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Abstract

The aim of this study was to examine the influence of glucosyltransferase-gene-negative (gtf-) Streptococcus mutans strains unable to synthesize water-insoluble or soluble glucan on the structure and macromolecular diffusion properties of in vitro grown mixed oral biofilms. Biofilms modeling supragingival plaque consisted of Actinomyces naeslundii OMZ 745, Candida albicans OMZ 110, Fusobacterium nucleatum KP-F2, Streptococcus oralis SK 248, Veillonella dispar ATCC 17748T and one of the S. mutans strains UA159, OMZ 966, OMZ 937 or OMZ 977. Biofilms were grown anaerobically on sintered hydroxyapatite disks for 64.5 h at 37 degrees C. To perform confocal laser scanning microscopy analyses, microorganisms were stained with Syto 13 and extracellular polysaccharides (EPS) with Calcofluor. Macromolecular diffusion properties were measured following timed biofilm exposure to Texas-Red-labeled 70-kDa dextran. Results showed that replacing wild-type S. mutans by a gtfC- mutant led to an increase in the volume fraction occupied by cells from 29 to 48% and a decrease of the EPS volume fraction from 51 to 33%. No such changes were observed when the S. mutans wild-type strain was replaced by a gtfB- or gtfD- mutant. The diffusion coefficient of 70-kDa dextran in biofilms containing the gtfC- S. mutans was 16-fold higher than in biofilms with the wild-type strain indicating a strong macromolecular sieving effect of GTF C-generated glucans. Our data demonstrate the influence of EPS on the structure and macromolecular diffusion properties of an oral biofilm model and uncover our still limited knowledge of the function of EPS in biofilms and plaque.
Effects of *Streptococcus mutans* *gtfC* Deficiency on Mixed Oral Biofilms in vitro

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**Key Words**

Biofilm · *Streptococcus mutans* · Glucosyltransferases · Confocal laser scanning microscopy

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**Abstract**

The aim of this study was to examine the influence of glucosyltransferase-gene-negative (*gtf\textsuperscript{−}*) *Streptococcus mutans* strains unable to synthesize water-insoluble or soluble glucan on the structure and macromolecular diffusion properties of in vitro grown mixed oral biofilms. Biofilms modeling supragingival plaque consisted of *Actinomyces naeslundii* OMZ 745, *Candida albicans* OMZ 110, *Fusobacterium nucleatum* KP-F2, *Streptococcus oralis* SK 248, *Veillonella dispar* ATCC 17748\textsuperscript{T} and one of the *S. mutans* strains UA159, OMZ 966, OMZ 937 or OMZ 977. Biofilms were grown anaerobically on sintered hydroxyapatite disks for 64.5 h at 37\degree C. To perform confocal laser scanning microscopy analyses, microorganisms were stained with Syto 13 and extracellular polysaccharides (EPS) with Calcofluor. Macromolecular diffusion properties were measured following timed biofilm exposure to Texas-Red-labeled 70-kDa dextran. Results showed that replacing wild-type *S. mutans* by a *gtfC\textsuperscript{−}*-mutant led to an increase in the volume fraction occupied by cells from 29 to 48\% and a decrease of the EPS volume fraction from 51 to 33\%. No such changes were observed when the *S. mutans* wild-type strain was replaced by a *gtfB\textsuperscript{−}*- or *gtfD\textsuperscript{−}*-mutant. The diffusion coefficient of 70-kDa dextran in biofilms containing the *gtfC\textsuperscript{−}*- *S. mutans* was 16-fold higher than in biofilms with the wild-type strain indicating a strong macromolecular sieving effect of GTF C-generated glucans. Our data demonstrate the influence of EPS on the structure and macromolecular diffusion properties of an oral biofilm model and uncover our still limited knowledge of the function of EPS in biofilms and plaque.

