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Streptococcus mutans and *Streptococcus sobrinus* biofilm formation and metabolic activity on dental materials

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Abstract

Objectives. To examine potential correlations between streptococcal biofilm formation and lactate production in streptococcal biofilms formed on the surface of dental materials with different surface characteristics. ***Materials and methods.*** Samples of a glass-ionomer cement (*Ketac Molar*) and a ceramic (*Empress 2*) were incubated with whole saliva and suspensions of *Streptococcus mutans ATCC 25175* or *Streptococcus sobrinus ATCC 33478* for initiating single-species biofilm formation for either 4 or 24 h. The relative amount of adherent, viable cells was determined using a Resazurin and a MTT assay. Metabolic activity was assessed by quantifying lactate production with a modification of the commercial *Clinpro Cario L-Pop* kit. ***Results.*** Both assays identified similar *S. sobrinus* biofilm formation on the two substrata; for *S. mutans,* the MTT test showed significantly fewer streptococci on the glass-ionomer cement than on the ceramic. Concerning metabolic activity, for *S. sobrinus,* significantly higher lactate production was observed for biofilms formed on the glass-ionomer cement in comparison to the ceramic, whereas similar values were identified for *S. mutans.* ***Conclusions.*** Within the limitations of the study, the results suggest that the pure amount of adherent streptococci does not *a priori* indicate the metabolic activity of the cariogenic bacteria organized in the respective biofilm. Thus, comparisons between the relative amount of adherent streptococci and their metabolic activity may allow for an improved understanding of the effect of dental material surfaces on the formation and metabolic activity of streptococcal biofilms.

Key Words: *lactate production, Streptococcus, biofilm, bacterial adhesion*

Introduction

Secondary caries is counted among the most frequent reasons for the replacement of dental restorations [1]. With regard to this aspect, plaque formation on the surface of dental restoratives may contribute to the occurrence of secondary caries lesions [2]; thus, it is wishful that dental materials yield low susceptibility to plaque formation on their surface. In the recent years, numerous studies have investigated biofilm formation on dental restorative materials, yet these studies merely focused on the quantification of adherent bacteria or biomass on the surface of different dental restorative materials. However, as caries are predominantly caused by the demineralization of enamel and dentin by acids produced by the metabolic activity of cariogenic bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus spp.*, the amount of adherent bacteria does merely give an impression of microbial colonization, but does not indicate their metabolic activity and thus the potential pathogenic impact of the microorganisms adherent to the surface. Although it might be assumed that high amounts of adherent streptococci correlate with high lactate production, this correlation has—to the best knowledge of the authors—not been analyzed.

With regard to these aspects, the objective of the present study was to examine potential correlations between the streptococcal biofilm formation on the surface of two distinct dental materials and the metabolic activity of the microorganisms organized in the biofilm. It was hypothesized that the higher the

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relative amount of adherent *S. sobrinus* and *S. mutans* cells, the higher the production of lactate in biofilms formed on the surface of two distinct dental materials.

Materials and method

Preparation of samples

Standardized samples (diameter 10 mm, thickness 1.5 mm) were prepared from a glass-ionomer cement (*Ketac Molar*; 3M ESPE, Seefeld, Austria), and a lithium-disilicate ceramic (*Empress 2*; IvoclarVivadent, Schaan, LIechtenstein). Samples made from the glass-ionomer cement were prepared by placing the material into standardized molds, whereas the ceramic samples were prepared from standardized wax blanks that were processed in accordance with manufacturer's instructions for use. All samples were smoothed using silicone carbide paper (grain 1000 and 4000, successively; Buehler GmbH, Düsseldorf, Germany) and a rotating grinding disc apparatus (Motopol 8, Buehler Ltd., Coventry, UK).

Surface roughness analysis

For determination of peak-to-valley surface roughness (R_a), a profilometric contact measurement device (Perthen S6P, Feinprüf-Perthen, Göttingen, Germany) was used; R_a was determined on three randomly selected spots on the surface of each of 10 samples from each material (one in central position, two at the margins).

