Clin Oral Invest (2010) 14:241–250
DOI 10.1007/s00784-009-0286-0

ORIGINAL ARTICLE

Effect of mouthrinses on *Aggregatibacter actinomycetemcomitans* biofilms in a hydrodynamic model

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Received: 4 August 2008 / Accepted: 8 May 2009 / Published online: 22 May 2009
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Abstract The aim of the study was to evaluate the effects of Listerine®, Meridol®, and Perioaid® on the viability and total number of bacteria in established biofilms using an in vitro model under hydrodynamic conditions. Biofilms of *Aggregatibacter actinomycetemcomitans* were placed in a modified Robbins device and rinsed twice daily during 4 days. Bacteria were quantified by culture and quantitative polymerase chain reaction. Visualization of the samples was performed by scanning electron and confocal laser scanning microscopy, combined with a fluorescent vital staining. All three mouthrinses caused a significant reduction in the number of cultivable *A. actinomycetemcomitans* in a biofilm. Perioaid® was significantly the most powerful in killing the biofilm-protected bacteria and also in counteracting the development of thick dense microbial communities. The total amount of bacteria was not significantly affected by Listerine® and Meridol®.

Keywords *Aggregatibacter actinomycetemcomitans* · Amine fluoride/stannous fluoride mouthrinse · Biofilm · Chlorhexidine mouthrinse · Essential oil mouthrinse · Modified Robbins device

Introduction

Several commercially available antiseptic products are used by clinicians to improve oral hygiene or the outcome of periodontal therapy. When comparing their efficacies, clinical studies often show contradictory results. This is probably due to patient-related factors (microbial constitution, host response factors, compliance factors) [1–3]. To elucidate the effects of antiseptics, it is desirable to compare them under identical conditions and under conditions which are relevant for the oral cavity. This can be achieved by the use of reliable in vitro models.

Certain bacteria are known to play a key role in the pathogenesis of biofilm-associated diseases like caries and periodontal diseases. The gram-negative coccobacillus *Aggregatibacter actinomycetemcomitans* is an important periodontal pathogen [4, 5]. This microorganism is also associated with certain systemic infections, such as: endocarditis [6], meningitis, osteomyelitis [7], and brain abscesses [8].

The aim of this study was to develop a reliable in vitro test to compare the antimicrobial effectiveness of mouthrinses on bacterial biofilms under hydrodynamic conditions. This model was used to evaluate the effects of three commercially available antiseptic mouthrinses on the viability and total amount of *A. actinomycetemcomitans* in biofilms.

Materials and methods

Bacterial strains and culture conditions

Two clinical strains of *A. actinomycetemcomitans*, strain 1398 (called SA 18) serotype b and strain 2751 (called SA 19) serotype e (both provided by S. Asikainen, University of Helsinki, Helsinki, Finland), were included in the study. Bacteria were grown on blood agar plates (Blood Agar Base II; Oxoid, Basingstoke, England), supplemented with 5 mg/mL hemin, 1 mg/mL menadione (Merck, Darmstadt,

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Germany), and 5% (*v*/*v*) sterile horse blood (Biotrading, Keerbergen, Belgium). Colonies were picked off and cultured overnight in tryptic soy broth (TSB; Oxoid) at 37°C in a 5% CO_2 environment. Bacteria were harvested by centrifugation and resuspended in "biofilm medium" which is a modification of the medium described by Kinniment and colleagues [9]. These researchers used a complex medium in which a model glycoprotein (gastric mucin) was the major carbon source to reflect the main carbon and energy source available to bacteria in the mouth. Our biofilm medium also presents a high concentration of proteins and contains: 2.5 g/L mucin (Sigma, St. Louis, MO, USA), 2.0 g/L sodium bicarbonate (Merck), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Merck), 30 g/L TSB (Oxoid), 1 g/L Lab Lemco (Oxoid), and 4 mL resazurin (Sigma) from a 25 mg/100-mL solution. The bacterial concentration was adapted based on spectrophotometrical measurements (outer diameter at 600 nm) to obtain a solution containing 1×10^8 colony-forming units (CFU) per milliliter.

Establishment of biofilms

Circular glass disks (7-mm diameter and 1 to 2-mm thick) served as the surfaces for *A. actinomycetemcomitans* biofilms. The sterile specimens were transferred into polystyrene 24-well plates (Iwaki microplate, Scitech, Diu, Japan), inoculated with 1-mL bacterial culture per well, and incubated at 37°C in a 5% CO_2 environment. After 1 day, half of the medium was refreshed and, on the second day, the disks were fitted into the specimen plugs of a modified Robbins device (MRD; Dentaid, Barcelona, Spain). This flow cell model consists of four rectangular stainless steel chambers which were filled with biofilm medium. A separate chamber was used for each rinse. Five plugs could be placed in each chamber. The bacteria on the disks were immediately in direct contact with the medium. The interior temperature of the chambers was maintained at 37°C. All chambers were connected with the same bioreactor by means of silicon tubes. The bioreactor, a double-walled flask, contained a stir bar and a continuous culture of *A. actinomycetemcomitans*, either strain SA 18 or strain SA 19, in biofilm medium at 37°C. Fresh medium was provided from a reservoir via a pump. Another peristaltic pump was used to push the bacterial culture from the bioreactor through the MRD with a flow rate of 200 µL/min [10]. The tubing and culture chambers were washed and autoclaved prior to each run. Anaerobic gas (10% H_2, 10% CO_2, 80% N_2) was delivered into the system via bacterial filters (pore size 0.2 µm).