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Microbial biofilms, consisting of single cells, cell aggregates and microcolonies embedded in an extracellular polymeric matrix, are a most common phenomenon on earth and have been the subject of intense experimental and theoretical scrutiny over the past decade [Costerton, 1995; Marsh, 1995; Wimpenny et al., 2000; Hermanowicz, 2003]. In human dental plaques, which are naturally occurring complex biofilms, two species of mutans streptococci, *Streptococcus mutans* and *Streptococcus sobrinus*, are thought to play a major role in the etiology of dental caries. Their pathogenicity appears related to their acidogenic potential and ability to form water-insoluble, extracellular and enzymatically virtually undegradable polysaccharides from sucrose [Guggenheim, 1970; Hama-
The synthesis of extracellular glucans is catalyzed by three glucosyltransferases (GTFs). GTF B synthesizes water-insoluble glucan (mutan) only, GTF C produces both water-insoluble and soluble glucan (dextran) [Aoki et al., 1986; Hanada and Kuramitsu, 1988], whereas GTF D exclusively synthesizes water-soluble glucan [Hanada and Kuramitsu, 1989]. These glucans, especially the water-insoluble mutan, are considered to promote adhesion and colonization of the organism and to mediate protection from antimicrobial agents and resistance to toxic compounds [Sutherland, 1999; Banas and Vickerman, 2003].

The use of a variety of S. mutans mutants has demonstrated that selective or total elimination of the GTF enzymes leads to decreased production of glucans [Munro et al., 1991] as well as decreased incidence of smooth-surface caries in rats [Yamashita et al., 1999]. In this study we used gtf^- strains of S. mutans, and in particular a gtfC^- mutant, to investigate the role inherent to S. mutans in the formation of mixed biofilms in vitro and in defining the biophysical properties of the extracellular matrix. Based on information from a previous study [Thurnheer et al., 2003] that demonstrated the exclusion of macromolecules exceeding approximately 10 kDa, we chose a fluorescence-labeled 70-kDa dextran to monitor mutant-associated changes in the biofilm’s macromolecular diffusion properties. This macromolecule may not be relevant to caries but it may help to reveal changes in biofilm structure due to loss of extracellular polysaccharides (EPS).

Materials and Methods

Construction of gtfB^- , gtfC^- and gtfD^- Mutants

A gtfC^- mutant was constructed by replacement mutagenesis as described [van der Ploeg and Guggenheim, 2004]. In brief, two fragments, encompassing the S. mutans gtfC gene and located between bp 94 and 734 and bp 1616 and 2201, respectively, were amplified by PCR and cloned in vector pFW15 [Podbielski et al., 1996] in such a way that the erythromycin resistance gene present on pFW15 was flanked by the two gtfC fragments. The resulting plasmid was linearized with BamHI and transformed into S. mutans UA159 (OMZ 918), selecting mutants by erythromycin resistance. One transformant strain was designated OMZ 937. gtfB^- and gtfD^- mutants were constructed essentially in the same way except that both genes were interrupted by the kanamycin resistance gene (aac-aph) harbored on plasmid pFW13 [Podbielski et al., 1996]. In the gtfB^- mutant (OMZ 966) bp 646–1537 of gtfB was deleted and replaced by the kanamycin resistance gene. In the gtfD^- mutant (OMZ 977) bp 1377–2152 of gtfD was deleted and replaced by the kanamycin resistance gene. The mutations were confirmed by PCR analysis.

The phenotype of each planktonically grown mutant, as well as that of the gtfB^- and gtfC^- mutants reisolated from biofilms, was verified by SDS-polyacrylamide gel electrophoresis followed by GTF activity staining (fig. 1). For this, 10-ml cultures grown at 37°C for 16 h in Todd-Hewitt broth with 0.3% yeast extract and 0.5% glucose were centrifuged and extracted in 500 µl of 6% SDS, 3% mercaptoethanol, 0.02% bromphenol blue and 8 µl of 3 M urea (90 min at 37°C). 15 µl of the soluble fraction of the extract were loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis, the gel was washed three times for 30 min in 50 m M Tris-HCl, pH 7.5, and then incubated overnight at 37°C in 50 m M acetate buffer, pH 5.5, containing 0.05% dextran, 2% Tween-100 and 5% sucrose.

