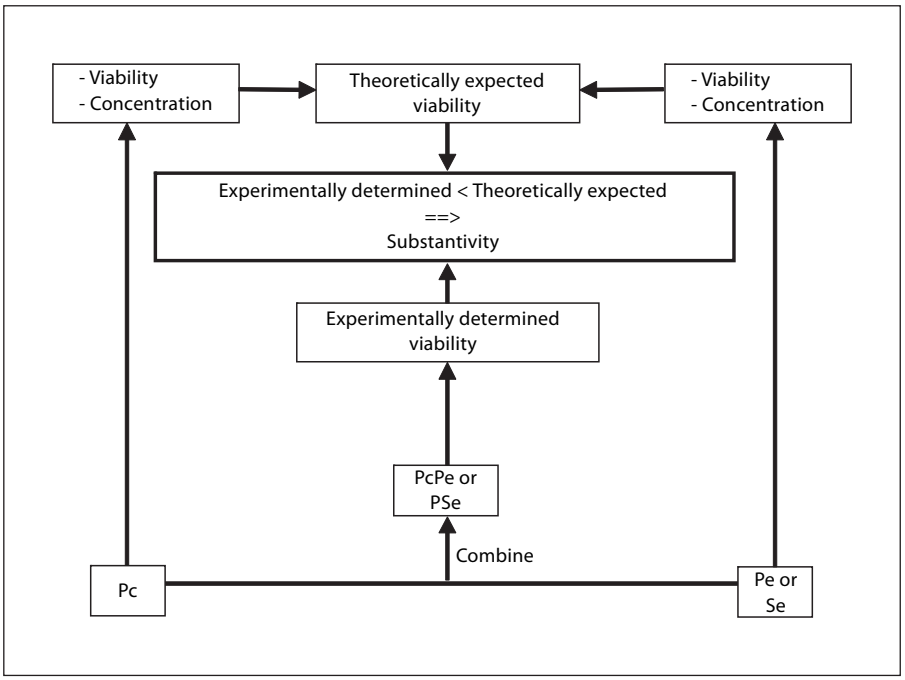

Fig. 2. Flowchart for combining control plaque (Pc) and experimental plaque (Pe) or saliva (Se) in order to reveal residual antimicrobial activity in plaque left behind or in saliva, according to the two routes outlined in figure 1. When the experimentally measured viability of Pc combined with Pe or Se was lower than theoretically expected on the basis of the individual Pc and Pe samples before combining, it was concluded that Pe or Se contained residual antibacterial activity.

1:1 mixtures were also made of Pc and Se samples and left for 2 h. These samples are denoted PcSe. Afterwards samples were analyzed. A time span of 2 h was chosen to create an opportunity for the antimicrobial activity in Pe or Se to perform their antimicrobial activity on the untreated plaque Pc, as also suggested in other studies [Welin-Neilands and Svensäter, 2007]. All samples were stored at room temperature.

Since both Pe and Se might exert an immediate antimicrobial influence on Pc, theoretically expected viabilities of the combined plaque (Pc + Pe) as well as plaque and saliva (Pc + Se) samples at t = 0 s were calculated using the viabilities of the samples prior to combining. Subsequently, the theoretically expected values of the combined samples were compared with the experimentally determined viabilities of the combined plaque (PcPe) as well as of the combined plaque and saliva (PcSe) samples. Experimentally determined viabilities lower than theoretically expected are an indication that the experimental plaque or saliva sample still contained residual antimicrobial activity, 6 h after the last use of the antimicrobial rinse (see fig. 2 for a schematic presentation of the experimental protocol).

Collection of Plaque and Saliva

Plaque was collected under the supervision of a professional dentist from the buccal, lingual, palatal and interproximal sides of the dentition with a sterile cotton swab stick and a dental instrument (Implant Deplaquer, KerrHawe, Switzerland) [Van der Mei et al., 2006]. The plaque collected was suspended in 2 ml sterile reduced transport fluid [Syed and Loesche, 1972]. Furthermore, approximately 2 ml of unstimulated saliva was collected. To suspend bacterial clumps, all individual plaque and saliva samples were vortexed and sonicated for 10 s at 30 W (Vibra Cell, model 375, Sonics and Materials Inc., Danbury, Conn., USA).

Analyses of Plaque and Saliva Samples

The bacterial concentrations in all samples were determined using a Bürker-Türk counting chamber in combination with a phase-contrast microscope (Olympus, Japan). In order to determine the experimental viability of the plaque and saliva samples, 20 μl of each sample was put on a microscope glass slide and stained for at least 15 min in the dark with 80 μl LIVE/DEAD stain (*Bac*Light™, Molecular Probes Europe BV). The LIVE/DEAD kit consisted of a 1:1 mixture of two nucleic acid stains: SYTO® 9, which penetrates most membranes freely, and propidium iodide, which permeates only through damaged membranes. Simultaneous application of both dyes results in green fluorescence of viable cells with an intact membrane, whereas membrane-damaged, dead bacteria produce red fluorescence. Images were collected using a fluorescence microscope (Leica DM4000 B, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). At least three images of each sample were randomly taken, and the total number of dead and live bacteria was counted. At least 100 bacteria per sample were counted. The viability per sample was expressed as percentage live bacteria (%L).