Exposure to oral rinses

Three commercially available oral rinses were tested in this study. Listerine® (Pfizer Consumer Healthcare, Morris Plains, NJ, USA) contains a fixed combination of four essential oils (EO) as active ingredients (thymol 0.064%, eucalyptol 0.092%, methyl salicylate 0.060%, menthol 0.042%). EO kill microorganisms by disrupting their cell walls and inhibiting their enzymatic activity. They prevent bacterial aggregation, slow down bacterial multiplication, and extract endotoxins [11]. Meridol® (GABA International, Basel, Switzerland) is an amine fluoride/stannous fluoride (AmF/SnF_2)-containing mouthrinse without alcohol. The combination of AmF/SnF_2 inhibits the accumulation of bacteria and reduces their general viability [12]. Perioaid® (Dentaid Benelux, Houten, the Netherlands) contains 0.12% chlorhexidine (CHX), a biguanide which appears to be the most effective chemical agent for plaque inhibition [13]. It binds to bacteria and hinders their adsorption onto teeth [14] and/or attacks the bacterial cell membrane, causing leakage and/or precipitation of the cellular contents [15].

A. actinomycetemcomitans biofilms were exposed twice daily for 30 s to one of the three test oral rinses or phosphate-buffered saline (PBS) as negative control. The time interval represented a person who would rinse in the morning and in the evening during 4 days [16]. The first rinse was applied 8 h after starting the flow in the MRD. Pumps were halted before each rinse, and 10-mL mouthwash or PBS was gently injected with a syringe into the chambers. After 30 s, 10 mL fresh (not bacteria containing) medium was injected in the same way to remove the oral rinse. After this procedure, normal flow was reestablished.

Bacterial quantification

Preparation of samples

After 4 days, the first disk was removed from each chamber prior to the last rinse for determination of the number of bacteria in the biofilms. Prior to removal, the disks were rinsed with 10-mL fresh biofilm medium to flush the nonadhered bacteria. By washing the biofilm like this, the devastating effect of an air/liquid interface was avoided, as this could give rise to severe errors in enumeration [17]. The four remaining disks in each chamber were rinsed one last time with the mouthwashes and flushed with fresh biofilm medium to remove the oral rinses. All disks were carefully removed from the MRD for further analysis. One disk per chamber was used for determination of the number of bacteria immediately after the last rinse. Disks 3 and 4 were used for visualization and the last disk was an extra disk. The disks used for quantification were placed in sterile tubes containing 1 mL PBS, vortexed vigorously for 1 min, sonicated for 15 min at 100 W, and vortexed again for 1 min before being tenfold diluted in PBS.

Quantification of cultivable A. actinomycetemcomitans

Survivors in the suspension were enumerated by colony counting on 5% horse blood agar plates after incubation for 3 days at 37°C in a 5% CO_2 environment. The efficacy of the disruption procedure was monitored by gram staining before and after the procedure and observation of the disks by a light microscope (×100).

Quantification of the total number A. actinomycetemcomitans

Bacterial DNA was extracted from the undiluted solutions by use of Instagene Matrix (Biorad, Hercules, CA, USA) according to the manufacturers' instructions. A quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assay based on the 16S ribosomal RNA gene of *A. actinomycetemcomitans* was performed with an ABI Prism 7700 system (Applied Biosystems) to quantify the total number of bacteria, dead or alive, in each sample, as it was described by Boutaga and colleagues [18]. The program was: 2 min at 50°C, 10 min at 95°C, followed by 45 repeats of 15 s at 95°C and 1 min at 60°C. The design of the primers and probe (Eurogentec, Seraing, Belgium) is shown in Table 1. The amplicon size was 80 bp. Quantification was based on a plasmid standard curve (slope −3.2, intercept 40.4, R^2=0.99).

Statistical analysis

In each experiment, the three mouthrinses and the control solution were tested together. The two *A. actinomycetemcomitans* strains were investigated separately. The experiment was repeated nine times for each strain, on different days and with fresh bacterial cultures. First, normal quantile plots were used to assess normal distribution of the residuals. A log-transformation was conducted on the numbers of bacteria, based on the results of these normal residual tests. Next, a linear mixed model was fit, taken into account the grouped data. Data were grouped per experiment for statistical evaluation of the effects of the different rinses and control. Treatment was modeled as a fixed factor. The grouping of the data in experiments was modeled as a random factor. Pairwise comparisons were calculated and *p* values of the differences were corrected for simultaneous hypothesis testing such that the overall alpha was 0.05 according to the simulation procedure described by Bretz et al. [19]. The level of significance was set at $p<0.05$.

