Biofilm and dental caries: application of the constant depth film fermentor
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Citation for published version (APA):

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Chapter 6

Susceptibility of Biofilm Cells to Chlorhexidine
Abstract

This study aims to compare bacterial cells in undisturbed biofilms with cells dispersed from biofilms for their susceptibility to chlorhexidine. *Streptococcus mutans* biofilms were grown in dentin specimens either in grooves or on the surfaces in a Constant Depth Film Fermentor (CDFF). After 15 days, the specimens were removed from the CDFF. The two types of biofilm cells were then treated with either sterile water, 0.05% or 0.2% chlorhexidine for 10 min. Viability of biofilm cells and acid production after a 10-min 1% glucose application were measured by plating and by capillary electrophoresis, respectively. The results showed that the effects of chlorhexidine on viability of biofilm cells were seen in all groups and were dose-dependent. The reduction in viability of the cells in undisturbed groove biofilms was much less than in the other groups for both chlorhexidine treatments. A significant reduction in lactic acid production was seen in all groups after 0.05% chlorhexidine treatment and no further reduction was observed using 0.2% chlorhexidine. The magnitude of the chlorhexidine effect on lactic acid production was not significantly different among the groups. In conclusion, the geometry of the substratum substantially affected the viability of the biofilms after antimicrobial treatments. The changes in acid production of biofilm cells did not always follow the changes in viability.

Introduction

Biofilms have been demonstrated to be more resistant to antimicrobial treatment than planktonic cells [Kinniment *et al.*, 1996b; Costerton *et al.*, 1999]. This phenomenon was observed by comparing cells grown in biofilms with those grown in broth. To further understand the mechanism of this resistance, cells dispersed from biofilms and cells in undisturbed biofilms were compared for their resistance to antimicrobials or to acid shock [Millward and Wilson, 1989; Li *et al.*, 2001]. It was shown that chlorhexidine was less effective against cells in *Streptococcus sanguis* biofilms than against cells dispersed from the same biofilms, regardless of the age of the biofilms and chlorhexidine application time [Millward and Wilson, 1989]. This indicated that the biofilm suprastructure might protect cells against antimicrobials. In another study,
where the acid tolerance response of *Streptococcus mutans* was investigated, disruption of the structure was shown to only reduce the acid resistance of biofilm cells when the biofilms were grown with sucrose. No effect was observed when biofilms were grown with glucose [Li et al., 2001]. In the abovementioned studies, only viability of the biofilm cells was used as the outcome variable.

In caries research, acid production by bacterial cells can serve as indicator for both bacterial metabolism and risk of lesion formation, since the initial event in caries is the acid induced dissolution of dental hard tissue. Due to the relationship between bacterial viability and acid production, in most studies only bacterial viability is assessed to evaluate the efficacy of antimicrobials. However, results from some clinical and *in vitro* studies, where the efficacy of antimicrobials on both viability and acidogenicity of dental bacteria was tested, indicated that the changes in acid production did not always follow the changes in viability. Several authors reported a significant reduction of acid production but no suppression of bacterial viability after chlorhexidine application [McDermid et al., 1985; Gerardu et al., 2003].

The aim of this study was to test the effects of chlorhexidine on viability and acid production of bacterial cells in undisturbed biofilms and cells dispersed from biofilms. For this, *Streptococcus mutans* biofilms were grown in a Constant Depth Film Fermentor. The substratum was dentin with or without grooves, which modeled retention sites and smooth surfaces of the teeth, respectively.

**Materials and Methods**

**Constant Depth Film Fermentor**

The equipment used for growing biofilms was a Constant Depth Film Fermentor (CDFF) (University of Wales, Cardiff). It is equipped with a rotating turntable containing 15 polytetrafluoroethylene (PTFE) pans. The turntable rotates under PTFE scraper bars that smears the incoming medium over the 15 pans. Each sampling pan has 5 cylindrical holes (5.0 mm in diameter), which contain dentin specimens placed on top of PTFE plugs.

**Preparation of Specimens**

Two types of dentin discs (n = 48) were used to grow biofilms. One type was dentin discs with three parallel grooves (330 µm wide and 650 µm deep). The other type was
dentin discs with smooth surfaces. The preparation of the specimens was described in detail in a previous study [Deng and ten Cate, 2004]. The dentin discs with grooves were placed flush with the surface of PTFE pans, while the dentin discs with smooth surfaces were recessed to a depth of 300 μm.

