

Real-Time Microsensor Measurement of Local Metabolic Activities in *Ex Vivo* Dental Biofilms Exposed to Sucrose and Treated with Chlorhexidine[∇]

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Dental biofilms are characterized by structural and functional heterogeneity. Due to bacterial metabolism, gradients develop and diverse ecological microniches exist. The aims of this study were (i) to determine the metabolic activity of microorganisms in naturally grown dental biofilms *ex vivo* by measuring dissolved oxygen (DO) and pH profiles with microelectrodes with high spatial resolution and (ii) to analyze the impact of an antimicrobial chlorhexidine (CHX) treatment on microbial physiology during stimulation by sucrose in real time. Biofilms were cultivated on standardized human enamel surfaces *in vivo*. DO and pH profiles were measured in a flow cell system in sterile human saliva, after sucrose addition (10%), again after alternative treatment of the sucrose exposed biofilms with CHX (0.2%) for 1 or 10 min or after being killed with paraformaldehyde (4%). Biofilm structure was visualized by vitality staining with confocal microscopy. With saliva as the sole nutrient source oxygen consumption was high within the superficial biofilm layers rendering deeper layers (>220 μm) anoxic. Sucrose addition induced the thickness of the anaerobic zone to increase with a concurrent decrease in pH (7.1 to 4.4). CHX exposure reduced metabolic activity and microbial viability at the biofilm surface and drove metabolic activity deeper into the biofilm. CHX treatment led to a reduced viability at the biofilm surface with minor influence on overall biofilm physiology after 1 min; even after 10 min there was measurable respiration and fermentation inside the biofilm. However, the local microenvironment was more aerated, less acidogenic, and presumably less pathogenic.

Biofilms are complex, surface-associated, microbiological communities (7) that are characterized by microscale spatial, structural, and functional heterogeneity (40). The biofilm consists of microorganisms that are embedded in an extracellular slime matrix consisting of biopolymers of microbial origin such as polysaccharides, proteins, and DNA (16). This extracellular polymeric slime is highly hydrated and influences both the structure and the diffusion behavior within the biofilm (39). Bacterial metabolism results in the development of chemical and physiologic/metabolic gradients within the biofilm (17). Due to different concentrations of oxygen, nutrients, and microbial metabolic by-products, local microecological niches are created, allowing the coexistence of microorganisms with different growth requirements in close proximity (30). For example, the growth of anaerobic microorganisms within a generally aerobic environment within the oral cavity is possible. Carbohydrates and sugar are the most important energy sources for microorganisms in dental plaque (23) and, in the case of a lacking external substrate supply, they are able to metabolize

salivary glycoproteins (5). Nutrient depletion causes the microorganisms to either grow very slowly or to stop growing completely, entering a dormant-like state.

Changes in the ecologic balance of the oral microflora and in dental biofilms are a causative factor for the development of dental caries (43), gingivitis, and periodontitis (1); thus, these diseases can be considered as biofilm mediated. Fundamental factors that may lead to a shift in the microflora and the predominance of pathogens are the local pH value, the redox potential, and the availability of nutrients and/or carbohydrates (30). Caries, for instance, is a multifactorial disease. However, its main cause is the bacterial carbohydrate catabolism and the release of organic acids by acidogenic bacteria in the biofilm. This promotes the predominance of cariogenic pathogens such as *Streptococcus mutans*, *Streptococcus sobrinus*, and other acidogenic microorganisms (28, 43). Consequently, this results in further acid production and a decreasing pH. Associated with this is the demineralization and lesion development of dental hard substance (54).

Next to individual improvement of mechanical oral hygiene (i.e., mechanical and manual brushing, as well as flossing), prevention and therapy of oral disease is achieved by adjunctive oral hygiene products containing antimicrobial agents (29, 47). A concentration of 0.2% chlorhexidine (CHX) in oral mouth rinses showed the best efficacy in clinical studies and is

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still considered a “gold standard” (45). The antiplaque effect of CHX is based on a broad antibacterial spectrum. During application it immediately shows bactericidal effect and continuous bacteriostatic effect due to its high substantivity in the oral cavity (20). Electron microscopic examinations showed that CHX binds to and damages bacterial cell membranes and leads to structural changes and leakage of cytoplasm (3). Furthermore, contraction of *in vitro* grown biofilms after CHX exposure was shown in addition to cell damage (19, 44), which could cause changes in the diffusion behavior by changes to extracellular polymeric slime density. However, in deep layers of oral biofilms not all bacteria were reached (49). Direct visualization by fluorescence microscopy of the CHX effect was described by Takenaka et al. (44) for a three-species oral biofilm grown *in vitro*. Cell damage started from the periphery of bacterial aggregates and slowly continued into the depths. Other authors have reported the inability of CHX to completely kill all bacteria in different *in vitro* biofilm systems (15, 19, 32) and *in vivo* studies (48, 55) when a normally used clinical concentration was applied at usual exposure times. The killing efficacy was dependent on the age of the biofilm and thus on its thickness and composition.

Detection of concentration gradients in oxygen, pH, and metabolites in undisturbed biofilms *in situ* requires a microsensor technique. Microelectrodes with a tip diameter of a few micrometers offer precisely localized measurements on the microscale in three dimensions under real-time conditions (9, 35). Thus far, microelectrodes have been applied for measuring plaque pH in dental research in a groove model *ex vivo* (53, 54) and in *in vitro*-grown *S. mutans* biofilms (13, 42). However, there is no information on direct measurements of oxygen distribution and consumption in dental biofilms grown *in vivo* in the human oral cavity. In the field of environmental microbiology, analysis of the microenvironment, local activities, and gradients by microelectrodes is correlated with microscopic examination of the biofilm structure, thus allowing a relationship to be made between biofilm physiology and structure (18, 37). We decided to take a similar approach to characterize the effects of sucrose and CHX on *ex vivo* plaque biofilm physiology. The goal of the present study was to examine the metabolic activity (oxygen consumption and acid formation) and viability and the effect of antimicrobial CHX treatment on the physiology of the dental biofilms during exposure to sucrose. The spatial distribution of live and dead biofilm cells was examined by confocal laser scanning microscopy (CLSM), as well as by microbiological culture.