Visualization of the effect of oral rinses

Scanning electron microscopy

Biofilms were removed from the MRD after 4 days and fixation was performed in 2.5% glutaraldehyde (Sigma) in 0.1 mol/L sodium cacodylate buffer (pH 7.4) at 4°C for 12 h. The specimens were rinsed in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 1 h with three changes and rinsed with distilled water for 1 min. Additionally, the samples were dehydrated by placing the disks in baths with an ascending concentration ethanol (25%, 50%, 75%, 95%, 100%) each time for 10 min, followed by a 5-min bath in hexamethyldisilazane (Sigma). Finally, the samples were air-dried, mounted on aluminum stubs with silver paint, sputter-coated with gold, and examined by scanning electron microscopy (SEM) with a magnification of 10,000 (Philips XL20 Fe-SEM; Philips Co., Eindhoven, the Netherlands) [20].

Confocal laser scanning microscopy combined with vital fluorescent staining

Biofilms were visualized by confocal laser scanning microscopy (CLSM) to analyze their structure and the bacterial vitality after treatment with the oral rinses for 4 days. Bacteria were stained with a fluorescent vital stain. A solution of Syto 9 (Molecular Probes, Eugene, OR, USA) and Sytox orange (Molecular Probes) dyes in physiological saline was applied on the *A. actinomycetemcomitans* biofilms. This mixture selectively stains the living bacteria green (excitation at 488 nm) and dead bacteria red (excitation at 543 nm). After incubation for 30 min at room temperature, the samples were washed with physiological saline. The biofilms were visualized with an Olympus IX 70 CLSM (Olympus, Aartselaar, Belgium) equipped with an argon and a helium–neon laser. To examine the bacterial biofilm structure and the vitality of the bacteria therein, samples were scanned. Series of optical sections with intervals of 1 µm were taken and reconstructed three-dimensional views were created with Fluoview 500 imaging software (Olympus).

For both microscopic techniques, three replicates were conducted.

Table 1 Primers and probe used in the qRT-PCR assay for *A. actinomycetemcomitans*

Primers and probe	Sequence (5′–3′)	Concentration (nM)
Aa-forward	GAACCTTACCTACTCTTGACATCCGAA	300
Aa-reverse	TGCAGCACCTGTCTCAAAGC	300
Aa-probe	AGAACTCAGAGATGGGTTTGTGCCTTAGGG	100

Probe was labeled 5′ FAM, 3′ TAMRA

Results

Effect of mouthrinses on the number of cultivable *A. actinomycetemcomitans* within biofilms

Bacterial quantification was performed before the last rinse step to show the "long-term" influence (8 h) and immediately after the last rinse step to include also the direct effect (Table 2). The effects of the mouthrinses on the number of cultivable bacteria of both investigated strains are shown in Fig. 1. Data were expressed as the proportion *A. actinomycetemcomitans* recovered from treated disks relative to the control disks. No significant strain-related differences were found. All three mouthwashes reduced the number of colony counts of *A. actinomycetemcomitans*, compared to the control solution. The effects were in general more pronounced immediately after the last rinse than prior to the last application. Immediately after the last rinse, these reductions were significant ($p<0.05$) for all mouthrinses. Before the last rinse, only Perioaid® caused significant ($p<0.05$) reductions. Perioaid® was, both before and after the last rinse, significantly ($p<0.05$) more potent than Meridol® and Listerine®.

Comparison of the number of cultivable bacteria immediately before and after the last rinse showed that the immediate-killing effect of Listerine® was more efficient than the immediate-killing effect of Perioaid®, but Perioaid® had a better "long-term" effect than Listerine®.

Effect of mouthrinses on the total number *A. actinomycetemcomitans* in a biofilm

To elucidate the effect of Perioaid®, Meridol®, and Listerine® on the total number of bacteria, next to their effect on bacterial viability within the biofilm, the influence of the mouthwashes was analyzed by qRT-PCR (Table 2). The results obtained for strains SA 18 and SA 19 are depicted in Fig. 2 as the relative change in the total bacterial number for the three mouthrinses compared to PBS (control).

When the three mouthrinses were applied on the biofilms, a statistically significant ($p<0.05$) reduction in the total number of bacteria was only observed for strain SA 18 after treatment with Perioaid®. The total number of bacteria was reduced with less than 20%. This reduction is considerably smaller than the reductions observed in the number of cultivable *A. actinomycetemcomitans* (Fig. 1). Statistically significant ($p<0.05$) interstrain differences could be noted, after treatment with Perioaid®, with strain SA 18 being more sensitive than SA 19. Immediately after the last application of Perioaid® on *A. actinomycetemcomitans* strain SA 18, a reduction in the bacterial cell number of 18.2% was detected. Meridol® caused a reduction of 15.5% and Listerine® caused nearly no changes (2.2% reduction). For SA 19 biofilms, more than 95% of the total number of bacteria stayed attached after all treatments (Fig. 2).