Preparation of Biofilms

Our standard procedures to produce 6-species biofilms have been described previously [Shapiro et al., 2002; Thurnheer et al., 2003]. In brief, Actinomyces naeslundii OMZ 745, Candida albicans OMZ 110, Fusobacterium nucleatum KP-F2 (OMZ 596), Streptococcus oralis SK 248 (OMZ 607), Veillonella dispers ATCC 17748 (OMZ 493) and S. mutans UA159 (OMZ 918; wild-type strain), or OMZ 937 (gtf^-), OMZ 966 (gtf^-) or OMZ 977 (gtf^-), were used for biofilm formation [Guggenheim et al., 2001a; Shapiro et al., 2002]. Biofilms were grown in 24-well polystyrene cell culture plates on sintered hydroxyapatite (HA) disks (ø 10.6 mm; Clarkson Chemical Company, Williamsport, Pa., USA) that had been preconditioned in whole unstimulated pooled saliva (pellicle-coated) on day 1 of a biofilm experiment (for the preparation of batches of processed, pasteurized saliva, see Guggenheim et al. [2001a]). To start a biofilm experiment, disks were covered with 1.6 ml of growth medium (saliva/mFUM) and 200 µl of a mixed cell suspension prepared from equal volumes and densities of each strain or in some experiments using a selection of the above-mentioned strains. mFUM corresponds to a well-established tryptone-yeast-based broth medium designated as FUM [Gmür and Guggenheim, 1983] and modified by supplementing 67 m M Sorensen’s buffer (final pH 7.2). The carbohydrate concentration in mFUM was 0.3% (w/v) and consisted of glucose for the first 16 h and from then on of a 1:1 (w/w) mixture of glucose and sucrose (see below). Biofilms were incubated anaerobically at 37°C for 64.5 h. In order
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Confocal Laser Scanning Microscopy and Image Analysis

Biofilms were examined using a DM IRB E inverted microscope (Leica Mikroskopie GmbH, Wetzlar, Germany) fitted with a UV laser (Coherent Inc., Santa Clara, Calif., USA), a He-Ne laser (Uniphase Vertriebs GmbH, Eching/Munich, Germany), an Ar laser (Coherent Inc.) and a TCS SP2 computer-operated confocal laser scanning microscopy (CLSM) system (Leica Lasertecnik GmbH, Heidelberg, Germany). Filters were set to 400–490 nm for detection of Calcofluor, 520–540 nm for Syto 13 and 595–660 nm for Texas Red. Confocal images were obtained using ×40 (numeric aperture 1.25) and ×100 (numeric aperture 1.4) oil immersion objectives. Each biofilm was scanned at 5 randomly selected positions not close to the edges of the disks. Z series were generated by vertical optical sectioning at each of these positions; the thickness of each section was 1.018 μm. Image acquisition was done in 6-fold line average mode and the data were processed on a Silicon Graphics 320 TM visual workstation (Mountain View, Calif., USA) fitted with Windows NT v4.0. Scans were recombined and volumes were estimated using Imaris TM 3.1 software (Bitplane AG, Zürich, Switzerland). Depths of probe penetration into biofilms were calculated by multiplying the number of stained z-sections by 1.018 μm.

Calculations and Statistical Analyses

The theoretical background for the calculation of diffusion coefficients has been described previously [Thurnheer et al., 2003]. It was deduced that net mass transport of dye into the biofilm followed Fick’s first law of diffusion and that the mean square displacement $\langle x^2 \rangle$ was related to the diffusion coefficient (D) by

$$\langle x^2 \rangle = 2D\Delta t.$$  

Accordingly, diffusion coefficients were calculated by relating $\langle x^2 \rangle$ (the square of the experimentally determined $\langle x \rangle$ values) with the corresponding penetration times ($\Delta t$) using weighted least squares regression [Thurnheer et al., 2003].

The unpaired t test was used to evaluate differences in total cell and EPS volumes between biofilms of different composition, considering 10 measurements per biofilm type (5 randomly selected spots of 2 biofilms). A p value ≤0.05 was considered to indicate a significant difference between two types. Statistical analyses were performed with StatView 5.01 (SAS Institute, Cary, N.C., USA).

