An active attachment biofilm model to develop anti-caries strategies
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Chapter 5

The Effect of Propidium Monoazide Treatment on the Measured Bacterial Composition of Clinical Samples after the Use of a Mouthwash.

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Abstract

Objectives
The use of an anti-microbial mouthwash results not only in a reduction of the number of viable cells in dental plaque but potentially also in a shift in the oral microbiome. DNA based techniques may be appropriate to monitor these shifts, but these techniques amplify DNA from both dead and living cells. Propidium monoazide (PMA) has been used to overcome this problem, by preventing the amplification of DNA from membrane-damaged cells. The aim of this study was to evaluate the use of PMA when measuring compositional shifts in clinical samples after mouthwash use.

Materials & Methods
On two consecutive days baseline samples from buccal surfaces, tongue and saliva were obtained from six volunteers, after which they used a mouthwash (Meridol, GABA, Switzerland) twice-daily for 14 days. Subsequently similar samples were obtained on two consecutive days. The microbial composition of the samples, with or without ex vivo PMA treatment, was assessed with 16S rRNA gene amplicon sequencing.

Results
Data showed a clear effect of mouthwash usage on the tongue and saliva samples. PMA treatment enhanced the observed differences only for the saliva samples. Mouthwash treatments did not affect the composition of the plaque samples irrespective the use of PMA.

Conclusions
The necessity to use a PMA treatment to block the DNA from dead cells in clinical studies aimed at measuring compositional shifts after the use of a mouthwash is limited to salivary samples.

Clinical Relevance
Measuring shifts in the oral microbiome could be hampered by the presence of DNA from dead cells.
Introduction

Most chronic oral infections are caused by microorganisms residing in a polymicrobial biofilm. Prevention and controlling of these infections may be achieved by the use of antibiotics and anti-microbials. The latter are designed to interfere with bacterial adhesion or bacterial metabolism, or to reduce bacterial, hence plaque, outgrowth (Marsh, 2012). Besides overall plaque reduction mouthwashes might induce a shift in the microbial composition of dental plaque (Mengel et al., 1996; Lingstrom et al., 2012).

In recent years the technologies to monitor the composition of biofilms have improved. Especially culture-independent DNA-based techniques have become available and are now widely used (Bizarro et al., 2013; Mason et al., 2013; Kistler et al., 2013; Crielaard et al., 2011; Ozok et al., 2012). A disadvantage of these technologies is that they do not differentiate between viable and dead bacterial cells. When evaluating the effects of anti-microbial compounds this might lead to an underestimation of the efficacy of treatments. Propidium monoazide (PMA) has been proposed to overcome this problem (Nocker et al., 2006). After PMA is covalently linked to DNA by photoactivation, this DNA cannot be amplified by PCR, which is required prior to analysis by amplicon-based sequencing methods. The intense light required for this photoactivation also inactivates the non-bound PMA. PMA does not penetrate cells that have an intact cell membrane and PMA treatment is therefore potentially suitable to distinguish between living and dead cells. This concept has been tested in a number of studies on single species (Nocker et al., 2006; Nocker et al., 2007), a study with oral pathogens (Loozen et al., 2011) and studies on more complex samples (Nocker et al., 2009; Lin et al., 2011). However, limited data are yet available on the necessity of including PMA treatments when analyzing compositional shifts after a relatively mild anti-microbial treatment. In a recent study we showed that PMA enhanced the observed differences after a chlorhexidine treatment in an in vitro polymicrobial saliva derived biofilm model (Exterkate et al., 2014). Yet, it remains unclear to what extend PMA enhances the observed compositional shifts also in samples from a clinical study.

The aim of this study was to evaluate the use of PMA when measuring compositional shifts in clinical samples after using a mouthwash for a short period of time.
Materials & Methods

Study design

The study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam. Six healthy volunteers participated in the study. They were asked to visit the laboratory four times. One week before the first visit they started to use a standard toothpaste (Prodent Cool Mint, Unilever, The Netherlands) twice daily, which was used throughout the study. The first and second visit were planned on two consecutive days after this one week washout period. After the second visit volunteers started to also use an AmF/SnF$_2$ mouth-rinse (meridol, GABA, Switzerland) twice daily, immediately following toothbrushing. They were asked to rinse with 10 ml mouthwash for 30 seconds using a timer. After using the mouthwash volunteers were asked not to drink or eat for 1 h. The third and fourth visit were held on two consecutive days after a 14-day period of mouthwash use.