Microscopic visualization

Microscopic observations of the *A. actinomycetemcomitans* biofilms after treatment with the mouthrinses for 4 days revealed a product-related disturbance in biofilm viability, density, and the total bacterial number. The SEM images, presented in Fig. 3, showed that *A. actinomycetemcomitans* strain SA 18 was able to form complex bacterial communities on the glass surfaces. The *A. actinomycetemcomitans* cells formed towering microcolonies, anchored to the surface and probably embedded in their exopolysaccharide matrix. Extracellular amorphous material covered the outer surface of the cells and appeared to combine adjacent cells by fibrous threads. Cell surface components, such as fimbriae-like structures, were also visible. Treatment of the *A. actinomycetemcomitans* strain SA 18 biofilms with Listerine® and Meridol® caused no clearly observable change in the amount of bacteria when compared to control biofilms. The CHX-containing mouthrinse Perioaid® did

Table 2 Mean number *A. actinomycetemcomitans* quantified with microbial culture and qRT-PCR

	Culture				qRT-PCR			
	Before last rinse		After last rinse		Before last rinse		After last rinse	
Strain	SA 18	SA 19	SA 18	SA 19	SA 18	SA 19	SA 18	SA 19
Perioaid®	1.17 (0.64)	1.81 (0.75)	1.36 (0.73)	0.55 (0.36)	6.73 (0.85)	8.80 (0.18)	6.98 (0.89)	8.57 (0.20)
Meridol®	4.20 (0.67)	4.17 (0.61)	3.01 (0.80)	3.53 (0.75)	8.01 (0.19)	8.64 (0.21)	7.36 (0.93)	8.76 (0.18)
Listerine®	5.12 (0.30)	5.48 (0.29)	3.92 (0.82)	3.71 (0.83)	8.17 (0.17)	9.04 (0.16)	8.45 (0.18)	8.80 (0.15)
Control	5.77 (0.26)	6.26 (0.31)	5.72 (0.26)	6.27 (0.33)	8.45 (0.26)	9.01 (0.18)	8.65 (0.23)	8.95 (0.17)

Log 10 of the number bacteria per milliliter (SD)

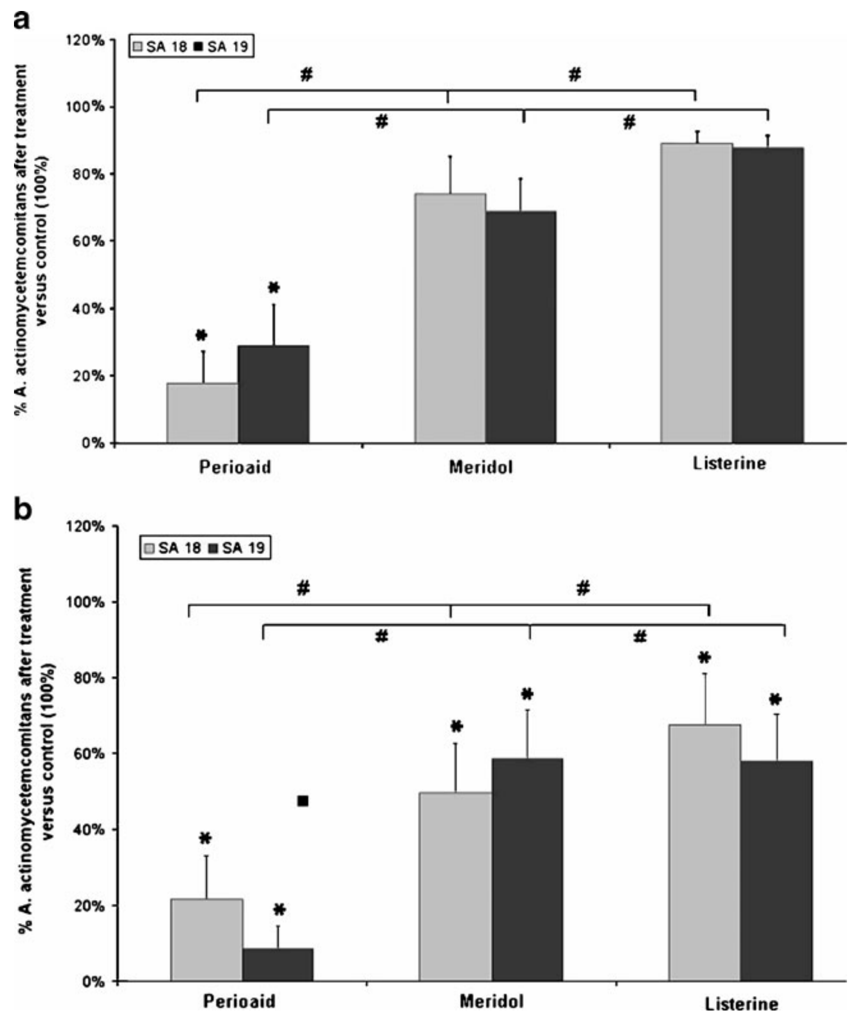

Fig. 1 Effect of mouthrinses on the number of cultivable *A. actinomycetemcomitans.* The effect of three mouthrinses (Perioaid®, Meridol®, Listerine®) and PBS (control) was tested in vitro in a modified Robbins device on biofilms of two clinical *A. actinomycetemcomitans* strains (SA 18 and SA 19). They were applied twice daily over a period of 4 days. The numbers of cultivable *A. actinomycetemcomitans* were determined by microbial culturing before the last rinse (**a**) and after the last rinse (**b**). Data were plotted as the relative proportion of *A. actinomycetemcomitans* recovered from a biofilm treated with a mouthrinse, compared to a biofilm treated with PBS (control). *Bars* represent standard deviations.*$p<0.05$, treated group versus control. #$p<0.05$, Perioaid®-treated group versus Meridol®- and Listerine®-treated groups

cause some observable changes in the structure and morphology of both the biofilm and the bacteria therein. The biofilms were thinner; less fibrous threads between the bacteria were observed and the bacteria had a more rounded appearance.