All samples were then stored in distilled water for 6 days prior to the experiments and were rinsed with 70% ethanol solution and deionized water prior to the experiments for surface equilibration, then each sample was inserted into a well of a 48-well plate (48-Well Cell Culture Cluster, Corning Inc, Lowell, MA).

Saliva preparation

Unstimulated whole saliva was collected directly prior to the experiments by expectoration from one volunteer healthy female donor aged 23 years, who refrained from ingestion and oral hygiene for at least 2 h. Saliva was sterilized using single-use filtration devices directly prior to the experiments (Vacuflo, Schleicher & Schüll Microscience GmbH, Dassel, Germany; 0.45 and 0.22 µm, successively).

Microbiological procedures

The species *Streptococcus mutans ATCC 25175* and *Streptococcus sobrinus ATCC 33478* (both by DSMZ; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) are routinely maintained in our laboratory with weekly sub-cultures in trypticase soy yeast extract medium (medium 92; DSMZ) and long-term storage at −70°C. For work with streptococcal suspensions, the bacteria were grown in static cultures at 37°C in medium 92 until the early-stationary phase of growth. Cells were harvested by centrifugation (3000 rpm, 18°C, 5 min; Hettich *Rotixa P*, Tuttlingen, Germany), washed twice with Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich, St. Louis, MO) and resuspended in sterile medium 92. The optical density of the suspensions was adjusted to 0.3 at 550 nm (*Genesys 10-S*, Thermo Spectronic, Rochester, NY), which corresponds to a microbial concentration of 3.65×10^8 cells/mL [3].

Biofilm formation

For simulating *S. sobrinus* and *S. mutans* biofilms, pellicle formation was initiated by incubating the sample-containing plates with 1 mL per well of sterile human whole saliva at 37°C in a thermo shaking device (*OrbitalShaker*, Thermo Forma, Marietta, OH). After 2 h, saliva was removed carefully and each sample was incubated with 1 mL of either *S. sobrinus* or *S. mutans* suspension for either 4 or 24 h.

Analysis of lactate production

Metabolic activity of the adherent streptococci was analysed by measurement of lactate production in biofilms formed on five samples of each material, bacterial species and incubation time; the experiments were repeated three times for validation. Directly prior the experiments, the reaction solution was transferred from commercially available *Clinpro Cario L-Pop* blisters (CCLP; 3M ESPE) to a light-proof beaker glass. Similarly, the enzyme solution was carefully transferred from the CCLP blister and mixed with glycylglycin buffer (AppliChem, Darmstadt, Germany) using a dilution of 3:65, i.e. 480 µl of enzymatic solution were mixed with 9.92 mL of glycylglycin buffer.

After incubation with streptococcal suspensions, samples were carefully removed from the plates, transferred to new well plates whose wells were filled with 1 mL of PBS each and gently washed twice by manual fluctuation. Subsequently, samples were carefully transferred to new well plates and incubated with 1.5 mL of a solution of 5% D(+)-saccharose (Carl Roth GmbH + Co KG, Karlsruhe, Germany) in PBS. After 1 h, 50 µl of each aliquot were transferred to a 96-well plate (Microtiter plate, Nunc, Kamstrup, Denmark) and mixed with 130 µl of *Clinpro* enzymatic solution, 30 µl of *Clinpro* reaction solution and 20 µl of a freshly prepared solution of β-NAD$^+$ (Carl Roth GmbH + Co KG, Karlsruhe, Germany; 21.5 mg β-NAD$^+$ per mL deionized water). Changes in absorbance (ΔE/min) were calculated from eight measurements at 570 nm each 15 s over a time span of 2 min using an automated plate reader (Fluostar Optima, bmg Labtech, Offenburg, Germany) and were averaged for further analysis.

For quantification of lactate production, a calibration curve was established using solutions of sodium L-lactate (Sigma-Aldrich) in deionized water, applying sodium L-lactate concentrations ranging from 0.1–3 mM as standards. Analysis of the calibration curve was repeated twice and the mean values were transferred to a trend line (Microsoft Excel 2003; Microsoft Corporation, Redmond, WA). The linear equation and the coefficient of determination were calculated.

