

XTT assay of ex vivo saliva biofilms to test antimicrobial influences

XTT-Test zur Testung von antimikrobiellen Einflüssen auf ex vivo Speichelbiofilme

Abstract

Objective: Many dental diseases are attributable to biofilms. The screening of antimicrobial substances, in particular, requires a high sample throughput and a realistic model, the evaluation must be as quick and as simple as possible. For this purpose, a colorimetric assay of the tetrazolium salt XTT (sodium 3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) converted by saliva biofilms is recommended. Cleavage of XTT by dehydrogenase enzymes of metabolically active cells in biofilms yields a highly colored formazan product which is measured photometrically.

Materials and method: The suitability of the XTT assay for detecting the vitality of ex vivo saliva biofilms was tested to determine the efficacy of chlorhexidine and ozone versus saliva biofilms grown on titanium discs.

Results: The XTT method lends itself to testing the vitality of microorganisms in saliva biofilms. The sensitivity of the arrays requires a specific minimum number of pathogens, this number being different for planktonic bacteria and those occurring in biofilms. The antibacterial effect after treatment with chlorhexidine or ozone was measured by XTT conversion that was significantly reduced. The antimicrobial efficacy of 60 s 0.5% and 0.1% chlorhexidine treatment was equal and comparable with 60 s ozone treatment.

Conclusion: The XTT assay is a suitable method to determine the vitality in saliva biofilms, permitting assessment of the efficacy of antimicrobial substances. Its quick and easy applicability renders it especially suitable for screening.

Keywords: biofilm model, saliva, *S. mutans*, ozone, chlorhexidine, XTT assay

Zusammenfassung

Ziel: Viele Zahnerkrankungen sind auf Biofilme zurückzuführen. Das Screening von antimikrobiellen Substanzen erfordert einen hohen Probendurchsatz mit einem realistischen Modell. Die Auswertung sollte hierbei so schnell und so einfach wie möglich durchführbar sein. Zu diesem Zweck wird ein kolorimetrischer Test mit dem Tetrazoliumsalz XTT (Natrium 3'-[1-[(phenylamino)-carbony]-3,4-Tetrazolium]-Bis(4-methoxy-6-nitro)benzol-sulfonsäurehydrat) empfohlen, das durch den Speichelbiofilm umgewandelt wird. Durch die Umsetzung von XTT durch Dehydrogenase-Enzyme von metabolisch aktiven Zellen in Biofilmen entsteht ein gefärbtes Formazanprodukt, das photometrisch nachgewiesen wird.

Material und Methode: Die Eignung des XTT-Tests zum Nachweis der Vitalität von ex vivo Speichelbiofilmen wurde getestet, indem die Wirksamkeit von Chlorhexidin und Ozon auf Speichelbiofilme auf Titanplättchen bestimmen wurde.

Ergebnisse: Die XTT-Methode eignet sich für die Testung der Vitalität der Mikroorganismen in Speichelbiofilmen. Die Empfindlichkeit des

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Tests erfordert eine bestimmte Mindestanzahl von Mikroorganismen, wobei sich diese Anzahl in planktonischen und Biofilmenkulturen unterscheidet. Die antibakterielle Wirkung nach der Behandlung mit Chlorhexidin oder Ozon wurde durch die Reduktion der XTT-Umsetzung nachgewiesen.

Die antimikrobielle Wirksamkeit von 60 s Behandlung mit 0,05% und 0,1% Chlorhexidin war vergleichbar mit 60 s Ozonbehandlung.

Fazit: Der XTT-Test ist eine geeignete Methode, um die Vitalität in Speichelbiofilmen zu bestimmen und erlaubt die Beurteilung der Wirksamkeit von antimikrobiellen Substanzen. Durch die schnelle und einfache Anwendbarkeit ist dieser Test besonders für Screenings geeignet.

Schlüsselwörter: Biofilmmodell, Speichel, *S. mutans*, Ozon, Chlorhexidin, XTT-Assay

Introduction

Bacterial infections play a specific role in dentistry. After the supragingival tooth surfaces and mucous membranes have been wetted with saliva, microbes settle there and form a biofilm [1]. This biofilm accommodates dental pathogens and protects them against environmental stress factors, such as chemotherapeutics, the immune system, acids, hunger periods, and reactive oxygen products [2], [3].