CLSM visualized the surface coverage by the bacteria and optical sectioning revealed additional information about biofilm density and thickness (Fig. 4). Z stacks of optical sections and reconstructed vertical cross sections from stacked Z series showed how *A. actinomycetemcomitans* strain SA 18 cells covered the majority of the surface in the control situation, with projections arising from the surface. Listerine® caused nearly no changes in biofilm structure and thickness and in the surface coverage. Only minor changes in biofilm density could be observed. Perioaid® and Meridol®, however, resulted in a thinner and less compact biofilm. The applied fluorescent vitality staining revealed changes in the proportions of living versus dead bacteria upon treatment with the rinses. Perioaid® caused a pronounced increase in the relative amount of dead bacteria compared to the control solution. Meridol® showed also a killing effect, but Listerine® did not cause a clear shift in the relative amount of living versus dead bacteria.

Discussion

In clinical trials, factors such as the compliance and general medical condition of the test persons could influence the study outcome. Additionally, as a result of individual differences in salivary flow rates and plaque levels, individuals vary substantially in the potential benefit they may attain from rinsing with an antiplaque agent [21, 22]. This might explain why similar studies sometimes give rather contradictory results. Riep et al. [23] for example found after a 4-day plaque-regrowth study no significant plaque reduction in the AmF/SnF_2 rinse group in contrast to Arweiler et al. [24]. Essential oils and 0.12% CHX showed comparable antiplaque activities in one 6-month clinical study [25], but 0.12% CHX had a more pronounced antiplaque effect than essential oils according to another

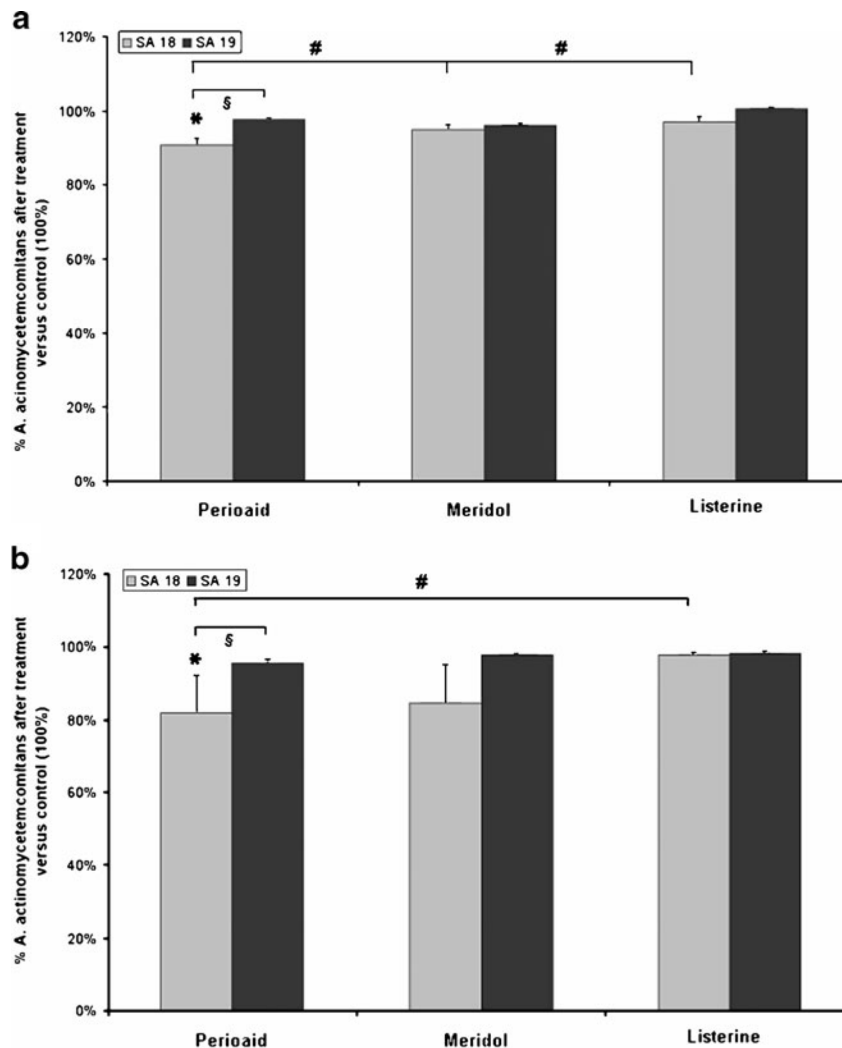

Fig. 2 Effect of mouthrinses on the total number of *A. actinomycetemcomitans.* The effect of three mouthrinses (Perioaid®, Meridol®, Listerine®) and PBS (control) was tested in vitro in a modified Robbins device on biofilms of two clinical *A. actinomycetemcomitans* strains (SA 18 and SA 19). They were applied twice daily over a period of 4 days. The total numbers of *A. actinomycetemcomitans* were determined by quantitative PCR analysis before the last rinse (**a**) and after the last rinse (**b**). Data were plotted as the relative proportion of *A. actinomycetemcomitans* recovered from a biofilm treated with a mouthrinse, compared to a biofilm treated with PBS (control). *Bars* represent standard deviations. *$p<0.05$, treated group versus control. #$p<0.05$, Perioaid®-treated group versus Meridol®- and Listerine®-treated groups. §$p<0.05$, strain SA 18 versus SA 19

6-month clinical trial [26]. The use of a standardized in vitro model can offer important advantages from this point of view as one can use a fixed controlled setup in different experiments.