The volunteers refrained from brushing in the 24-h period before the visit(s). The use of the mouthwash was continued and the last mouthwash was 2 h before the scheduled time of the visit, after which volunteers refrained from eating and drinking.

During each visit six separate samples were taken, two plaque samples, two tongue samples and one saliva sample, which was aliquoted. Each plaque sample was collected from the buccal surfaces of two molars in contralateral quadrants. One plaque sample was taken from the 16 and 27 with a sterile plastic ash and collected in a sterile 1.5 ml Eppendorf® tube containing 100 µl sterile PBS. The other sample was collected from the buccal surfaces of the 26 and 17 in the same way. One sample was treated with PMA while the other sample was left untreated. At visits 1 and 3 the samples from the 16/27 were treated with PMA and at the visits 2 and 4 the samples from 26/17 were treated with PMA.

For the tongue samples the tongue was virtually divided in half. A sterile micro-brush (Microbrush International, Grafton, USA) was used to collect one sample from each half. The tip of the micro brush was cut-off and stored in a sterile 1.5 ml Eppendorf®
tube containing 50 µl sterile PBS. On visits 1 and 3 the left-half sample was treated with PMA on visits 2 and 4 the right-half sample was treated with PMA.

Volunteers were asked to donate Parafilm® stimulated saliva (appr. five ml). After thorough mixing two 500 µl samples were taken. One sample was treated with PMA and the other sample was left untreated.

This overall design resulted in 24 samples per subject. The samples obtained from the subjects before the two weeks mouthwash period will be referred to as baseline samples. The samples after the two week mouthwash period will be referred to as post-rinse samples. The samples that were treated with PMA after collection will be referred to as PMA-treated samples.

**PMA treatment**

One mg Propidium monoazide (Biotum Inc., Hayward, USA) was dissolved in 100 µl 20% DMSO to give a 20 mmol/L stock. All samples were treated with 50 µmol/L PMA (Nocker et al., 2006; Loozen et al., 2011). In order to obtain this concentration 1.3 µl of PMA stock was added to 500 µl saliva. For the tongue and plaque samples the PMA stock was diluted ten times. To 100 µl plaque sample 2.5 µl diluted PMA-stock was added and 1.3 µl diluted PMA-stock was added to the 50 µl tongue samples.

The samples were incubated in the dark for five minutes and then exposed to intense light for two minutes using a 650 W halogen lamp at 25 cm from the samples. The samples were kept on ice during this procedure (Nocker et al., 2006). After PMA treatment the samples were stored at -80 °C until further use.

**DNA extraction, amplicon preparation and pyrosequencing**

Bacterial DNA was extracted as described previously (Crielaard et al., 2011), quantified (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Molecular probes inc, Willowcreek) and stored at -20°C until further analysis.

Barcoded amplicon libraries of the small subunit ribosomal RNA gene V5-V7 hypervariable region were generated for each of the samples as described previously.
Sequencing data analysis

The sequencing data was processed using QIIME (Quantitative Insights Into Microbial Ecology) version 1.5.0 (Caporaso et al., 2010) as described previously (Kraneveld et al., 2012). To allow comparisons among different samples from the same source, the dataset was randomly subsampled to a standardized number of reads/sample (3830 for plaque, 4040 for tongue and 960 for saliva samples). Then an OTU-based β diversity approach was used. For this, a normalized OTU abundance table was used in Principal Component Analysis (PCA), using PAST software (Hammer et al., 2001). In addition to an OTU-based approach, phylogenetic measures of community β diversity - weighted UniFrac, a quantitative measure (Lozupone et al., 2007) was applied. Principal coordinates analysis (PCoA) was used to compare groups of samples based on weighted UniFrac distance metrics.

Calculating distances between samples

The Unifrac distances data allow for the calculation of the weighted distance between two samples using the phylogenetic tree. The distances between samples from the same subject were used for the following comparisons for each of the sample sources. 1) “Baseline_noPMA vs duplicate baseline-noPMA”, which is an indication for the variation between the baseline samples. 2) “Baseline_PMA vs Baseline_noPMA” would show the effect of the PMA treatment on baseline samples. 3) “Baseline_noPMA vs Post-rinse_noPMA” shows the effect of the rinse period. And finally, 4) the “Baseline_PMA vs Post-rinse_PMA”, which shows the effect of PMA on the measured effect of the rinsing period.