Results

Mutants were confirmed successfully by PCR and analyzed for GTF B and GTF C activity (GTF D cannot be detected because its water-soluble product, dextrin, is washed out) after SDS-polyacrylamide gel electrophoresis (fig. 1). Although the insertion of the kanamycin resistance gene is expected to be polar owing to the presence of a transcriptional terminator downstream of the aac-aphD gene [Podbielski et al., 1996], the gtfC gene was still expressed in the gtfB- mutant OMZ 966. This is not surprising, since it has been shown before that gtfC is transcribed from a promoter located downstream of the gtfB gene [Goodman and Gao, 2000]. In addition, from each

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biofilm, 12 mutants were recovered as single colonies from a mitis-salivarius agar plate and analyzed for GTF B and GTF C activity as above. All 12 S. mutans colonies isolated from the biofilm with OMZ 937 exhibited the same GTF activity patterns as the original OMZ 937 used to inoculate the biofilm. Among the 12 mutants that were isolated from the biofilm containing OMZ 966, 11 had retained the phenotype of OMZ 966, whereas 1 colony had lost GTF C activity.

Figure 2a and b show representative CLSM images of mixed biofilms containing the wild-type and the gtfC-decient strain of S. mutans, respectively. A shift in the ratio of total bacteria to EPS is evident. The total volume covered by cells, EPS and nonstained extracellular matrix was defined as 100%. Calculations based on 10 z-series showed that in biofilms containing the wild-type strain UA159 the volume fraction occupied by bacteria was 29.4 ± 6.7% (mean ± SD) and that of EPS 48.3 ± 12.4%; in biofilms with the gtfC-mutant OMZ 937 the respective volumes were 50.8 ± 13.3 and 32.6 ± 6.0% (fig. 3). The increase in cell volume fraction and the decrease in EPS volume fraction in these biofilms were statistically significant (p = 0.0003 and 0.002, respectively). On the other hand, neither the incorporation of a gtfB- nor a gtfD- S. mutans strain into the biofilm resulted in significant changes of the total cell volume (p = 0.3125 and 0.7411, respectively) or the EPS volume fraction (p = 0.7047 and 0.6434, respectively) in comparison to control biofilms with UA159 (fig. 3). Biofilm thickness after 64.5 h was on average 28 ± 8 μm and 32 ± 12 μm for biofilms containing the wild-type strain or the gtfC-mutant, respectively (n = 70; 14 biofilms with 5 measurements each). Although a striking reduction of the EPS volume fraction occurred when S. mutans OMZ 937 replaced UA159 in biofilm formation, a 33% EPS volume fraction remained. To clarify the source of this EPS, we grew biofilms without S. mutans or without streptococci, and in addition, single-species biofilms consisting of only the wild-type or the gtfC-mutant of S. mutans. Multi-species biofilms grown without S. mutans showed in comparison to biofilms with OMZ 937 neither significant differences in the cellular (p = 0.4739) nor the EPS volume fraction (p = 0.3879; fig. 3). Neither did the cellular fraction in biofilms with no streptococci differ from the one seen with biofilms containing OMZ 937 (p = 0.5173), but the EPS volume fraction had decreased to 26% (p = 0.015;
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Fig. 3. Comparison of volumes occupied by cells (●) and EPS (■) in in vitro oral biofilms of variable composition. 100% was defined as the sum of the volume fractions filled by cells, EPS and nonstained extracellular space. wt = Wild-type; only wt = monospecies biofilm with wild-type S. mutans.

Table 1. Time-dependent mean distance of penetration ± SD of 70K-Dex into 6-species biofilms containing either the wild-type strain (UA159) or the gtfC-deficient strain (OMZ 937) of S. mutans

<table>
<thead>
<tr>
<th>Time, s</th>
<th>Distance of penetration, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. mutans UA159 (wild-type)</td>
</tr>
<tr>
<td>2</td>
<td>7.12 ± 1.63</td>
</tr>
<tr>
<td>30</td>
<td>NT</td>
</tr>
<tr>
<td>60</td>
<td>9.36 ± 1.40</td>
</tr>
<tr>
<td>120</td>
<td>12.14 ± 1.98</td>
</tr>
<tr>
<td>300</td>
<td>16.48 ± 2.38</td>
</tr>
<tr>
<td>600</td>
<td>24.83 ± 1.95</td>
</tr>
</tbody>
</table>

Data are based on 15 measurements. NT = Not tested.

Diffusion kinetics of 70K-Dex into multi-species biofilms containing either the wild-type strain or the gtfC<sup>−</sup>-strain of S. mutans are depicted in figure 5. In biofilms incubated for 2 s, the probe just stained the superficial layer of the biofilm. After a 60-second incubation, staining was confined to the upper quarter of the biofilms with the wild-type strain, whereas in biofilms containing the mutant strain the probe penetrated about halfway through the biofilms. After 120 s, 70K-Dex was confined to the upper half of the ‘wild-type’ biofilms but had reached the bottom of mutant-containing biofilms.