In vitro screening of oral antimicrobials was often performed on planktonic suspensions [27]. Recently, more researchers have implied biofilms in their in vitro testing of oral antimicrobials because of the increased resistance of sessile bacteria [28–30]. To produce in vitro predictive findings for clinical activity, it is also important to incorporate hydrodynamic forces which are continuously present in the oral cavity [31]. These forces can exert important shear and clearance effects [21]. The contact time between oral rinse and bacteria in vivo is maximum 30–60 s per application. This aspect should be incorporated in an in vitro test setup to make it representative for the in vivo situation. Studies about the efficacy of antimicrobials on biofilms are, however, often performed under static conditions. Some researchers did include fluid flow in their model but neglected the clearance effect since the test substance was pumped continuously through the system for prolonged periods [32]. The model described in this study allows repeated short exposures of biofilms to antimicrobial solutions. A rather similar setup was the artificial throat model described by Busscher et al. [33]. Bacteria collected from voice prostheses were allowed to form biofilms on hard surfaces under static conditions. Then, these samples were rinsed three times a day for 12 days with certain test products (buttermilk). The time schedule was representative for having breakfast, lunch, and diner. Samples were removed from the system and analyzed by microbial plating and SEM.

The MRD described in the presented study has been used previously to study the colonization of hard and soft surfaces by *A. actinomycetemcomitans* under hydrodynamic conditions [34] and to evaluate the influence of streptococcal species on *A. actinomycetemcomitans* colonization [35]. The effects of the three oral rinses were never compared in this kind of in vitro model. However, a recent study tested the susceptibility of *Porphyromonas gingivalis* to three oral

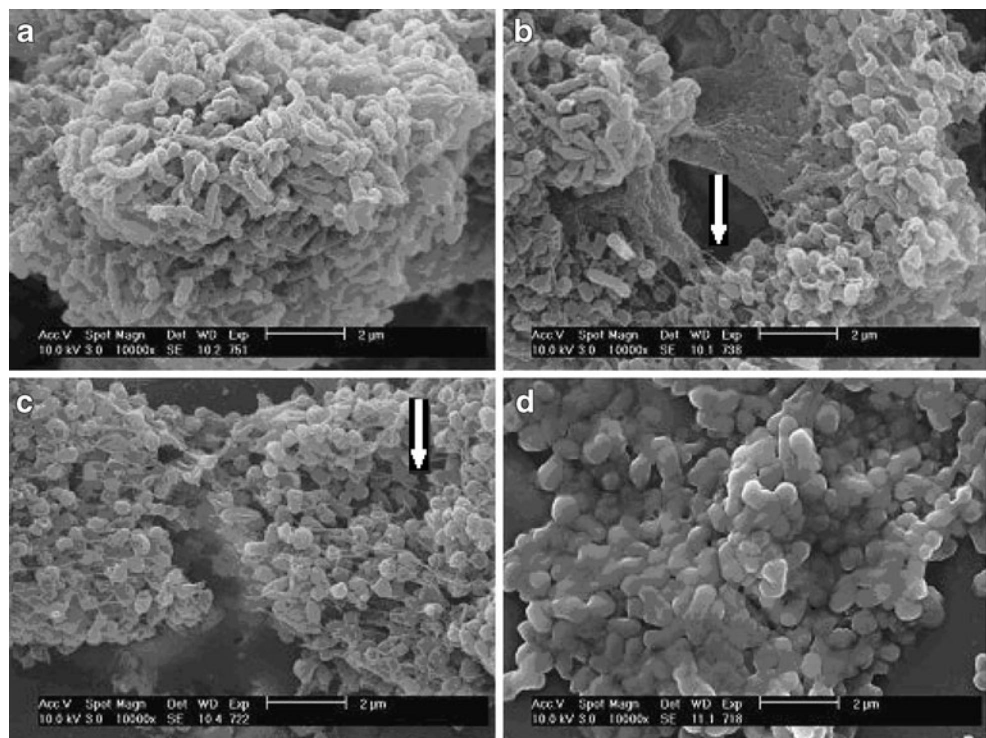

Fig. 3 Scanning electron micrographs of *A. actinomycetemcomitans* strain SA 18 biofilms on glass disks. Samples were visualized after exposing them twice a day during 4 days to PBS (control; **a**), Listerine® (**b**), Meridol® (**c**), and Perioaid® (**d**). *Arrows* indicate the fibrous threads between bacteria in the biofilm. *Scale bars* represent 2 µm