Antimicrobially effective substances and techniques should be tested on a suitable biofilm model, as the efficacy against planktonic pathogens has little predictive value for the efficacy against biofilms.

For a number of years, several mono-species biofilm models have been available which accommodate typical oral microbes [4], [5]. Streptococci are frequently used as a caries model, although other scientists found out that they do not represent the etiological pathogens of the disease [6]. For the treatment of periodontal diseases, anaerobic periodontal marker pathogens are important [7]. But *in vivo* plaque microbiota is highly diverse and complex [8]. The oral cavity harbors more than 1,000 different microorganisms, which join to form multispecies biofilms [9]. Guggenheim et al. used biofilms consisting of a maximum of six different pathogens [10]. The oral fluid, too, is an essential component in the formation of dental biofilms. The proteins in the saliva are a significant source of food for microbes. Pellicle proteins settle on the dental surfaces forming the so-called conditioning film. This conditioning film forms the basis for the development of biofilms, as the adhesins of the bacteria directly bind to these oligosaccharide-containing glycoproteins [11], [12]. In addition salivary antimicrobial factors are important stressors that can enhance biofilm formation [13], [14]. Many artificial saliva formulations have been designed that attempt to imitate this process in order to ensure realistic biofilm formation. In most cases, however, artificial saliva fails to provide all the organic and inorganic components that exist in natural saliva. Moreover, no evidence has been supplied that artificial saliva promotes biofilm formation [15].

For their biofilm models, other researchers used volunteers' saliva which was filtered under sterile conditions, diluted and centrifuged [16].

A simple, more realistic multispecies biofilm model can be obtained by culturing the saliva of volunteers, without prior filtration under sterile conditions [17]. Of course, this is only a model because the circadian rhythm of salivation and the correspondingly variable composition of saliva cannot be imitated, nor can regular food intake [18], [19]. Furthermore, the oral conditions are different in each patient [20]. A subsequent detection of biofilm formation is difficult. Even when using a non-specific agar, such as Columbia blood agar, it is not possible to definitely detect every species in the culture. Alternatively, colorimetric methods, e.g., the XTT assay, are appropriate to measure metabolic activity and vitality. XTT is a yellow salt that is reduced by dehydrogenases of metabolically active cells to a colored formazan product. Colorimetric methods are attractive because they have the potential to generate clear-cut endpoints based on a visible color change.

The objective of this study, therefore, was the development of a method suitable for testing saliva biofilms using XTT.

Materials and method

Cultivation of biofilms

Biofilms were cultured on titanium discs 5 mm in diameter and 1 mm thick (Straumann, Basel, Switzerland). The sterile titanium discs were positioned in 96-well microtitre plates (Techno Plastic Products AG, Trasadingen, Switzerland), covered with 100 µl fresh, unstimulated saliva of healthy volunteers (aged between 20 and 30 years, non-smokers), and incubated aerobically at 37 °C. The donors did not take any medication three months prior the study and did not have active carious lesions or periodontal disease. After 24 h, the saliva was drawn off and replaced by sterile brain-heart infusion broth (BD, BBL™, Heidelberg, Germany), as a highly nutritious general-purpose growth medium. After 48 h, the medium was drawn off, and the discs were washed with 0.9% NaCl

solution and transferred onto a new, sterile microtitre plate.

Antiseptic treatment with chlorhexidine

Chlorhexidine digluconate was used as a 0.1% and 0.05% aqueous solution. The discs were covered with 100 µl of the antiseptic and incubated for 1 min.

After this exposure, the chlorhexidine was drawn off, and the antiseptic effect was stopped by adding 1 ml of inactivator (Lipofundin MCT 20%, B.Braun, Melsungen, Germany). The inactivation of chlorhexidine by the inactivator was validated by the quantitative suspension test according to EN 1040. Physiological saline was used for control.