Mean penetration depths of 70K-Dex into different multi-species biofilms are summarized in table 1. Because 70K-Dex traveled so quickly through biofilms with the gtfC<sup>−</sup> strain, diffusion was also measured at 30 s, but not beyond 120 s. The calculation of 70K-Dex diffusion coefficients from the data listed in table 1 resulted in values...
Fig. 4. CLSM images as described in figure 2 of 64.5-hour biofilms formed by *S. mutans* UA159 (wild-type, a) or *S. mutans* OMZ 937 (gtfC− mutant) alone (b). Images were obtained by using a ×40 oil immersion objective.

Fig. 5. Diffusion of 70K-Dex into 64.5-hour biofilms as a function of time (up to 120 s). CLSM images of vertical cross-sections through biofilms containing either the *S. mutans* wild-type strain (left) or the gtfC−-deficient mutant (right). Images were obtained by using a ×100 oil immersion objective.
of 0.46 ± 0.15 μm²/s for biofilms containing the wild-type S. mutans strain and 7.15 ± 1.83 μm²/s for biofilms with the gtfC⁻ mutant.

Discussion

Abundance of mutans streptococci paired with frequent intake of dietary sucrose seem to be cornerstones in the development of human dental caries [Guggenheim, 1970; Hamada and Slade, 1980; Loesche, 1986]. It was postulated and widely accepted that the synthesis of water-insoluble glucans by S. mutans and S. sobrinus is a prerequisite for their ability to colonize tooth surfaces efficiently [Hamada and Slade, 1980; Loesche, 1986]. If this is true, incorporation of a gtfB⁻ or gtfC⁻ S. mutans strain into in vitro polyspecies biofilms should result in less S. mutans and less mutan and thus reveal major effects on biofilm structure and diffusion properties. We tested this hypothesis experimentally in vitro using a modification of our established biofilm model [Shapiro et al., 2002; Thurnheer et al., 2003]. The so-called ‘feast-and-famine’ model is characterized by directed fluctuations in available nutrients and reflects the supply of nutrients in dental plaque [Carlsson, 1984; McBain et al., 2003]. Characteristic features of the model are enlarged production of EPS and slow biofilm growth. Presumably, secondary modification of glucans in the biofilm matrix by glucanas produced by mutans streptococci [Guggenheim and Burckhardt, 1974] are the main reason for these changes in biofilm properties, leading to tenaciously adherent biofilms, which can only be removed completely by mechanical forces (data not shown). The ‘feast-and-famine’ model produces in several respects the most plaque-like biofilms, but, due to its quantitatively high demand for saliva, is used only for a restricted spectrum of experimental questions.