**Inoculation and Operation of the CDFF**

The bacterium used in this study was *Streptococcus mutans* C180-2 [De Stoppelaar et al., 1967]. Prior to inoculating the CDFF, 100 ml sterile stimulated human saliva was pumped into the CDFF for 3.5 h to form pellicles on the surfaces. Then, 15 ml of an overnight culture of *S. mutans* was mixed with 500 ml growth medium and pumped into the CDFF for 8 h. After 8 h, the inoculum flask was disconnected. Sterile growth medium or 2% (w/v) sucrose solution was dripped into the system alternately by a peristaltic pump (Type MS-4/6-100, Ismatec, Zürich, Switzerland) at a rate of 0.5 ml/min. The sucrose was applied 4 times per day for periods of 30 min. The growth medium contained 3.7 g/L BHI medium (Difco, Laboratories, Detroit, Michigan, USA), 1.5 mM CaCl2 and 25 mM PIPES buffer. pH was adjusted to 7.0 by the addition of 5 M KOH. The whole experiment lasted 15 days.

**The Regime of the Treatments**

On day 15, specimens were removed from the CDFF before the start of a next sucrose pulse. *S. mutans* biofilms, either left undisturbed or removed from the dentin discs, were treated outside the CDFF. The treatment solutions included sterile Milli-Q water, 0.05% and 0.2% chlorhexidine digluconate solutions. Four groups were defined according to the status of the biofilm cells and the type of the substratum. They were undisturbed biofilms in grooves (GU), undisturbed biofilms on surfaces (SU), dispersed biofilm cells from grooves (GD), and dispersed biofilm cells from surfaces (SD). The regime of the treatments is illustrated in figure 1.

For groups GU and SU, the specimens with biofilms were placed in 4 ml treatment solutions for 10 min. Then all samples were rinsed with Milli-Q water to remove the treatment solutions and kept in 40 ml cysteine peptone water (CPW) for 60 min at 37°C. Then the samples were transferred to 100 μl 1% glucose solutions for 10 min in order to test the acidogenicity of the biofilms. The reaction was stopped by placing the samples on ice. The biofilms were removed from the respective surface into the glucose solution for viability and acid production analysis.
For groups GD and SD, *S. mutans* biofilms were first removed from the grooves or the surfaces of dentin and mixed thoroughly with 100 μl of treatment solutions for 10 min. Treatments were stopped by adding 10 ml Milli-Q water. Supernatants were discarded after centrifugation (10 min, 13000 rpm, 20°C). The cells were kept in 1 ml CPW for 60 min at 37°C, after which cells were harvested by centrifugation (10 min, 13000 rpm, 20°C). Then 100 μl 1% glucose solution were added to the cells for 10 min, the reaction was stopped by placing the glucose solution with the cells on ice.

Removing bacterial cells from the dentin grooves was done by repeated scraping with 3 consecutive sterile paper points (size #20, QDENT; NDO Leeflang, the Netherlands). This method was discussed in detail by Deng et al. [2004a]. The biofilms on the surfaces were removed by microbrushes (regular size, Microbrush International Ltd, Ireland).

**Assessment of Viability and Acidogenicity**

The biofilm samples were dispersed by sonicating on ice for 30 s and vortex mixing for 30 s. Aliquots of each sample (10 μl) were plated on BHI agar plates after serial dilution. The plates were incubated anaerobically at 37°C for 3 days.

The remainder of each of the samples was placed in a water bath at 80°C for 5 min and centrifuged for 15 min at 13000 rpm at 4°C (Heraeus centrifuge, Dijkstra bv, the Netherlands). The supernatants were analyzed for organic acids by capillary electrophoresis. The pellets were determined for protein content by Bradford’s method [Damen et al., 2002].

**Statistic Analysis**

All statistical analyses were performed with SPSS (Version 9.0). The output variables were viable counts and lactic acid production of the biofilm cells. The efficacies of the treatments on these among the four groups were compared by two-way Analysis of Variance (ANOVA). The data of the four groups under water application were also compared by one-way ANOVA.
Fig. 1. The regime of the treatments. GU: undisturbed biofilms in dentin grooves; SU: undisturbed biofilms on dentin surface; GD: dispersed biofilm cells from dentin grooves; SD: dispersed biofilm cells from dentin surface. The treatments included sterile water, 0.05% and 0.02% chlorhexidine digluconate.