Statistical analysis

The OTU data were analysed statistically using analysis of similarity (ANOSIM) (Clarke, 1993) using PAST software (Hammer et al., 2001). The Bray-Curtis similarity index was used and p-values were corrected using Bonferroni correction for multiple comparisons.
Weighted Unifrac Distance data were analysed statistically using ANOVA with Tukey’s post-hoc test (IBM SPSS version 20).

Results

Bacterial composition

The relative abundance of the 15 most abundant bacterial genera in three types of samples is shown in Figure 1. The microbiome data varied considerably between the six subjects. Therefore data are shown for each individual. For each individual the averaged data from the two samples per time point are shown.

Even considering the large inter-individual variation, some initial observations could be made. The main bacterial genera accounted for 92.4% (SD 4.4%) of the composition, indicating that the other genera accounted for, on average, 7.6% of the total flora (indicated by the bottom area ‘others’). The (general) composition of the plaque samples was neither affected by the rinse period nor by the PMA-treatment. However for subjects 1 and 5 the use of the mouthwash did result in a difference in the profiles from the baseline and post-rinse samples. The profile of the saliva samples differed in the post-rinse samples treated with PMA. Also the tongue samples showed a different profile in the post-rinse samples compared to the baseline samples.

Principal component analysis

In Figure 2 the principal component analysis (PCA) plots are shown and Table 1 shows the outcome of the Anosim analysis of the OTU data for the three types of samples. The data labels in Figure 2 indicate the subject number. The plaque baseline samples (fig. 2, panel A) clustered close to each other for each individual (filled symbols), it is also apparent that after PMA treatment samples from the same subject still clustered relatively close to each other. The post-rinse samples (open symbols) did not form clusters which were clearly separated from the baseline samples. The PMA treatment on the post-rinse samples did not create a separation (open squares vs open circles). Also the Anosim analysis did not reveal any significant differences between the plaque data (Table 1).
Figure 1. Relative abundance of the 15 most abundant bacterial genera in samples from the individual subjects from three different sources. From top to bottom: plaque, saliva and tongue samples. From left to right per subject: baseline samples without PMA treatment, baseline samples with PMA treatment, post-rinse samples without PMA treatment and post-rinse samples with PMA treatment. A version in colour is available in Appendix 1, page 124.
Figure 2. Principal component analysis plot of the first two components of samples from the different time points with and without PMA treatment. From top to bottom: plaque samples, saliva samples and tongue samples. Each marker represents a single sample (● baseline samples without PMA, ■ baseline samples with PMA treatment, ○ post-rinse samples without PMA treatment, □ post-rinse samples with PMA treatment). Data labels indicate the subject number.
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Table 1. p-values and r-values from analysis of similarity (ANOSIM) of the OTU-data. P-values indicate which groups were significantly different. R-values signify dissimilarity between groups, the higher the R-value the more dissimilar the compared groups are.

The PCA data for the saliva samples showed a different pattern (fig. 2, panel B). The baseline samples clustered per subject close to each other (filled symbols) while the PMA treatment did not result in a separation between the two groups of samples (filled squares vs filled circles). The post-rinse samples clustered away from the baseline samples (open squares vs filled symbols). This difference was enhanced by a PMA treatment (open squares vs filled squares). Differences between baseline and post-rinse samples were significant (Table 1). Also the post-rinse PMA treated samples were different from the post-rinse non-PMA treated samples, whereas the effect of the PMA treatment did not reach a statistically significant difference in the baseline samples (p=0.064). The main loadings for the first axis in the PCA plots (i.e. the main contributors to explaining the variance) were from OTU’s *Veillonella* and *Haemophilus*. Both groups showed a relative increase after the rinse period and after a PMA treatment. In contrast, the *Streptococcus* and *Porphyromonas* OTU’s both showed a decrease after the rinse period followed by the PMA treatment. For the second PCA axis the main loadings were also determined by *Haemophilus* and *streptococci*. 
For the tongue samples PCA analysis showed a distinct effect of mouthwash usage (fig. 2, panel C). The post-rinse samples clustered away from the baseline samples (open symbols vs filled symbols). This data also showed a broader cluster for the post-rinse samples. PMA treatment of the baseline samples did separate these samples from the non-treated baseline samples (filled squares vs filled circles). This was also the case for the post-rinse samples (open squares vs open circles). Anosim analysis confirmed the findings from the PCA. The use of the mouthwash caused a significant difference in the tongue samples (baseline vs post-rinse samples, Table 1). The PMA treatment did not result in significant differences in the baseline nor in the post-rinse samples.