The theoretically expected viabilities of the combined PcPe or PcSe mixtures were calculated from the viabilities of the individual samples, while correcting for possible differences in bacterial concentrations. Accordingly, the theoretically expected viability for combined plaque samples PcPe was calculated using the following formula:

$$\%L_{PcPe} = \frac{\left(\%L_{Pc} \times C_{Pc}\right) + \left(\%L_{Pe} \times C_{Pe}\right)}{C_{Pc} + C_{Pe}} \quad (1)$$

where $\%L_{Pc}$, $\%L_{Pe}$, C_{Pc} and C_{Pe} represent the experimental viabilities and bacterial concentrations of Pc and Pe, respectively. Sim-

Table 2. Bacterial viability (%L) and bacterial concentration (C) in plaque and saliva collected 6 h after brushing with a toothpaste only or brushing followed by the additional use of an antimicrobial mouthrinse

	Toothpaste only		Followed by additional rinse					
	Prodent Coolmint		Listerine		Meridol		Crest Pro Health	
	%L	C, 10^9/ml	%L	C, 10^9/ml	%L	C, 10^9/ml	%L	C, 10^9/ml
Plaque	$47 \pm 8^{a, b, c}$	2.8 ± 1.1	$26 \pm 13^{d, c}$	3.4 ± 0.7	$30 \pm 6^{d, c}$	$1.7 \pm 0.5^{d, a}$	$20 \pm 4^{d, b, c}$	2.4 ± 0.6^{a}
Saliva	$55 \pm 7^{a, c}$	4.8 ± 1.9	$39 \pm 13^{d, c}$	4.0 ± 2.1	49 ± 6^{c}	$2.9 \pm 1.0^{d, a}$	47 ± 11^{c}	3.1 ± 1.3^{a}

Values are presented as averages ± standard deviations; n = 24 for the toothpaste and n = 5 for the rinses. [a] Statistically different from Listerine at $p < 0.05$, Student's t test. [b] Statistically different from Meridol at $p < 0.05$, Student's t test. [c] Statistically significant differences between the products at $p < 0.05$, ANOVA. [d] Statistically different from Prodent Coolmint only at $p < 0.05$, Student's t test.

Table 3. Experimentally measured and theoretically expected bacterial viabilities (%L) of combined plaque samples (PcPe), consisting of plaque collected after brushing with a toothpaste only (Pc) and plaques collected after brushing followed by the additional use of an antimicrobial mouthrinse (Pe) and combined plaque and saliva samples (PcSe), consisting of plaque collected after brushing with a toothpaste only (Pc) and saliva collected after brushing followed by the additional rinse with one of the antimicrobial mouthrinses (Se)

Rinse	Bacterial viability			
	combined plaque samples		combined plaque and saliva samples	
	experimentally determined	theoretically expected	experimentally determined	theoretically expected
Listerine	19 ± 4^{e}	34 ± 8^{f}	39 ± 6^{e}	40 ± 7^{g}
Meridol	31 ± 6^{e}	40 ± 6^{f}	50 ± 4^{e}	50 ± 2^{g}
Crest Pro Health	19 ± 7^{e}	32 ± 4^{f}	39 ± 8^{e}	45 ± 5^{g}

Values are presented as average bacterial viability (%L) ± standard deviation, with n = 5. [e] Statistically significant differences between the experimental values at $p < 0.05$, ANOVA. [f] Statistically different from experimental values at $p < 0.05$, Student's t test. [g] Statistically significant differences between the expected values at $p < 0.05$, ANOVA.

ilarly, for the combined plaque with saliva samples, PcSe, theoretically expected viability was calculated according to:

$$\%L_{PcSe} = \frac{\left(\%L_{Pc} \times C_{Pc}\right) + \left(\%L_{Se} \times C_{Se}\right)}{C_{Pc} + C_{Se}} \quad (2)$$

where $\%L_{Pc}$, $\%L_{Se}$, C_{Pc} and C_{Se} represent the experimental viabilities and bacterial concentrations of Pc and Se, respectively.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 software for Windows (SPSS Inc., Chicago, Ill., USA). The data were analyzed using one-way analysis of variance (ANOVA) to indicate significant differences between bacterial viabilities and concentrations in plaque and saliva samples obtained after brushing only or after brushing followed by the use of an antimicrobial rinse. The Student t test was used for statistical comparison between the individual samples. P values <0.05 were considered to indicate significant differences.

Results

Bacterial viabilities of plaque and saliva samples after mechanical cleaning and mechanical cleaning followed by the additional use of an antimicrobial mouthrinse are summarized in table 2. Interestingly, the viabilities of the plaque samples were significantly ($p < 0.05$) lower 6 h after additional use of an antimicrobial mouthrinse, compared to only brushing with a toothpaste without antimicrobial claims. The viabilities of bacteria in saliva 6 h after additional use of an antimicrobial mouthrinse were slightly lower than after toothbrushing only, although this effect was only significant ($p < 0.05$) for Listerine.