In studies with planktonically grown bacteria, Tsumori and Kuramitsu [1997] investigated mutants defective in GTF C activity and described crucial changes in their ability to grow on glass in vitro in the presence of sucrose. In contrast, our results show that the incorporation of a gtfC⁻ S. mutans strain did not affect the ability to form biofilms on HA disks, neither in mixed-species nor in single-species biofilms. We proved the stability of the gtfC⁻ and gtfB⁻ mutants by reisolating them from the biofilms. The loss of GTF C activity in 1 reisolated gtfB⁻ mutant is probably the result of recombination between gtfC and gtfB which has been observed before [Ueda and Kuramitsu, 1988]. Since only 1 out of 12 reisolated mutants exhibited this phenotype, we have further not investigated this phenomenon. The thickness of biofilms was generally similar for both S. mutans wild-type and gtfC⁻ multi-species biofilms. Notably, the incorporation of the gtfC⁻ strain, but not of gtfB⁻ or gtfD⁻ mutants, caused a more than twofold increase in volume fraction occupied by microbial cells and a concomitant decrease in EPS volume fraction. These findings profile GTF C as the primary producer of S. mutans EPS and downstage the role of mutan in terms of supporting HA colonization by S. mutans. Interestingly, we found that biofilms grown without S. mutans or without streptococci at all had almost the same low cell-to-EPS volume ratio as multi-species biofilms containing the gtfC⁻ strain, and in single-species biofilms formed by the gtfC⁻ strain the EPS volume fraction was reduced to 4% (fig. 3). These observations suggest that the contribution of GTF B to the production of mutan in biofilms in vitro must be very small. These findings contrast with those reported by Yamashita et al. [1993] who, assessing supernatant fluids of planktonic cultures of various S. mutans mutants, found GTF B to be of greater importance for mutan synthesis than GTF C. In terms of the water-soluble glucan (dextran) thought to be produced primarily by GTF D, we assume that the GTF D enzyme produced in our biofilms either had a low production rate, produced dextran that was hydrolyzed by dextranase and/or was lost during washing steps. Our data do not allow to distinguish between these possibilities and therefore this question will require further analyses. How much did other species than S. mutans contribute to the EPS matrix in our biofilms? Biofilms formed in the absence of streptococci contained only slightly less EPS than S.-mutans-free S.-oralis-positive biofilms. Hence, our data allow to extrapolate that the contribution to EPS of S. oralis was only minor (approx. 7% of the total biofilm volume) with the reservation of the above-mentioned possibilities of secondary enzymatic degradation and washout. It follows that a substantial portion of the EPS formed by these biofilms (almost 30%) must have derived from other species than streptococci. We suspect that this material was produced primarily by A. naeslundii, known to synthesize a heteropolysaccharide composed of N-acetylglucosamine and a water-soluble, biofilm-degradable levan [Rosan and Hammond, 1974; van der Hoeven et al., 1976; Bergeron and Burne, 2001].

The importance of water-insoluble glucan for adherent growth in dental plaque biofilms has been consolidated by many reports in the past [Guggenheim, 1970; Hamada and Slade, 1980; Loesche, 1986]. However, in this in vitro biofilm model this was not manifest, perhaps indicating
that the growth conditions for *S. mutans* were still far more favorable than under in vivo conditions. The incorporation of the *gtfC* − *S. mutans* may not have affected bacterial proliferation in multi-species biofilms but had impacts on biofilm structure as discussed above. To investigate this subject further we performed macromolecular diffusion experiments. In a previous study, Thurnheer et al. [2003] had shown that macromolecules bigger than 3 kDa were strongly retarded possibly due to the limiting pore size of EPS. In view of those observations, we was of interest to investigate the diffusion of macromolecules in a biofilm with reduced EPS content. In our biofilm model, culture analyses of biofilms grown with the wild-type (UA159) or the *gtfC* − mutant (OMZ 937) strain showed no differences in terms of the total number of CFU and the number of CFU detected for each of the individual species [van der Ploeg and Guggenheim, 2004], whereas, as discussed above, the EPS volume fraction was markedly reduced in OMZ-937-positive biofilms. Considering the above described observation that only a small difference in the biofilms’ thickness (and thus in total volume) was observed, it follows that the cellular fraction must be less densely packed in biofilms with the mutant than in biofilms with the wild-type strain and that EPS cannot fill all the extra void volume fraction. Thus, it would seem logical that diffusion of macromolecules must be much faster in biofilms containing the *gtfC* − mutant OMZ 937. This was confirmed experimentally by demonstrating that in *gtfC* − biofilms with OMZ 937 and reduced EPS volume fraction diffusion of 70K-Dex was 16 times faster than in biofilms with UA159. These results also agree with the hypothesis that tortuosity due to EPS and its sieving effect must be the principal reason for hindered diffusion of macromolecules in mixed oral biofilm in vitro [Thurnheer et al., 2003]. Another reason for altered diffusion properties could be related to differences in the spatial distribution of *S. mutans* wild-type and mutant strains in the biofilms. Testing this hypothesis in detail is the subject of future experiments.

In summary, our data demonstrate the influence of EPS on the structure and macromolecular diffusion properties of an oral biofilm model. These results uncover our still limited knowledge of the function of EPS in biofilms and plaque. The role of EPS on the antimicrobial susceptibility of in vitro biofilms remains to be studied. Similarly, the probably decisive impact of interactions between various EPS components and between the different biofilm species and cells on structure, composition and properties of biofilms requires further investigation. Together, such investigations may lead to a more balanced and integrated approach in the assessment of caries pathogenesis.

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