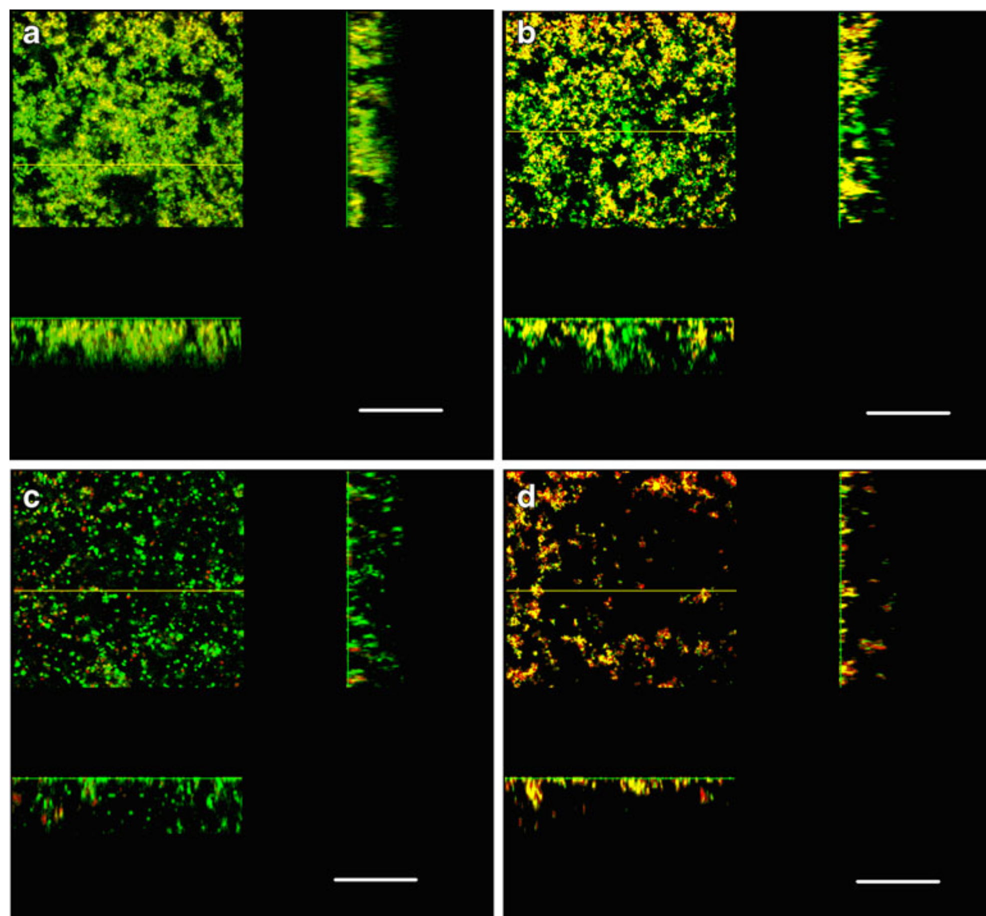

Fig. 4 Confocal images of *A. actinomycetemcomitans* strain SA 18 biofilms upon exposure to four test solutions: PBS (control; **a**), Listerine® (**b**), Meridol® (**c**), and Perioaid® (**d**). Biofilms were rinsed twice a day during 4 days. Visualization was performed immediately after the last rinse step. Samples were stained with two fluorescent dyes which selectively stain vital bacteria green and dead bacteria red. *Scale bars* represent 40 µm

antiseptics (chlorhexidine 0.2%, povidone–iodine 1%, and Listerine®) also using an MRD setup [36].

The MRD model was used here to evaluate the effect of three mouthrinses on *A. actinomycetemcomitans* biofilms. Perioaid® reduced the number of bacteria more profound than Meridol® and Listerine®, compared to the control solution. CHX-containing solutions appear to be in general the most effective against plaque and gingivitis in vivo and are considered as the golden standard [37, 38]. Based on these observations, it could be expected that the CHX-containing solution Perioaid® would give the best results in our in vitro test model against monospecies bacterial biofilms.

In different clinical trials, CHX-containing mouthrinses seemed superior to EO- and AmF/SnF_2-containing ones in the ability to maintain low plaque scores and gingival health [39] and in increasing the relative proportion of dead bacteria on teeth [40]. All three mouthrinses reduced biofilm viability when compared to the control solution based on the quantitative data and, although less obvious for Listerine®, on the CLSM observations. This is in line with in vivo studies which show that oral hygiene combined with the use of CHX-, EO-, or AmF/SnF_2-containing mouthrinse is more beneficial for plaque control than oral hygiene alone [41–43]. Another similarity seen with clinical studies is that the total number of bacteria was less influenced by the mouthrinses than the viability of the biofilm [40].

The total bacterial cell number was quantified using a qRT-PCR assay for *A. actinomycetemcomitans*. Boutaga and colleagues developed this assay and determined its sensitivity, which was 1 CFU. Their assay also proved to be highly specific, amplifying only DNA extracted from *A. actinomycetemcomitans* and none of the DNA from other tested species [18].