Table 3 compares the experimental and theoretically expected viabilities of combined plaques (PcPe) and of

control plaque combined with saliva (PcSe), respectively. The percentages of live bacteria theoretically expected in combined plaque samples (table 3) were significantly ($p < 0.05$) higher than the experimentally determined viabilities for all three mouthrinses. This indicates that plaque can be an effective reservoir for oral antimicrobials up till at least 6 h after rinsing. Similarly, it can be concluded from table 3 that saliva obtained 6 h after the use of an antimicrobial mouthrinse does not show residual antimicrobial activity, regardless of the mouthrinse considered. Consequently, for the antimicrobial rinses included in this study, saliva did not contribute to their substantive action.

Discussion

This study is the first to demonstrate that plaque left behind after mechanical cleaning and use of an antimicrobial mouthrinse can contribute to the substantivity of oral antimicrobials. The antimicrobial mouthrinses Listerine, Meridol and Crest Pro Health not only yielded a reduction in bacterial viability in plaque up to 6 h after mechanical cleaning (table 2), but moreover their active components were absorbed in plaque left behind. The amount of antimicrobials absorbed in plaque turned out to be sufficient to yield significant residual antibacterial activity (table 3) against untreated plaque, supporting our hypothesis that plaque left behind can contribute to the substantivity of oral antimicrobials. The results of this study, however, should not be taken as a case for incomplete oral hygiene, but do indicate that negative effects of plaque left behind can be reduced by absorption of antimicrobial mouthrinse components from oral health care products. In this respect, it is important to note that plaque is mostly left behind at places where antibacterial activity is needed most urgently, i.e. in fissures, interproximal spaces and gingival margins [Rylander and Lindhe, 1997]. Along similar lines, it has been argued that plaque can act as a reservoir for fluoride [Cenci et al., 2008].

Absorption of antimicrobials in plaque left behind was demonstrated by a novel approach of mixing test plaque and saliva samples with control plaque samples, rather than by more conventional chemical analyses of the presence of specific agents, as these chemical analyses would also involve antimicrobials bound to plaque constituents. Our novel mixing assay has as a major advantage that only bioavailable antimicrobials are included to the extent that they are indeed desorbed from treated plaques in concentrations high enough to kill new bacteria, which is exactly how this works in the oral cavity.

The contribution of plaque left behind to substantivity was demonstrated here for three antimicrobial mouthrinses with widely different active ingredients. Listerine is a formulation containing antimicrobial phenols and essential oils [Brecx et al., 1990, 1992]. Meridol is based on a combination of stannous fluoride and amine fluoride [Brecx et al., 1990, 1992], while Crest Pro Health contains cetylpyridinium chloride, an antimicrobial compound with similar antiplaque properties as chlorhexidine, but with faster intraoral clearance from saliva [Addy, 1997; Witt et al., 2005]. A chlorhexidine-containing antimicrobial mouthrinse was explicitly not included in this study, because its use in combination with a toothpaste containing sodium lauryl sulfate causes inactivation of the chlorhexidine [Barkvoll et al., 1989]. Also long-term use of chlorhexidine-containing rinses is rare as it causes staining of the teeth and altered taste sensation [Addy, 1997; Lorenz et al., 2006].

Remarkably, the experimental viabilities of combined plaque samples containing active components from Listerine or Crest Pro Health both amounted to 19% (table 3), which is slightly lower than the viabilities observed 6 h after rinsing with Listerine or Crest Pro Health, amounting to 26 and 20%, respectively (table 2). Although antimicrobial mouthrinses in general contain broad-spectrum antimicrobial efficacy, selective killing of specific bacterial strains cannot be ruled out and might in fact be suggested by the above observation. Upon combining experimental plaques with control plaques (PcPe), live bacteria of the most susceptible strains, already killed in experimental plaque, are added again via the control plaque and might be preferentially killed by antimicrobials present in the experimental plaque samples [Sekino et al., 2004].

No contribution of saliva toward the prolonged substantivity of any of the antimicrobial mouthrinses was found (table 3), but this could be due to insufficient sample size. Note that Listerine did decrease the bacterial viability in saliva samples taken 6 h after its use (table 2), although apparently saliva collected 6 h after rinsing with Listerine did not contain enough antimicrobial agent to exert an ongoing killing effect on control plaque samples. Evidently, the use of neither Crest Pro Health nor Meridol decreased the bacterial viability in saliva samples.

In conclusion, plaque left behind after mechanical cleaning can absorb oral antimicrobials from mouth-

rinses. Even after 6 h, the concentration of antimicrobials in plaque left behind is high enough to yield prolonged bacterial killing of new plaque. Plaque may thus contribute to the substantive action of an antimicrobial mouthrinse.

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