The main loadings for the first axis in the PCA plots were from OTU *Haemophilus* which increased after the rinse period and the PMA treatment and OTU’s *Veillonella* and *Prevotella* which decreased after the rinse period. For the second axis the main loading was from *Streptococcus* which showed an increase after the rinse period. This increase was smaller when a PMA treatment was applied. *Neisseria* and *Porphyromonas* were among the main loadings for the second axis. Both decreased after the rinse period.

*Principal coordinate analysis*

In Figure 3 the principal coordinate analysis (PCoA) of UniFrac distances data are shown for the three types of samples. These results were in agreement with OTU-based PCA analysis (fig. 2).

Panel A shows the plaque data. The data points for the baseline and post-rinse samples (filled symbols vs open symbols) did not separate, indicating that there were no differences. The PMA treatment did not create separate clusters either (filled squares vs filled circles and open squares vs open circles).

Panel B shows that the post-rinse saliva samples form a separate cluster from the baseline saliva samples (open symbols vs closed symbols). The post-rinse samples without PMA treatment (open circles) did not separate completely from the baseline samples (filled circles). The separation was enhanced by the PMA treatment (open squares vs filled squares). The post-rinse PMA treated samples (open squares) separated from the other samples.
Figure 3. Principal coordinate analysis plot of the first two components of samples from the different time points with and without PMA treatment. From top to bottom: plaque samples, saliva samples and tongue samples. Each marker represents a single sample (● baseline samples without PMA, ■ baseline samples with PMA treatment, ○ post-rinse samples without PMA treatment, □ post-rinse samples with PMA treatment). Data labels indicate the subject number.
The tongue samples (panel C) showed that the baseline samples (filled symbols) separated from the post-rinse samples (open symbols). PMA treatment did not create a separate cluster for either the baseline samples (filled squares vs filled circles) nor for the post-rinse samples (open squares vs open circles).

**Distances between samples**

The averaged distances between the respective groups in the PCoAnalysis on UniFrac weighted distance data are shown in Figure 4. The data shows that there were no significant differences between the plaque samples. The saliva data showed that the PMA treated baseline samples separated from the non-treated baseline samples. Also the post-rinse samples showed a significant difference in the distance compared to the non-PMA treated baseline samples. Moreover, the comparison between the PMA-treated baseline samples and PMA-treated post-rinse samples showed that the distance between samples as a result of the rinse period increased when a PMA treatment was applied.

The tongue samples showed a clearly increased distance between the baseline samples and the post-rinse samples. The data also showed that a PMA treatment of the baseline samples or the post-rinse samples did not result in an increased distance between the samples.

**Discussion**

The current study shows that a two-week period of twice daily rinsing with a mild anti-microbial mouthwash affected the bacterial composition of saliva and tongue samples, while dental plaque samples did not show a significant shift in composition. A PMA treatment enhanced the observed differences only for the saliva samples.
Figure 4. Averaged weighted distances between groups of samples in the phylogenetic tree. For each subject the distances between samples were calculated. For each source four comparisons were made. Each bar represents the averaged distance for the samples of the six subjects. Bars with the same symbol within each sample source are not statistically different (p<0.05).

As a result of the daily use of mouthwashes not only the amount of plaque on tooth surfaces and tongue but also the composition of these plaques might change. Mouthwashes should shift plaque towards a more healthy composition. Such shifts are now being assessed by DNA-based techniques. A disadvantage of these methods is that vital and non-vital bacterial cells cannot be distinguished. Several studies have already shown that PMA treatments enable the discrimination between live and dead cells both in in vitro studies (Loozen et al., 2011; Nocker et al., 2009; Lin et al., 2011) and in clinical samples (Kim et al., 2013; Yasanuga et al., 2013). Most studies using PMA treatments were performed using Quantitative-PCR and were aimed at determining the numbers of selected species. Assessing the effect of a PMA treatment on compositional shifts has been limited. Alvarez (Alavarez et al., 2013) showed in a 5-species biofilm model that PMA treatments allowed for the determination of viable cells after a treatment with cetylpyridinium chloride (CPC). Exterkate (Exterkate et al., 2104), showed in vitro that PMA treatments enhanced the observed differences in biofilm composition after a Chlorhexidine rinse. This
implies that PMA treatments may be necessary to obtain a correct picture of the bacterial composition after antibacterial treatments.