Quantification of adherent viable streptococci

For quantification of adherent viable streptococci, Resazurin reduction (Alamar Blue) was used as introduced earlier [4–9]. After pellicle formation, samples were incubated either with *S. mutans* or *S. sobrinus* suspension (1 mL) and Resazurin (15 µL, 0.007536 g/10 mL, Resazurin; Sigma-Aldrich) for either 4 or 24 h and subsequently carefully rinsed twice with PBS to remove unbound cells using a standardized protocol for minimizing the risk of removing adherent bacteria. Adherence of streptococci was quantified by measuring the dimensionless relative fluorescence intensity [10]. A total of 15 samples were used for each material, bacterial species and incubation time, including five samples for control purposes. The five control treatments included incubation of test samples with Resazurin dye but no bacteria (dye control; $n = 3$), incubation of test samples with bacteria but no Resazurin dye (bacteria control; $n = 1$) and incubation of bacteria with medium 92 (medium control; $n = 1$). The relative fluorescence intensity values for the test samples ($n = 10$) were calculated by subtracting the average relative fluorescence intensity measured for the dye controls from the relative fluorescence intensity values measured for each of the 10 test samples.

Due to the high standard deviations for data gathered with the Resazurin test, biofilm formation was repeated and analyzed using the MTT test as introduced previously [11]. Analogously to the Resazurin-based approach, a total of 15 samples were used for each material, bacterial species and incubation time, including five samples for control purposes. MTT solution was prepared by dissolving 5 mg/mL thiazolyl blue tetrazolium bromide (Sigma-Aldrich) in sterile PBS and PMS solution was prepared by dissolving 0.3 mg/mL phenazine methosulfate (Sigma-Aldrich) in sterile PBS. Biofilm formation was simulated analogously as for the Resazurin-based approach, including the formation of the acquired salivary pellicle (2 h) and incubation with *S. mutans* or *S. sobrinus* suspension for either 4 or 24 h. MTT reaction solution was prepared by freshly mixing 1 mL of MTT solution, 1 mL of PMS solution and 8 mL of sterile PBS. Subsequent to biofilm formation and careful rinsing, 200 µL of the MTT reaction solution were added to each sample well and incubated under light-proof conditions at 37°C. After 5 h, the reaction solution was gently removed from each well and 200 µL of a lysing solution consisting of 10% V/V sodium dodecyl sulfate and 50% V/V dimethylformamide (both Sigma-Aldrich) in distilled water was added to each well. After 1 h of further incubation under light-proof conditions at 37°C, 180 µL of the aliquot was transferred to new sterile well plates and the absorbance was measured at a wavelength of 550 nm (Fluostar Optima). Control treatments included dye controls ($n = 2$), medium controls ($n = 2$) and bacteria controls ($n = 2$).

Statistical analysis

All calculations and graphic display were carried out using SPSS 16.0 for Windows (SPSS Corporation, Chicago, IL). Means and standard deviations were calculated for R_a and relative fluorescence intensities and for lactate production applying the linear equation for the calibration curve. Normal distribution of data set was verified prior to further analysis using the Shapiro-Wilk test; homogeneity of variances was also preliminarily checked using Bartlett's test. Statistical analysis was performed using one-way (R_a) and two-way analysis of variance (ANOVA; relative fluorescence intensities, absorbance and lactate levels), setting incubation time and substratum as fixed factors for each bacterial species. Pearson correlation analysis was used to determine the correlation between the relative amount of adherent streptococci and lactate levels. The level of significance (α) was set to 0.05.

Results

Surface roughness (R_a)

For the glass-ionomer cement (mean R_a 0.18 (±0.08) µm), one-way ANOVA indicated significantly higher R_a than for the ceramic (0.04 (±0.01) µm; $p < 0.001$).

Biofilm formation

Values for relative fluorescence intensity (Resazurin test) and absorbance (MTT test) are indicated in Table I.