MATERIALS AND METHODS

Biofilm formation. An individually fabricated intraoral acrylic stent supplied with standardized human enamel slivers was worn by one healthy person for 3 days, starting in the morning on different occasions to obtain *in vivo* generation of mature dental biofilms. A preceding dental examination revealed no clinical and radiological signs of gingivitis, periodontitis, or caries. The enamel specimens were prepared from freshly extracted human third molars. After sterilization, they were processed to a surface roughness of $0.16 \pm 0.02 \mu\text{m}$ according to natural interproximal enamel-enamel contact areas (52). The area (in mm^2) of the enamel surface was calculated following digitalization (Scion Image Rel. 4; Scion Corp.). Afterward, the specimens were fixed to the stent in the region of the upper premolars and molars toward the natural teeth to simulate retention areas (Fig. 1). During the time of biofilm formation, a regular diet was maintained and personal oral hygiene was performed using a toothbrush, dental floss, and water. During meals and tooth brushing the stent was stored in a humid

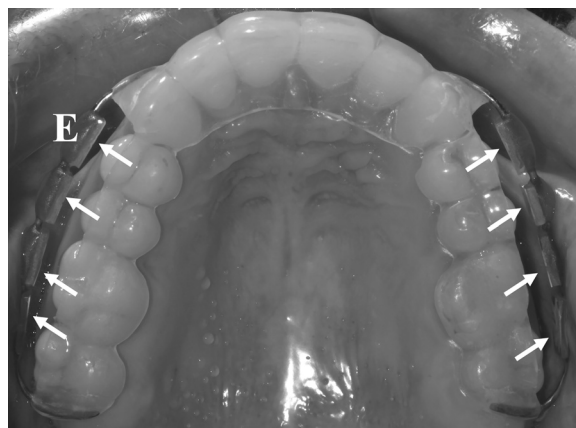


FIG. 1. Clinical picture of the upper jaw with the intraoral acrylic stent *in situ*. The enamel slivers (E) were fixed in the region of the premolars and molars toward the natural teeth to simulate retention areas (arrows).

chamber. At least 2 h before the biofilm samples were taken, the subject refrained from eating and drinking. At the end of each period the specimens were carefully removed from the device for further analysis without interfering with the adhering biofilm. The procedure was approved by the ethical committee of the medical faculty and informed consent was given.

Antimicrobial treatments. Untreated native dental plaque biofilms with no sucrose amendment served as control (–suc). The antimicrobial treatment of previously sucrose exposed biofilms (+suc) was performed *ex vivo* using a 0.2% CHX formulation (Chlorhexamed Forte; Glaxo SmithKline, Bühl, Germany). The biofilms were exposed to 10 ml of the CHX solution for either 1 min (CHX_1) or 10 min (CHX_10). Afterward, they were carefully dipped into sterile saliva. As a negative control, biofilms were killed by fixation with 4% paraformaldehyde (PFA) overnight. After fixation, the biofilms were cleaned twice in sterile saliva. The effective CHX concentration was calculated within different layers of the biofilm based on a simplified model (semi-infinite medium, the CHX surface concentration c_0 equals the concentration in the liquid of 0.2%) (8).

Microelectrode measurements. (i) Saliva. For saliva samples, 30-ml portions of whole saliva were collected by paraffin stimulation from two healthy volunteers 2 h after breakfast. Sterilization was performed immediately (12). After sonification and separation of debris by filtration, each saliva sample was centrifuged at $25,524 \times g$ for 30 min at 3°C (Biofuge 22R; Heraeus, Hanau, Germany). The supernatant was filtered by two Millex low binding protein filters (0.45 and $0.22 \mu\text{m}$; Millipore, Eschborn, Germany). The sterile saliva was buffered weakly and adjusted to a pH of 7.1 to 7.2 by 0.2 mM Na_2HPO_4 and 0.3 mM KH_2PO_4 , stored at 4°C , and used within 48 h. For experiments, saliva was diluted 1:10 (vol/vol) using sterile water.

(ii) Ex vivo flow cell setup. After removal from the stent, the enamel coupons with biofilm were immediately placed in a small flow cell (6.75 cm^3) for subsequent microelectrode measurements. The flow cell was fed from a total volume of 250 cm^3 of 1:10 saliva by use of two peristaltic pumps connected to both the inlet and outlet of the flow cell at a flow rate of 3 ml/min. The sterile human saliva as medium was aerated, and the temperature was kept constant at 24°C (room temperature). The limited volume of saliva due to the harvesting and handling procedure necessitated recirculation. Each set of measurements were collected within ~60 min and no longer than 90 min, after which the spent saliva was replaced with fresh saliva. Dental biofilm samples were subjected to the salivary medium with no added sucrose (–suc) and with added sucrose (25 g; 10% [wt/vol]) to stimulate microbial activity (+suc). Antimicrobial treatment was performed by removing the previously sucrose exposed specimens carefully from the flow cell and immersing them into the CHX formulation (CHX_1 and CHX_10) or PFA. After each treatment, the samples were gently dipped in 1:10 saliva and immediately returned to the flow cell that contained the sucrose-supplemented 1:10 saliva. After removal from the oral cavity, the thickness of the *ex vivo* biofilms was measured from microelectrode experiments, as well as being estimated from a stereomicroscope.

(iii) Microelectrodes. Microelectrodes were used to measure dissolved oxygen (DO) concentration and pH. They were fabricated as described by Revsbech (36)

and deBeer et al. (10) with tip diameters of 10 and 5 μm . Measurements were performed by using a semiautomated setup. Sensors were connected to a picoamperemeter and millivoltmeter, respectively, and positioned by using a micro-manipulator (model MM33; Maerzhaeuser, Wetzlar, Germany) with a motor-controlled z-axis (model VT-80; Micos, Eschbach, Germany). Amplifier signals were recorded by a data acquisition system (model DAQCard AI16XE50; National Instruments, Austin, TX) on a portable computer. Positioning during profiling and data acquisition were controlled by using custom-made software. Profile measurements were started 30 min after the biofilms were placed into the flow cell, allowing the biofilm to equilibrate and come to steady state (taken as no significant change to the profile or a depth measurement within a 2-min period). We measured depth profiles at high spatial resolution (25- μm increments) within the biofilm before and during exposure to sucrose (at least 20 min upon sucrose addition) or after additional antimicrobial treatment (CHX and PFA). Each profile took about 5 min. Also, the dynamic response (time series) of the biofilm to sucrose addition was recorded by leaving the microelectrode at one depth within the biofilm. For time series, the tip of the sensor was positioned either at the surface of the biofilm (DO) or near the biofilm base (pH). For flux calculations it is important that the profiles were at steady state. Steady state was confirmed by repeated profiling. Approximately 2 min after steady-state conditions had been achieved repeated profiles were taken at slightly different locations. Each set of measurements was collected within ~ 60 min. Profiles, aerial rates, and local conversion rates were calculated from at least four to six profiles. Profile data were reported as means ± 2 (standard errors [SE]).