Changes in the total number of bacteria showed strain-related differences after application of Perioaid®. The total number *A. actinomycetemcomitans* in strain SA 19 biofilms remained unaffected upon rinsing with the three mouthrinses. SA 19 biofilms showed, however, a significant decrease in the number of cultivable bacteria after application of the three mouthrinses. On SA 18 biofilms, Perioaid® had a significant effect on both the cultivable and total bacterial number. These interstrain differences could possibly be related to the different phenotypic properties of both strains [44]. It could be observed that SA 18 is a more autoaggregating strain compared to SA 19. During culture, SA 18 was moderately adhesive on an agar-plate and in broth; it was growing both in solution and in autoaggregation particles. On the glass disks, this strain showed pronounced three-dimensional projections and bulbous microcolonies of cells. When the linkage of such a bulbous autoaggregation particle with the surface was broken by an oral rinse, a large number of cells were detached. SA 19 cells formed a "lawn" over the surface. Thick cell layers with high cell density were formed and attached bacteria were generally uniformly distributed. This strain adhered strongly on the agar plates during culture and in broth it formed a thorough adherent film to the recipient, but no autoaggregation particles were seen. The strain-related differences could also be correlated with the serotype [45]. *A. actinomycetemcomitans* strain SA 18 belongs to serotype b which is strongly associated with periodontal disease [46] and is the most common serotype in aggressive periodontitis. The pronounced presence of cell surface appendages in serotype b, as seen by SEM, shows the high pathogenic potential of this serotype [47]. Serotype e (strain SA 19) was not detected in aggressive periodontitis [48]. For other infections, it has been reported that bacterial serotype and sensitivity can be correlated. The sensitivity of clinical *Candida albicans* isolates to fluconazole in vitro correlated with their serotype [49]. Other researchers showed that the sulfonamide resistance of meningococci from clinical material was serotype dependent [50].

Microbial culturing was performed to quantify the effects of the mouthrinses on the amount of cultivable bacteria within the biofilms. The control solution caused no changes, while all three oral rinses did have an immediate effect on the *A. actinomycetemcomitans* viability within biofilms. Immediately before the last rinse, only Perioaid® caused a significant reduction. Perioaid® was more able to maintain its antimicrobial effect, compared to the other rinses, while Listerine® showed a better immediate-killing effect. Previous studies also showed that some oral antimicrobials containing CHX can maintain their antimicrobial activity over a prolonged period of time [51]. Clinically effective antiplaque agents are characterized by good intrinsic antibacterial activities and oral retention properties [21].

Microscopic techniques were utilized to visualize the effects of the test products on biofilm structure, density, viability, and total bacterial number. SEM and CLSM images showed tenacious *A. actinomycetemcomitans* biofilms upon rinsing with the control solution. Application of Listerine® also allowed the formation of thick dense biofilms. Treatment with Meridol® and mainly Perioaid® did, however, show some effect on biofilm structure, density, and thickness. Some structural changes in the biofilms were observed in the microscopic images; however, the quantitative PCR data revealed only minor changes in the total bacterial cell number. An explanation for this event could be that genetic material of killed and detached bacteria remains and is detected by the very sensitive PCR technique. *A. actinomycetemcomitans* biofilms grown in vitro consist of tightly packed cells enmeshed in a self-synthesized extracellular polymeric matrix. The biofilm matrix contains, next to polysaccharide and other compo-

nents, extracellular DNA [52–54]. If bacterial cells are disrupted, DNA could possibly remain in the matrix.

One could argue the clinical relevance to test mouthrinses for their effects towards subgingival bacteria; however, *A. actinomycetemcomitans* can also be found in supragingival plaque and saliva. Studies have moreover shown that the nature and amount of supragingival plaque can influence the composition of the contiguous subgingival flora and that rinsing with an antiplaque/antigingivitis mouthrinse can have significant antimicrobial activity against subgingival periodontopathogens [55].

A biofilm-based model in which the intraoral situation was imitated by the introduction of controlled hydrodynamic conditions was used in this study to assess the influence of three commercially available oral rinses (Perioaid®, Meridol®, and Listerine®) on in vitro monospecies biofilms of the periodontal pathogen *A. actinomycetemcomitans*. This model takes into account the biofilm mode of growth of oral bacteria and the fluid flow present in the oral cavity. The above-mentioned conclusions were drawn within the limitations of this in vitro study. One should always be cautious to extrapolate results from in vitro studies to the in vivo clinical situation. The described model offers, however, an array of possibilities, although further improvements should be carried out to ameliorate the comparability with the in vivo situation even more. It could be used in the future to determine the relative ability of other oral rinses to interfere with bacterial biofilms and biofilm viability.

Acknowledgements The authors thank Dr. Anca Margineanu and Rudy de Vos for assistance with, respectively, CLSM and SEM visualization. This research was funded by the following grants: OT 03/52 and FWO G/0240/04. Isabelle Sliepen was supported by a Ph.D. grant of IWT Flanders and Wim Teughels by the KU Leuven Research Fund and the Research Fund-Flanders. This study was supported by Pfizer (Pfizer Consumer Healthcare, Morris Plains, NJ, USA).

Conflict of interest This study was supported by Pfizer (Pfizer Consumer Healthcare, Morris Plains, NJ, USA).

The MRD was supplied by DentAid (Spain).

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