For the Resazurin test and *S. sobrinus*, two-way ANOVA indicated no significant differences between the two substrata, indicating the presence of similar amounts of streptococci ($p = 0.498$). A significant impact was observed for the incubation times ($p = 0.028$), as significantly higher relative fluorescence intensities were identified after 24 h biofilm formation than after 4 h biofilm formation, which indicates higher levels of viable S. *sobrinus* after

Table I. Relative fluorescence intensities (Resazurin-based approach; no unit), absorbance values (MTT-based approach; no unit) and lactate levels (mmol/L) in *Streptococcus mutans* and *Streptococcus sobrinus* biofilms on the surface of glass-ionomer cement (*Ketac Molar*) and ceramic (*Empress 2*); means and standard deviations are indicated.

	Relative fluorescence intensity				Absorbance			
Bacterial strain	*S. mutans*		*S. sobrinus*		*S. mutans*		*S. sobrinus*	
Biofilm formation	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
Glass-ionomer cement	16 571 (9421)	20 068 (5127)	17 196 (6751)	26 862 (7085)	0.05 (0.02)	1.08 (0.15)	0.07 (0.03)	1.93 (0.18)
Ceramic	22 723 (11 527)	19 909 (11 970)	17 275 (5760)	22 372 (16 939)	0.04 (0.02)	1.69 (0.44)	0.08 (0.03)	1.84 (0.46)

	Lactate production			
Bacterial strain	S. mutans		S. sobrinus	
Incubation time	4 h	24 h	4 h	24 h
Glass-ionomer cement	0.20 (0.20)	0.70 (0.47)	0.23 (0.20)	0.91 (0.34)
Ceramic	0.09 (0.09)	0.52 (0.30)	0.17 (0.17)	0.64 (0.36)

24 h biofilm formation. For *S. mutans*, no significant differences in relative fluorescence intensities concerning the different substrata ($p = 0.344$) and incubation times ($p = 0.914$) were found, which indicates similar amounts of viable *S. mutans* cells.

For the MTT test and *S. sobrinus*, two-way ANOVA identified no significant differences between the two substrata ($p = 0.608$), which indicates similar amounts of viable streptococci. A significant increase in absorbance values was measured after 24 h biofilm formation in comparison to 4 h biofilm formation ($p < 0.001$), which indicates higher amounts of viable streptococci after 24 h biofilm formation. For *S. mutans*, significantly higher absorbance values were measured for biofilms formed on the ceramic in comparison to those formed on the glass-ionomer cement ($p < 0.001$), which indicates significantly higher levels of viable *S. mutans* cells on the ceramic. After 24 h biofilm formation, a significant increase in absorbance was identified in comparison to 4 h biofilm formation ($p < 0.001$), which indicates a significant increase in the amount of adherent viable streptococci.

Lactate production

The coefficient of determination found for the standard lactate solutions was 0.967. Values for lactate production are indicated in Table I. For *S. sobrinus* biofilms, two-way ANOVA showed significant influences of the substratum material ($p = 0.035$) and the incubation time ($p < 0.001$) for lactate levels, which indicates significantly lower lactate levels for biofilms formed on the ceramic than on the glass-ionomer cement and significantly higher lactate levels after 24 h of incubation than after 4 h. For *S. mutans* biofilms, two-way ANOVA showed significant influences of the incubation time on lactate values ($p < 0.001$), indicating significantly higher values after an incubation of 24 h, but no significant influence of the substratum material on lactate levels ($p = 0.067$).

Correlation analysis

Pearson correlation coefficients for relative fluorescence intensities (Resazurin-based approach) and lactate production ranged between −0.347 and 0.432; no significant correlations between relative fluorescence intensities and lactate levels were observed.

Pearson correlation coefficients for absorbances (MTT-based approach) and lactate production ranged between −0.260 and 0.670; a significant correlation was identified for absorbances measured for 4 h *S. mutans* biofilm formation on the glass-ionomer cement and the corresponding lactate levels ($p = 0.034$).

Discussion

The results of the present study suggest rejection of the research hypothesis which proposed a simple correlation between streptococcal biofilm formation and metabolic activity.