In order to obtain constant and reproducible experimental conditions for all microelectrode measurements, the *ex vivo* measurements were conducted at room temperature (24°C). In this way we ensured that there were no temperature fluctuations of the microelectrodes, the standardization solutions, or the media during measurements despite the various rapid medium exchanges, allowing the profiles to more rapidly come to steady state.

(iv) Rate calculations. Under the conditions of the setup, the biofilm surface is covered by a stagnant medium layer, through which transport occurs only by diffusion. The diffusive transport of solutes through this diffusive boundary layer (DBL) is proportional to the concentration gradient in the DBL according to Fick's first law of diffusion: $J = D_0 (\partial c/\partial x)$, where J is the flux (in $\text{nmol cm}^{-2} \text{s}^{-1}$), D_0 is the molecular diffusion coefficient (in $\text{cm}^2 \text{s}^{-1}$), and $\partial c/\partial x$ is the concentration gradient (in nmol cm^{-4}). Assuming a molecular diffusion coefficient of oxygen of $2.3634 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (4) for oxygen at 24°C, aerial transport rates can be calculated that represent the total biofilm activity. Assuming diffusion as the dominant transport process within the biofilm, the curvature of the profiles within the biofilm was used to calculate the local activities according to a modified version of Fick's second law of diffusion: $D_{\text{eff}} (\partial^2 c/\partial x^2) = r$, where D_{eff} is the effective diffusion coefficient within the biofilm, $\partial^2 c/\partial x^2$ is the local change in concentration gradient, and r is the local conversion rate (in $\text{mol m}^{-3} \text{ s}^{-1}$) (35). We used a stepwise algorithm (18) to calculate local conversion rates (oxygen) as a measure for local oxygen consumption. To determine where acidification was occurring within the biofilm, we calculated rate data in terms of H^+ production or consumption from the pH data. However, since $[\text{H}^+]$ is a function of buffering (21), our concentrations should only be considered for purposes of relative comparison in this system and not as absolute concentrations. First, we converted the pH to proton concentration $[\text{H}^+]$ in nmol cm^{-3} by using the equation $[\text{H}^+] = 1/10^{\text{pH}}$. For the D_{eff} of H^+ we used $1.86 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, which was based on a D_{aq} of $9.31 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 25°C (26) and a $D_{\text{eff}}/D_{\text{aq}}$ ratio of 0.2, which was estimated by Stewart (39) from diffusion measurements in dental plaque. Negative uptake rates indicate net H^+ production (acidification) and positive uptake rates indicate net consumption.

CLSM. The CLSM investigation was performed by using a Leica DM RXE microscope connected to a TCS SP2 AOBs confocal system (Leica Microsystems) equipped with an argon laser (488 nm) and an He-Ne laser (543 nm, 633 nm).

Analysis of microbial viability. The biofilm samples (–suc, +suc, CHX_1, CHX_10, and PFA) were stained by using a Molecular Probes Live/Dead BacLight bacterial viability kit (Invitrogen). Each biofilm sample was incubated in 500 μl of staining solution for 15 min. The stained biofilms were subsequently examined by CLSM. The green fluorescent DNA-binding dye SYTO 9 labeled all MO (detection, 500 to 535 nm), while the red fluorescent propidium iodide (PI) labeled MO with harmed bacterial membranes (detection, 600 to 720 nm). Sequential scanning mode was used to eliminate “bleed over.” Z-series of confocal images (xy scan) were acquired with a 10×0.3 (NA) magnifying objective at 2- μm intervals. We chose low magnification to provide a larger field of view and a more general representation of the biofilm distribution in plan view at the top of the biofilm and the base. The thickness of the optical sections was $\sim 6 \mu\text{m}$; thus, we oversampled in the z dimension by a factor of 3 to ensure a continuous

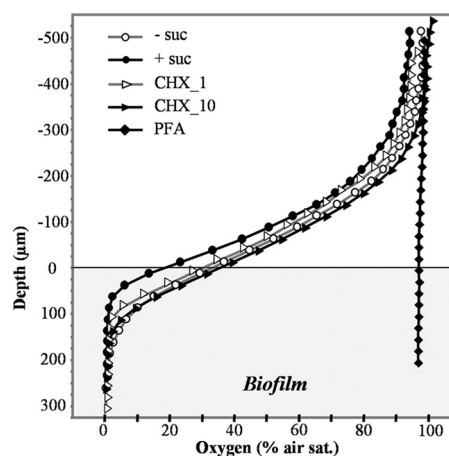


FIG. 2. Averaged oxygen profiles measured *in situ* on dental biofilms in salivary solution (1:10) without sucrose (–suc, $n = 11$), with 10% (wt/vol) sucrose (+suc, $n = 6$), and with 10% sucrose after 1 min (CHX_1, $n = 4$) or 10 min (CHX_10, $n = 5$) of treatment with CHX and in a PFA-killed control (PFA, $n = 3$). Error bars were omitted for clarity.

data set. Additionally, the biofilm thickness (L_f) was measured as the distance between the substratum and the highest cell cluster of the entire biofilm. The biofilms were examined at two different locations in the middle of the enamel surface. Digital image analysis of the optical sections was performed by using a custom-made software based on Axiovision (Zeiss, Germany). The analysis of the microbial viability (V) was based on the fluorescently labeled membrane integrity. After thresholding, V was calculated as the percentage of green (vital) voxels related to the whole number of red (dead) plus green voxels in every optical section of the biofilms. For each biofilm, the mean viability of all layers was calculated.