The current study assessed shifts in the bacterial composition of clinical samples as a result of mouthwash usage. Such compositional shifts will only be observed if various bacterial species are affected differently by the mouthwash. We note that, in general, compositional shifts after mouthwash applications may be due to either selective killing or selective outgrowth of specific species. Moreover, PMA treatment will only lead to different measured composition if the ratio between dead and live bacterial cells is different for different species.

In the current study the most pronounced effect of the mouthwash on bacterial composition was measured for the tongue samples. A clear compositional shift occurred after two weeks mouthwash use. This shift was apparently not affected by the presence of non-viable cells in the tongue samples, as PMA treatment did not enhance or reduce the observed rinse effect. This finding suggests that the composition of the tongue samples shifted towards increased numbers of species that were less susceptible to the AmF/SnF containing mouthwash. Another possibility would be that the observed shift is caused by selective outgrowth of specific species. From the lack of a PMA effect we conclude that DNA from cells killed during antimicrobial treatment is quickly removed i.e. within the two hour timeframe between the last rinse and the collection of the samples, unless such killing was not selective.

The salivary microbiome changed as a result of the two weeks mouthwash period. Bacteria in saliva are derived from biofilms from all oral surfaces (both hard and soft tissues). A shift in the composition of saliva therefore reflects changes in the oral biofilms. PMA treatment showed a clear effect on the measured composition of both baseline and post-rinse salivary samples. This finding indicates that for both types of saliva samples the ratio’s between living and dead cells were different for the various species. A possible explanation for the PMA effect could be that, as salivary bacteria are derived from all oral surfaces, saliva typically contains shedded cells from oral biofilms, and therefore contains a higher proportion of dead cells. Also salivary anti-microbial peptides could influence the ratio between live and dead cells. This effect
could be more pronounced in saliva than in plaque or tongue samples. The shift in composition after a PMA treatment was more apparent for the post-rinse samples than for the baseline samples, which is also an indication that the post-rinse samples contained a higher amount of dead cells than the baseline samples.

In two out of six subjects the bacterial composition of the plaque samples changed after the two week mouthwash period. In the other subjects no changes could be observed. The buccal dental surfaces were brushed 24 hours before the visit to the clinic and the mouthwash use was continued up to 2 h prior to the visit in the dental clinic. Any plaque accumulation occurred by outgrowth of the bacteria that were left after brushing or from those species that newly adhered and grew out in that 24h timeframe. The fact that no shifts in the composition were observed for the buccal plaque samples indicates that the mouthwash did not influence outgrowth or recolonization of these surfaces. The PMA treatment did not induce shifts in the measured composition, indicating that either the cells that were killed by the mouthwash were removed quickly or that killing of the cells was not selective.

The current study was not aimed at determining the overall efficacy of a mouthwash, as would be evidenced by reduced plaque scores or bacterial cell counts in saliva. Instead, the rationale for this study was whether PMA treatments have an effect on the measured bacterial composition, presumably resulting in a more accurate account of the viable bacteria in the respective microbiomes.

From the current study we conclude that measuring compositional shifts in tongue and plaque samples using DNA-based techniques is not affected by DNA from dead cells.

In contrast, the measured composition of salivary samples was influenced by the presence of DNA from dead cells. The necessity to use a PMA treatment to block the DNA from dead cells in clinical studies studying mouthwashes and aimed at measuring compositional shifts seems to be limited to salivary samples.

In general, we conclude that caution is indicated when DNA based methods are used to determine bacterial composition of (in vivo) biofilms. In the current study sample type and effect of rinse differed in the extent to which dead cells affected the
outcome. Therefore including a treatment step in the sample analysis, such as PMA binding to the DNA from dead cells, should always be considered.

Acknowledgement

Conflict of interest
The study was financially supported by GABA International AG (Therwil, Switzerland). There were no restrictions with respect to the design of the study or the publication of the results.

Author Contributions
Study design: RE, WC, JC. Performed the experiment: RE, MB, JK. Analyzed the data: RE, EZ, BB. Wrote the paper: RE, EZ, WC, JC.

Ethical Standards
This study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam.

References