As the salivary pellicle may influence microbial adhesion and metabolic activity of microorganisms significantly [12,13], the test specimens were incubated with human whole saliva for 2 h prior to incubation with streptococci as previous studies have shown that the pellicle reaches its maximum thickness after 2 h of saliva exposition [14,15]. Both *S. mutans* as well as *S. sobrinus* have been identified in dental plaque and, due to their acidogenicity and acid tolerance, they are counted among the most important species responsible for caries formation [16,17]. As organic acids such as lactate demineralize tooth tissues, enamel might be regarded as an ideal substrate for studies investigating biofilm formation and lactate production. Nevertheless, from a material scientist's point of view, design and invention of innovative dental materials contributing to the minimization of biofilm formation and lactate production on their surface is wishful. Moreover, standardization of enamel samples is complex in terms of size and surface condition; thus, two commonly used and characteristic dental materials with distinct surface properties have been chosen for analysis in this study.

Data gathered with the Resazurin test for quantification of adherent streptococci showed high standard deviations, which made it difficult to identify differences in biofilm formation between the substrata. Although high standard deviations were anticipated for 4 h biofilm formation due to potential influences of the salivary pellicle on early microbial adhesion, standard deviations even increased for 24 h biofilm formation and, surprisingly, values indicated no significant increase in the relative amount of adherent *S. mutans* after 24 h in comparison to 4 h biofilm formation. As these results did not comply with the significantly increased values identified for the production of lactate after 24 h incubation, biofilm formation was repeated employing a different quantification approach based on the formation of formazan (MTT test), which identified a significant increase in the relative amount of both *S. mutans* and *S. sobrinus* after 24 h biofilm formation, coinciding with lower standard variations than observed for the Resazurin test. These results correspond to the results of other researchers [18] and indicate that the Resazurin test as employed in the present study may not be the most effective means for analyzing 24 h streptococcal biofilm formation. Previous investigations showed that extensive reduction of Resazurin during prolonged incubation times may lead to the formation of the non-fluorescent hydro-resorufin [19], which directs to an under-estimation of the number of adherent cells. Despite the reproducible preliminary tests that had been performed for the present study, this phenomenon might serve as an explanation for the high standard deviations observed. However, concerning the very low values detected with the MTT test after 4 h biofilm formation, it appears that the Resazurin test might be more sensitive for the quantification of few adherent cells.

Previous studies have demonstrated that ceramic materials yield low amounts of bacteria and plaque on their surfaces both *in vitro* [6,20–22] and *in vivo* [23]. In contrast, for glass-ionomer cements, some researchers identified similar or even higher amounts of plaque on their surface than conventional dental materials [24–28] and other researchers underline the anti-adhesive behaviour of glass-ionomer cements due to the inhibitory effect of released ions [29–31]. Taking into consideration that the values identified for 24 h biofilm formation with the Resazurin test and those identified for 4 h biofilm formation with the MTT test should be interpreted with caution, the results of the present study indicate that, for 4 h biofilm formation, *S. mutans* and *S. sobrinus* adhere in similar amounts to the ceramic and the glass-ionomer cement. This phenomenon might be accounted to the simulation of an experimental salivary pellicle, which reduces differences in bacterial colonization among materials with originally distinct physico-chemical properties [32–34]. For 24 h biofilm formation, analysis of data gathered with the MTT-assay indicated different results for the two species: for *S. mutans*, significantly lower relative amounts of viable cells were detected on the glass-ionomer cement than on the ceramic, whereas similar amounts were identified for *S. sobrinus*. These results suggest a species-dependency concerning streptococcal proliferation on the surface of materials used in the current study. However, it should be borne in mind that only a limited number of aspects concerning biofilm formation were simulated in this pilot study, excluding, for instance, clinically relevant phenomena such as coaggregation between genetically distinct cell types [35] or the continuous production of extracellular polysaccharides (EPS) due to the lack of sucrose in the nutrient broth.