Culture method. In addition to the fluorescence-based microscopic viability assessment, conventional cultivation was also performed. The biofilm was mechanically detached from the enamel surface by scraping and transferred into sterile saline, followed by sonification on ice to disrupt larger clumps and chains, and then homogenized by vortexing. The total bacterial counts (BC) were measured by using a bacterial counting chamber (System Helber, Saaringia, Germany; counting volume, $0.8 \times 10^{-3} \text{ mm}^3$) under a dark-field microscope (magnification, 500 \times). For this purpose the number of single cells and particles (small remaining chains were counted as one count) were enumerated. Thus, it is possible that our BC were slightly underestimated. The BC were reported per mm^2 of the substrate surface (BC/ mm^2). Cultural viability was examined by plating the serially diluted biofilm samples on Schaedler agar plates supplemented with sheep blood and vitamin K_1 (Becton Dickinson, Heidelberg, Germany) using a spiral plater (Meintrup, Löhden, Germany). The CFU were counted after anaerobic incubation for 48 h at 37°C and reported as CFU/ mm^2 .

Statistics. BC and CFU values were log transformed. The data were reported as mean and corresponding 95% confidence intervals (CI) unless otherwise indicated. Statistical comparisons between data sets were made by using analysis of variance (Microsoft Excel 2000). Differences were considered significant for $P < 0.05$. Each experiment was performed three times.

RESULTS

Microelectrode measurements. (i) Profiles of DO and pH. The biofilms were $\sim 300 \mu\text{m}$ thick. Repeated profiles over the course of the experiment indicated no measurable increase in biofilm thickness over this time. All biofilms except the PFA-killed group showed an anaerobic zone in deep biofilm layers. Here, we define anaerobic regions according to the criteria of Loesche (27) as oxygen saturation values of $<0.5\%$ (Fig. 2 and 3). With no external substrate present (–suc), DO penetrated into the biofilm to a depth of 220 μm . Upon sucrose addition (+suc), DO penetration was reduced to the upper 150 μm ,

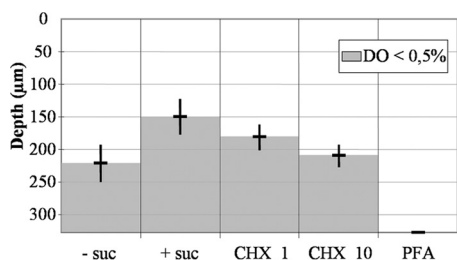


FIG. 3. Anaerobic regions (DO < 0.5%, gray) in dental biofilms *in situ* (1:10 buffered saliva) without sucrose (-suc), with 10% (wt/vol) sucrose (+suc), and with 10% sucrose after 1 min (CHX_1) or 10 min (CHX_10) of treatment with CHX and in a PFA-killed control. Mean (black line) and corresponding 95% confidence intervals (error bars).

with a concomitant increase in the thickness of the anaerobic layer. Treatment with CHX reversed this effect and, despite the presence of sucrose (10% [wt/vol]), penetration of DO increased to 180 and 210 μm. PFA-killed biofilms were fully aerated. Repeated profiling at the same location ensured that the procedure of removing the biofilm from the flow cell, and replacing it without any treatment outside had no effect on the profiles (data not shown). In the biofilms without sucrose (-suc) and the PFA-killed control group, the pH within the biofilm and in the salivary solution (pH 7.1 to 7.2) were more or less the same, with a minimum pH of 7.03 (-suc; CI = 6.99 to 7.08) and 7.15 (PFA) (Fig. 4). Sucrose addition (+suc) resulted in a strong pH decrease, particularly at the base of the biofilm with the lowest values reaching 4.32 (CI = 3.97 to 4.66) at a depth of 220 μm. Thus, the lowest pH occurred in the anoxic layer. Compared to the +suc situation the antimicrobial CHX_1 treatment did not result in remarkable differences in pH in the different layers of the biofilm (minimum, 4.87; CI = 4.53 to 5.20), CHX_10 had a significant effect. The lowest values now only dropped to pH 5.87 (CI = 5.05 to 6.70).

(ii) **Time series of oxygen and pH.** Sucrose supplementation of the saliva solution led to an immediate decrease of DO concentration at the biofilm surface (Fig. 5). A measured re-

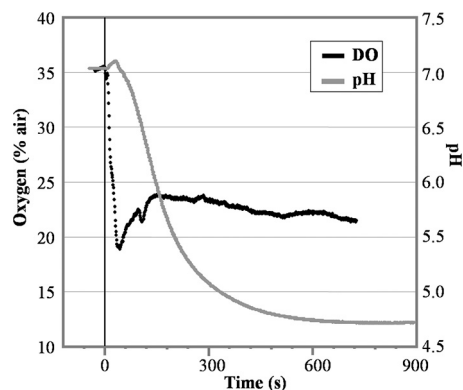


FIG. 5. Representative time course of oxygen (DO, black line) measured *in situ* at the surface of a dental biofilm and time course of pH (gray line) measured *in situ* at the biofilm base (250 μm depth) before ($t < 0$) and after ($t > 0$) sucrose addition (10% [wt/vol]) to the medium (1:10 buffered saliva). $t = 0$ corresponds to the time when the sucrose-containing medium got into first contact with the biofilm surface.

sponse was discernible within seconds, and a new steady state was reached within about 3 min. During that time the oxygen concentration decreased from ca. 35% air saturation to 23%. At the biofilm base the pH also decreased immediately from 7.1 to 4.7 within less than 10 min (Fig. 5). Fifty percent of this response occurred within the first 2 min.