Results

Viability of the Biofilms

After 15 days, biofilms were visible in the dentin grooves and on the dentin surfaces. The viability of biofilm cells after water treatment was similar among the groups (data not shown). These values served as baseline for each group to calculate the
percentage of survival of the biofilm cells after treatment (Figure 2). In all groups a significant effect of chlorhexidine was observed, which was generally dose-dependent. The magnitude of this effect was different among the experimental groups. In group GU, the 0.2% chlorhexidine treatment resulted in only 94% reduction in viability, while the other groups showed more than 99.8% reduction. Since the viable counts in the latter three groups had all dropped to or under the detection limit, the precise efficacy of the treatments could not be compared.

The difference between the groups was also seen, though less pronounced, after 0.05% chlorhexidine treatment, where no reduction was found for group GU, and around 90% reduction for the other three groups. No difference in reduction of viability was found between group SU and SD.

**Fig. 2.** The percentage survival of biofilm cells after 0.05% and 0.2% chlorhexidine treatments in four groups. The percentage was calculated by considering the viable cells after water treatment at 100%. GU: undisturbed biofilms in dentin grooves; SU: undisturbed biofilms on dentin surface; GD: dispersed biofilm cells from dentin grooves; SD: dispersed biofilm cells from dentin surface. The dark horizontal line marks the detection limit (n = 4).

### Lactic Acid Production of the Biofilms

Various organic acids were produced by biofilm cells after 1% glucose applications. Lactic acid was the predominant acid. There were also traces of acetic acid and propionic acid. As viability, lactic acid produced after the chlorhexidine treatments was calculated as percentage of the values found after water treatment (Figure 3). In
all groups, a significant reduction of lactic acid production was seen after 0.05% chlorhexidine treatment and no further reduction was observed after 0.2% chlorhexidine treatment. When the effects of the chlorhexidine were compared among the groups, it was observed that they increased in the order: GD > SD > SU > GU, although the differences did not reach significance.

**Fig. 3.** The percentage of lactic acid produced by biofilm cells after 0.05% and 0.2% chlorhexidine treatments in four groups, when compared to those after water treatment. GU: undisturbed biofilms in dentin grooves; SU: undisturbed biofilms on dentin surface; GD: dispersed biofilm cells from dentin grooves; SD: dispersed biofilm cells from dentin surface (n = 4).

**Discussion**

In this study, cells dispersed from biofilms and cells in undisturbed biofilms were compared for their susceptibility to chlorhexidine in terms of both viability and acidogenicity. The effect of the geometry of the substratum, with or without grooves, on this susceptibility was also investigated.

In a previous study, when growing *S. sanguis* biofilms on filter membranes, Millward and Wilson [1989] found a significant resistance to chlorhexidine for the cells in undisturbed biofilms when compared to the dispersed cells. It was assumed that the suprastructure of biofilms protect the cells from the killing effect of

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chlorhexidine. However, in the current study, no different reduction in viability was observed between the two types of cells when the biofilms were grown on the surface. Only when the biofilms were grown in the grooves, significantly less reduction in viability was seen for the cells in undisturbed biofilms than for the disrupted cells. It seemed that the suprastructure of biofilms *per se* could not protect cells but the geometry of the grooves might restrict the access of chlorhexidine and therefore have protected the cells. The different results between our study and the cited study might be caused by the different growth conditions and different bacterial species in the two studies.

We noticed that biofilm cells in the current study, even after being dispersed, were more resistant to chlorhexidine than cells grown in broth. In a pilot study, we grew *S. mutans* in brain heart infusion overnight and even 0.001% chlorhexidine induced 1-1.5 log reductions (data not shown). In this study, to achieve the same reduction in the dispersed biofilm cells, 0.05% chlorhexidine treatment was needed. Since dispersed cells can come in close contact with chlorhexidine after being dispersed, we presume that the increased resistance might be related to a phenotypic change of the cells when they are grown as biofilm.

The results from group GU showed a reduction in lactic acid production induced by 0.05% chlorhexidine treatment when there was no decrease in viability, which confirmed the findings from clinical and other *in vitro* studies [McDermid *et al.*, 1985; Gerardu *et al.*, 2003]. It is documented that the damage in bacterial cells by chlorhexidine was reversible when the concentration of chlorhexidine was low [Denton, 2000]. Therefore, we hypothesize that the metabolic activity of cells was impaired by the treatment, evidenced by a reduction of acid production, but that the cells recovered during growing on the agar plates for three days, resulting in no observable decrease in viability.

In conclusion, the differences in the susceptibility to chlorhexidine between the cells dispersed from biofilms and the cells in undisturbed biofilms were influenced by the structure of the substratum. The changes in acid production of bacterial cells after chlorhexidine treatment did not necessarily follow the changes in viability.