It has been argued that anti-adhesive properties of a dental material do not necessarily coincide with anti-metabolic properties [26,36]. Thus, it is obvious that the relative amount of streptococci adherent to a substratum does not necessarily give a sufficient impression of their metabolic activity; as a result, the analysis of metabolic activity of lactate production in biofilms of cariogenic streptococci may be regarded as a step towards increasing the clinical relevance of studies investigating biofilm formation on the surface of dental materials. Although some researchers studied acid production in dental biofilms [37–39], they did not quantify acid levels directly but by titration of pH-shifts in the surrounding bacterial suspension. The approach introduced in the present study was based on a commercial chair-side assay for the evaluation of clinical caries risk (*Clinpro Cario L-Pop*; CCLP). It can be applied for the quantification of lactate production in biofilms formed on the surface of dental restorative materials *in vitro*, with high lactate values correlating with high metabolic activity of cariogenic bacteria. The CCLP kit is based on the enzymatic oxidation of lactic acid by lactate dehydrogenase, which is coupled to a cascade of redox indicators generating a blue colour signal [40–42]. This signal may either be analyzed qualitatively using a semi-quantitative scale as used in the chair-side assay, or, in a modified approach, be employed for a quantitative estimation of lactate production. The high coefficient of determination observed for the analysis of standard lactate solutions underlines the accuracy of this approach. Nevertheless, for valid interpretation of the results gathered in the present pilot study, it should be taken into consideration that only a limited number of factors with potential impact on the production of lactate in streptococcal biofilms have been simulated. These factors include the use of sucrose (which is not completely transferred into lactate due to its particular role in the formation of extracellular polysaccharides by oral streptococci) and the use of a single sucrose concentration. A clinical study identified only poor correlations between the CCLP readings and lactic acid production in oral biofilms as measured by capillary electrophoresis [41]. With regard to this aspect it should be taken into consideration that quantification of lactate produced in biofilms with enzymatic approaches may suffer from inhibition; however, for the present study, no inhibition of lactate dehydrogenase could be identified. For the results of the present study, Pearson correlation coefficients suggested only little correlation between the relative amounts of adherent streptococci and lactate levels. This phenomenon might be due to the circumstance that biofilm formation and streptococcal acidogenicity are dependent on different factors, with adhesion being more dependent on the surface characteristics of the substratum and incubation parameters, and acidogenicity being more dependent on the pH of the surrounding medium, the presence of nutrients or anti-metabolic agents. Moreover, it may be possible that constituents of the acquired salivary pellicle influence adhesion and metabolic activity of streptococci to different extents; thus, adhesion and metabolic activity of oral streptococci should be treated as separate variables and analysis of both factors should be performed for gaining comprehensive insights in streptococcal biofilm formation and pathogenicity. Previous studies demonstrated that glass-ionomer cements may actually inhibit the acid production of oral streptococci, which has been attributed to the release of fluoride [43–45]. However, the results of this study suggest at least similar or higher lactate production in biofilms formed on the glass-ionomer cement than on the ceramic. Although the release of fluoride from the glass-ionomer cement has not been analyzed in the present study, the findings might be attributed to the fact that the release of fluoride from glass-ionomer cements diminishes with time [46], with a fluoride burst directly after preparation that decreases continuously until it is recharged by exposure to fluoride [47]. As all samples were stored in distilled water for several days prior to the experiments, it is likely that the residual release of fluoride was too low for showing an inhibitory effect on streptococcal metabolism. However, further studies are necessary to evidence these considerations. Nevertheless, for both streptococcal species a tendency towards lower lactate production in biofilms formed on the ceramic than for those formed on the glass-ionomer cement could be observed.

Conclusions

Judging within the above discussed limitations of this *in vitro* study, it might be assumed that the sole amount of cariogenic streptococci adherent to a dental substratum does not *a priori* allow for an estimation of the metabolic activity of the bacteria organized in the respective biofilm. Thus, for future laboratory and clinical studies, comparisons between the relative amount of adherent streptococci and their respective metabolic activity might improve the clinical significance of laboratory studies on biofilm formation and, from a material scientist's point of view, contribute to the development of novel dental materials with both anti-adhesive and anti-metabolic properties.

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