(iii) **Areal and local activities of the biofilm.** The areal uptake rates of oxygen inside the biofilm, calculated from the concentration gradient through the DBL, were not affected significantly by the addition of sucrose or the CHX treatments (Fig. 6). The killed negative control (PFA) showed no aerobic activity. However, the addition of sucrose and exposure of the biofilms to CHX did have a significant impact on acidification and the H^+ production rate (Fig. 6). Sucrose addition caused an increase in H^+ production rate by a factor of almost 1,000. This production rate was subsequently reduced by a factor of 2.5 after a 1-min exposure to CHX and by a factor of ~30 after

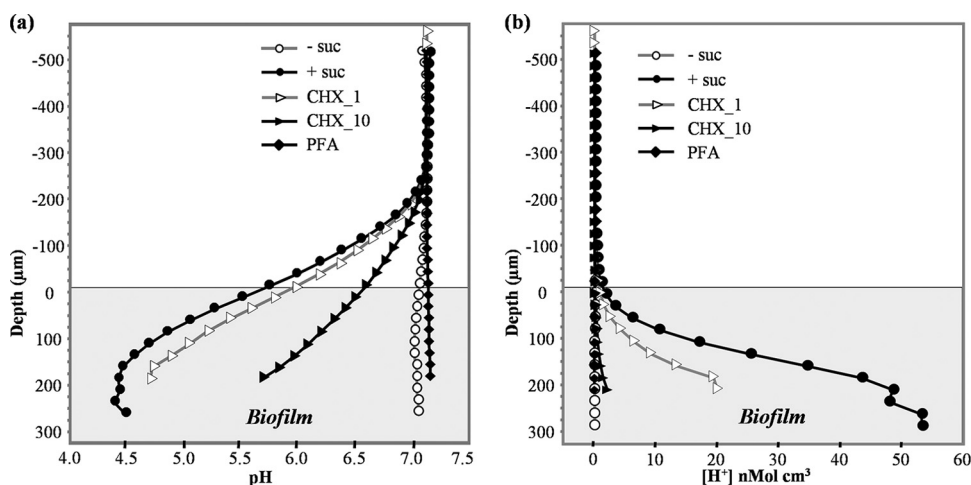


FIG. 4. pH (a) and corresponding $[H^+]$ (b) profiles measured *in situ* on dental biofilms in salivary solution (1:10) without sucrose (-suc, $n = 5$), with 10% (wt/vol) sucrose (+suc, $n = 6$), and with 10% sucrose after 1 min (CHX_1, $n = 4$) or 10 min (CHX_10, $n = 4$) of treatment with CHX and in a PFA-killed control (PFA, $n = 2$). Error bars were omitted for clarity.

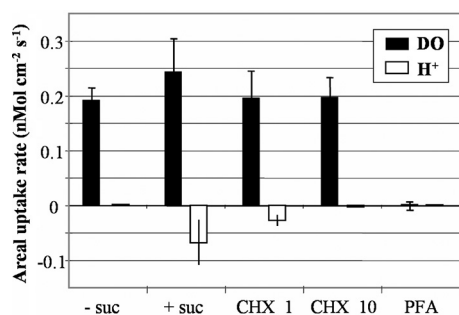


FIG. 6. Areal biofilm uptake rates of oxygen (■) and H⁺ (□) in dental biofilms *in situ* (1:10 buffered saliva) without sucrose (-suc), with 10% (wt/vol) sucrose (+suc), and with 10% sucrose after 1 min (CHX_1) or 10 min (CHX_10) of treatment with CHX and in a PFA-killed control. Error bars correspond to 95% confidence intervals. For H⁺ negative uptake rates indicate proton production (acidification).

a 10-min exposure. The PFA-killed control showed no acidification.

The local reaction in separate biofilm layers to the different treatments, however, showed a more detailed picture and varying influence of CHX on respiration and acidification inside the dental biofilm (Fig. 7). With saliva alone, oxygen was consumed to a small extent in all biofilm layers down to 220 μm . Although the oxygen flux into the biofilm was marginally changed by sucrose addition, the distribution of local uptake rates at different depths within the biofilm was altered. The highest local uptake rates within the biofilm were localized close to the surface in the top 50 μm of the biofilm. CHX_1 inactivated the biofilm surface but led to increased oxygen uptake rates between depths of 50 to 120 μm . After CHX_10 treatment, the aerobic heterotrophic activity was depressed deeper in the biofilm to between 75 and 175 μm ; thus, the overall oxygen consumption remained constant. The PFA-killed control showed no aerobic activity in any of the biofilm layers.

With unsupplemented saliva, there was little acid production anywhere in the biofilm. However, when sucrose was added acid was produced in the bottom region of the biofilm between

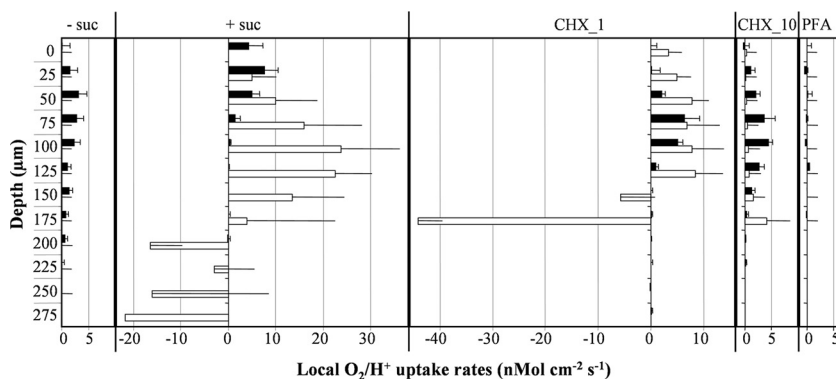


FIG. 7. Local oxygen (■) and H⁺ (□) uptake rates in different layers of dental biofilms *in situ* (1:10 buffered saliva) without sucrose (-suc), with 10% (wt/vol) sucrose (+suc), and with 10% sucrose after 1 min (CHX_1) or 10 min (CHX_10) of CHX treatment and in a PFA-killed control (PFA). Error bars correspond to 95% confidence intervals. A negative uptake rate indicates net production, and a positive uptake rate indicates net consumption.

TABLE 1. Structure and viability of *in situ* dental biofilms assessed by CLSM^a

Treatment	L _f (μm)		V (%)	
	Mean	95% CI	Mean	95% CI
-suc	226.7	148.7–304.6	63.4	53.6–73.08
+suc	217.3	201.4–233.2	70.7	63.0–78.4
CHX_1	230.0	106.3–353.7	2.0	-1.6–5.6
CHX_10	228.0	116.1–339.9	0.7	-0.2–1.6
PFA	232.7	143.0–322.3	18.9	8.0–29.9

^a Biofilms were assessed without antimicrobial treatment (-suc, +suc) and after 1 min (CHX_1) or 10 min (CHX_10) of CHX treatment or after PFA exposure (PFA). The mean biofilm thickness (L_f), the mean viability of all investigated dental biofilm layers (V), and the corresponding 95% confidence intervals (CI) are indicated.

175 and 275 μm . After exposure to CHX_1, acid production was elevated in the biofilm to 150 μm . After exposure to CHX_10 and after PFA killing, local acidification was suppressed throughout the biofilm.

CLSM. Microbial viability. CLSM analysis revealed a comparable mean L_f of 230 to 240 μm for all treatment modes (Table 1). Depending on the high density of *in vivo*-created dental biofilms, the laser penetration depth was limited to the outer 40 to 100 μm ($65.6 \pm 24.1 \mu\text{m}$) of the biofilm cell clusters. Thus, deeper areas could not reliably be visualized. The viability of untreated biofilms was ca. 60 to 70%. The distribution of live and dead or damaged bacteria showed no distinct distribution pattern. After CHX treatment, the mean viability was reduced to 2.0% (CHX_1) and 0.7% (CHX_10), respectively. The microorganisms at the biofilm surface were killed or damaged by CHX (Fig. 8). The PFA-“killed” control indicated a viability of almost 19% based on the Live/Dead stain.

Total bacterial counts and CFU/mm². The log BC/mm² values ranged between 7.27 (+suc) and 6.57 (PFA) and were similar for dental biofilms without and after treatment with CHX and PFA (Fig. 9). Almost 38% of the native biofilm microorganisms were culturable (log CFU/mm² 6.81; -suc). After sucrose exposure, the number of CFU increased (log CFU/mm² 7.13). CHX_1 treatment had a minor influence

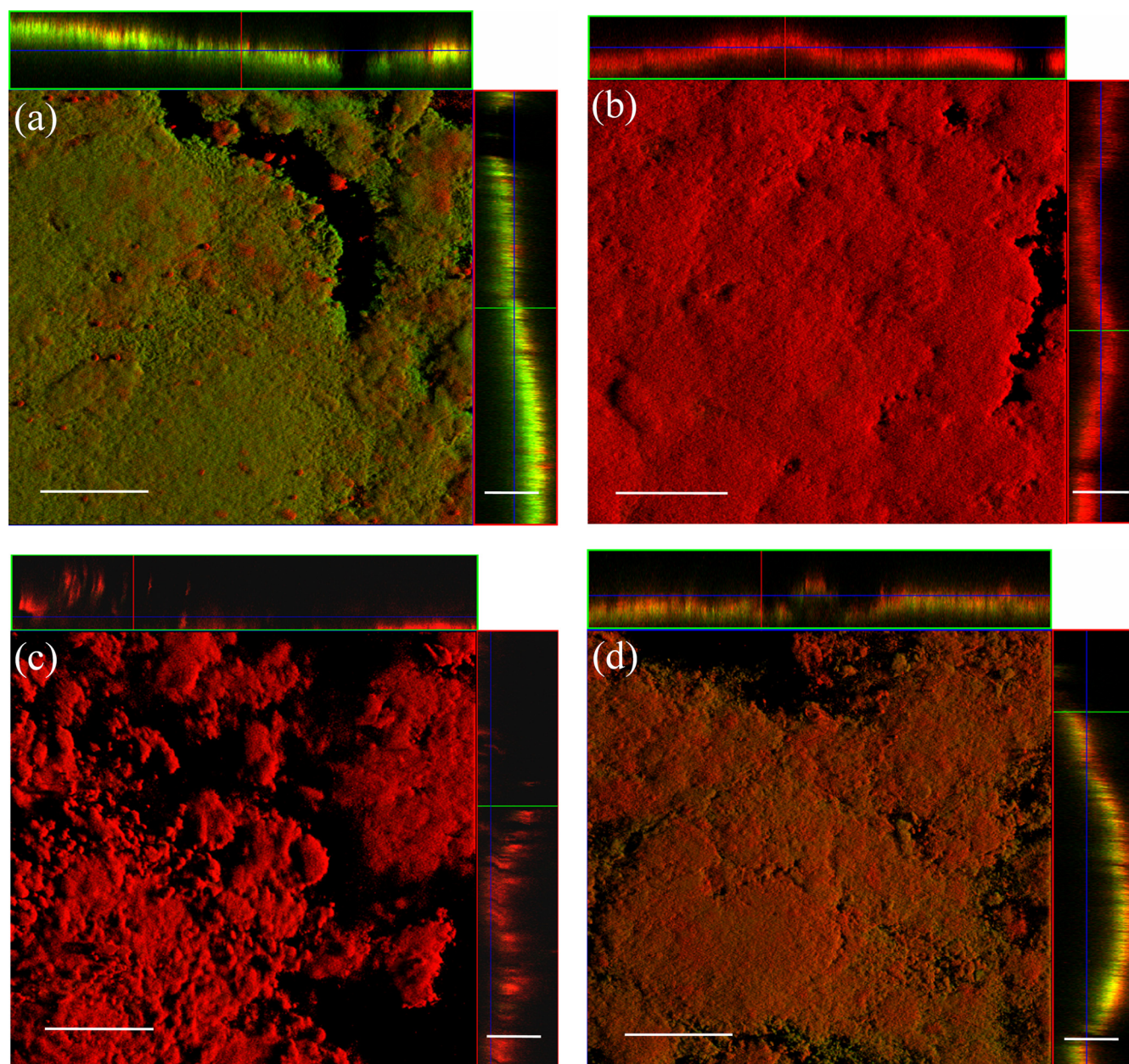


FIG. 8. Micrographs depicting the dental biofilms after staining with Live/Dead BacLight. Live or vital bacteria are green, and dead or damaged bacteria are red. Three-dimensional reconstructed confocal images and corresponding optical sections (xz and yz) at low magnification are shown. Scale bar, 200 μm . (a) Native 3-day-old dental biofilm showing a haphazard arrangement of vital MO and damaged cells with unstained areas in between; (b) biofilm after 1 min of CHX treatment (CHX_1) displaying only damaged bacteria at the surface; (c) biofilm after 10-min CHX treatment (CHX_10) with a similar appearance; (d) PFA-killed control.

(log CFU/mm² 6.45), whereas CHX_10 led to a reduction to log 4.93 CFU/mm². The PFA exposure reduced the CFU by almost 6 log units.

Diffusive transport of CHX within the biofilm. Assuming the transport of CHX from the solution into the biofilm is driven by diffusion, concentrations could be modeled along biofilm depth for different incubation times (Fig. 10). An assumed critical concentration of 0.1% CHX was reached only in a depth of 70 μm after 1 min and also after 10 min in a biofilm depth of about 230 μm , but not in the deepest biofilm layers.

Statistics. A summary of the statistical analysis results for the different investigation variables is given in Table 2.

DISCUSSION

Changes in the gradients of oxygen and pH in response to sucrose and additional CHX treatment in complex dental biofilms formed *in vivo* were measured by microelectrodes under real-time nondestructive conditions. The local effects on respiration and acidification inside dental biofilms were docu-

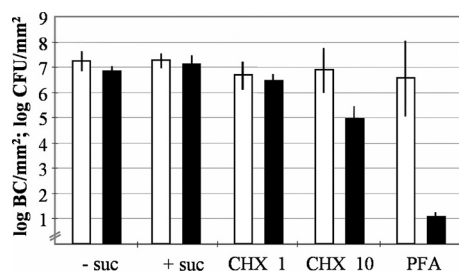


FIG. 9. Total bacterial cell counts (log BC/mm², □) and CFU (log CFU/mm², ■) in native dental biofilms (–suc), after sucrose exposure (+suc) or after 1 min (CHX_1) or 10 min (CHX_10) of treatment with CHX and in a PFA-killed control. Error bars correspond to the 95% confidence intervals.

mented. With unsupplemented saliva, oxygen consumption in the upper biofilm layer resulted in the bottom half of the biofilm being anoxic. Microbial respiration activity was obviously driven by endogenously produced substrates or salivary components, as became evident from the complete lack of activity in the PFA-killed control. Similar gradients have been documented in *Pseudomonas aeruginosa* (51), *Streptococcus epidermidis*, and *Staphylococcus aureus* (33) biofilms. Oxygen consumption in human dental biofilms has been found to be caused by Gram-positive microorganisms and especially oral streptococci (28, 46), not only for respiration but also to produce H₂O₂, which is presumed to be a mechanism by which to attack competing bacteria (23, 31). The result is that an anoxic niche conducive to the survival of anaerobic or acidogenic bacteria is created in deeper layers of the biofilm (22, 32). After sucrose addition the thickness of the anoxic layer increased, presumably due to increased respiration and other oxygen consuming metabolic activity in the upper layer. Concomitantly, a strong decrease in pH occurred in the anoxic region. The dynamic response of the dental biofilm physiology to environmental changes occurred instantly, and a new equilibrium was established within minutes. Comparable results were shown by Stoodley et al. (42) in *S. mutans* biofilms.

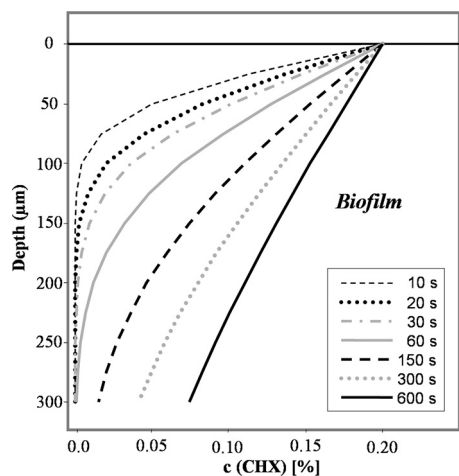


FIG. 10. Modeled concentration profiles of a 0.2% CHX formulation in dental biofilms at different incubation times (t) between 10 and 600 s.

TABLE 2. Results (P values) of the statistical comparison between different treatment modes determined using ANOVA^a

Compared treatments	P value for:						
	Biofilm conditions			Biofilm structure and viability			
	Flux		Anaerobic depth	L_f	V	Log BC/mm ²	Log CFU/mm ²
	O ₂	H ⁺					
–suc/+suc	0.03	0.01	<0.01	0.85	0.33	0.91	0.20
+suc/CHX_1	0.22	0.08	0.12	0.85	<0.01	0.14	0.04
+suc/CHX_10	0.14	0.01	<0.01	0.86	<0.01	0.45	<0.01
+suc/PFA	0.01	0.04	<0.01	0.76	<0.01	0.42	<0.01

^a Differences were considered significant for $P < 0.05$. Dental biofilms were assessed *in situ* (1:10 buffered saliva) without sucrose (–suc), with 10% (wt/vol) sucrose (+suc), after 1 min (CHX_1) or 10 min (CHX_10) of CHX treatment, or after PFA exposure (PFA). Boldfacing indicates a statistically significant difference ($P < 0.05$). L_f , biofilm thickness; V , mean microbial viability; BC, total bacterial count.

Without supplemental sucrose, almost no acid formation was detectable in the biofilms, but after sucrose addition the fermentation activity increased immediately in the anaerobic biofilm layers (>200 μ m). After about 10 min a minimal pH value of 4.3 was obtained. The range of pH drop we measured (from pH 7 to between pH 5.5 and 4.3) was in agreement with the results of microelectrode measurements in a dentine groove model reported by other authors (13, 53, 54). The degree of acidification in the biofilms will be expected to vary between individuals, as a function of both host and bacterial factors such as biofilm thickness, concentration of acidophilic bacteria, and saliva chemistry.

Because the temperature during the electrode measurements was below the physiological intraoral temperature (ca. 36°C), it can be assumed that the metabolic activity of the biofilms would have been higher at a higher temperature. Nevertheless, valuable information on microbial activity in dental plaque has been gathered from experiments performed on *ex vivo* specimens conducted at room temperature (53, 54), and we also point out that during the course of eating and drinking hot and cold foods the hard surfaces of the teeth experience a wide range of temperatures.

CHX effect on dental biofilm physiology and structure. An ideal prerequisite for a successful antimicrobial chemical treatment, such as CHX, is that all bacteria within the biofilm are exposed to an adequate concentration for an adequate time in order to achieve a clinically relevant reduction in pathogenesis. We used a simple diffusion model to calculate CHX concentrations as a function of depth and time. The model did not account for adsorption, release, or consumption, which will also influence transport (39). We assumed that a concentration of 0.1% CHX would be a clinically relevant concentration based on (i) the reported MIC range of 8 to 500 mg/liter by Stanley et al. (38) for different strains of oral bacteria and (ii) the finding that for some oral bacteria the BIC (biofilm inhibiting concentration) was up to 500 times (*Streptococcus sanguinis*) (25) that of the MIC. Furthermore, Vitkov et al. (49) were able to verify damage to bacterial cell membranes by electron microscopy when using a concentration of 0.1%. The calculated concentration profiles showed that when using a 0.2% CHX solution with an exposure time of 1 min a concentration

of 0.1% or greater was achieved only to a depth of 70 μm . Interestingly, this matches well with our experimental data, which showed that oxygen consumption had shifted from the top 50 μm to below 75 μm after 1 min of CHX exposure. The diffusion model serves well for orientation where the diffusion behavior of CHX in biofilms is concerned and provides insight into optimization of routine hygiene maintenance. The model predicts that it would take 17.5 min to achieve 0.1% CHX at the base of a 300- μm -thick biofilm, e.g., interproximal or at other retention sites. However, if the thickness of the biofilm was reduced to 100 μm the time will be reduced to <2 min; if it was reduced to 30 μm , then it would only take 12 s. These observations illustrate the clinical importance of limiting the physical thickness of the biofilm by mechanical means (e.g., brushing or scaling) prior to exposure to rinses and that dental biofilms are unlikely to be controlled by rinsing alone, unless the penetration time can be increased or the activity is more aggressive than CHX.

Although the customary recommended exposure time for daily CHX use is 1 min (2), we found limited effect by viability staining, oxygen consumption, acid formation, and culturability over this time period. Although CHX reduced the viability and metabolism on the biofilm surface, it did not significantly reduce the overall oxygen metabolism. Even after a 10-min treatment the biofilm was still physiologically active, demonstrating incomplete killing, and there was a relocation of activities deeper in the biofilm.

The acidifying fermentative processes of the biofilm were also relocated by treatment with CHX. With sucrose stimulation, acid production occurred at depths of >200 μm and after 1 min of CHX treatment higher up in the biofilm, at 150 μm . This upward shift in activity might reflect an ecological shift caused by CHX more adversely affecting competitors. After 10 min, CHX exposure acid fermentation was almost completely abolished throughout the biofilm, and the overall acidifying activity was reduced to 3%. Based on the local rate calculations and the prevailing CHX concentrations, it could be demonstrated that CHX inactivated bacteria from the top down and layer by layer of dental biofilms were killed with the highest efficacy in the outer regions. Presumably, this is caused by a combination of several mechanisms: (i) the deeper penetration of oxygen, reducing the appearance of anaerobic processes; (ii) the higher CHX concentrations and increased toxicity effects in deeper layers strongly affecting the fermentative processes; and (iii) a differing susceptibility to CHX of the functional group of organisms (i.e., aerobic heterotrophs versus anaerobic fermentative bacteria) could also exist, since oral microorganisms show diverse natural resistance toward CHX (14).

Since the primary effect of CHX on the bacterial structure is based on damaging the cell membrane (3, 49), the effect of CHX can be visualized by vital staining based on membrane integrity of cells. General mechanisms and limitations concerning the application of SYTO 9 and PI for the detection of bacterial cell viability are reported by Stocks (41). If the limitations are taken into account, the results of the Live/Dead stain should be seen as semiquantitative with regard to viability. In growth curves of oral streptococci (*S. mutans* and *S. sanguinis*), Decker (11) has demonstrated that staining with SYTO 9/PI reflected the microbial viability well according to the conventional microbiological investigation (optical density,

BC, and CFU). A ratio of 60% vital bacteria in untreated dental biofilms has been reported in other studies of 2- to 3-day-old biofilms (48, 50, 55). Compared to that, the percentage of culturable microorganisms in our study was only 38%; we speculate that there were many bacteria in the biofilm that were either in an inactive metabolic state or nonculturable. However, sucrose exposure (+suc) led to an increased rate of cultivable bacteria to almost 70%, suggesting that many bacteria in native dental biofilms may be in an inactive state during periods between eating. Similarly, for staphylococci it has been shown that there is an active zone on the biofilm surface, with only 10% of the bacteria being indeed dead, while the rest were live but inactive (33). In contrast to the viability of 2% estimated by Live/Dead staining, the culturability after 1 min exposure to CHX was 58%, so it might be assumed that for short exposures some bacteria were still viable, even if they appeared to be membrane damaged. It was only after 10 min of exposure that the number of CFU was significantly reduced compared to the corresponding BC. In all groups the applied cultivation conditions had influenced the growth of diverse bacterial species (streptococci versus nonstreptococci) and thus the difference between the counts and the CFU. After PFA fixation the viability based on the Live/Dead staining was 19%. It is anticipated that the PFA fixation affected the permeability of the cell membranes, and PI might not have been able to penetrate the fixated membranes. Compared to the absence of metabolic activity by microelectrode measurements and the almost 6-log reduction in CFU, we interpreted that the 19% viability by Live/Dead staining included false-positive results. This phenomenon is reflected in contradictory literature. On one hand, it was reported that PFA did not interfere with the staining properties of SYTO 9/PI (15). On the other hand, it could be shown that treatment or fixation with formaldehyde affected the culturability and metabolic activity but not membrane integrity based on the Live/Dead stain (6, 24, 34).

Our study demonstrates the utility of using microelectrodes to measure the influence of nutrients and antimicrobial agents on the physiology of human dental biofilms nondestructively and in real time. The microelectrode data can be corroborated with microscopy and culture techniques. The effect of CHX on sucrose-exposed metabolically active dental biofilms was investigated. It was not possible to kill the *in vivo* formed dental biofilms by CHX completely. CHX had a more pronounced effect on acidification than respiration, thereby creating a more aerated and less acidogenic environment. The data suggest that the microecology might be changed to one that was less pathogenic. Sustaining this microecology longer might inhibit the selection of pathogenic microorganisms like cariogenic pathogens such as *S. mutans* and other acidogenic bacteria or periodontopathogenic anaerobes and influence the composition of the oral microbial flora favorably.

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