Application of ozone

The test objects were direct treated for 20 s, 30 s, 40 s, or 60 s with gaseous ozone provided by a HealOzone device (KAVO, Biberach, Germany). The ozone is delivered via a hose into a disposable sterile cup at a concentration of 2,100 ppm ± 10%. The ozone gas is refreshed in this disposable cup at a rate of 615 cc/minute changing the volume of gas inside the cup over 300 times every second [21].

Inactivation was unnecessary as the device suctions off any residual ozone after application.

Viability measurement by XTT assay

Bioreduction of XTT could be potentiated by addition of electron coupling agents such as phenazine methosulfate (PMS) or menadione (Men) [22].

To optimize the staining solution, 200 µl of the XTT solution was added to each disc bearing the grown biofilm after 24 h and 48 h, respectively. The added XTT solution was composed of the following:

1. XTT (180 mg/l) (AppliChem, Darmstadt, Germany) and menadione (0.688 mg/l) (Sigma-Aldrich, Munich, Germany) (hereafter called „XTT+Men“)
2. XTT (180 mg/l) and phenazine methosulfate (20 mg/l) (PMS, AppliChem, Darmstadt, Germany) (hereafter called „XTT+PMS“)
3. XTT (180 mg/l), menadione (0,688 mg/l) and phenazine methosulfate (20 mg/l) (hereafter called „XTT+Men+PMS“)

To determine the measuring range, saliva was diluted with physiological saline and incubated with the XTT staining solution specified in test 1 (XTT+PMS+menadione).

To test the antimicrobial efficacy of chlorhexidine and gaseous ozone, the treated discs were also incubated with 200 µl of the staining solution (XTT+PMS+menadione).

After 3 h of incubation while shaking (Titramax, Heidolph Instruments, Schwabach, Germany) at 37 °C, 100 µl of all solutions were transferred onto a new sterile microtitre

plate and analyzed at 450 nm (reference wavelength 620 nm) using a photometer (anthos Mikrosysteme, Krefeld, Germany) [23].

Determination of CFU

After treatment, the titanium discs were placed into wells with 200 µl 0.9% NaCl solution and the biofilm was removed by ultrasonic scaling (Branson 2510, 130 W, 42 kHz, Dietzenbach, Germany). Serial dilutions were made by transferring 0.1 ml of the resultant suspension to 0.9 ml of fresh 0.9% NaCl solution. After that, an aliquot portion of 0.1 ml from each dilution was plated on Columbia sheep blood agar (BBL™, BD, Heidelberg, Germany) and incubated aerobe at 37 °C for 48 h. The colonies were counted and expressed as colony forming units (CFU). The CFU values were log transformed.

Confocal laser scanning microscopy

Live/Dead-Staining (BacLight, Invitrogen, Darmstadt, Germany) was used for microscopical analysis of the bacterial vitality. The biofilms were incubated immediately with the dye according to manufacturer's instructions. After incubation the discs were rinsed with 0.9% NaCl solution to remove dye residues from the biofilm. The samples were evaluated in the confocal laser scanning microscope (CLSM510 Exciter, Zeiss, Jena, Germany).

Scanning electron microscopy

For electron microscopy, the biofilms were prepared as follows: after a fixation step (1 h in 1% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, 5 mM HEPES (pH 7.4), and 50 mM NaN₃), the samples were treated with 2% tannic acid for 1 h, 1% osmium tetroxide for 1 h, 1% thiocarbonylhydrazide for 30 min, 1% osmium tetroxide at 4 °C overnight, and with 2% uranyl acetate for 2 h with washing steps in between. The samples were dehydrated in a graded series of acetone solutions (10 to 100%) and then critical-point dried. Finally, samples were mounted on aluminum stubs, sputtered with gold-palladium and examined in an EVO LS10 (Zeiss, Oberkochen, Germany) (Figure 1).

Analysis

For all experiments, at least eight test objects each were used. The chlorhexidine treatment required 23 discs. In addition, eight test objects each were available for control tests. Continuous data are presented as mean ± standard deviation. Statistical analyses were performed with STATVIEW® 5.0 (SAS, Cary, NC) software. Graphs were also created using STATVIEW. Medians are given with their standard errors. Nonparametric correlations (Mann-Whitney U-test) were estimated for comparison of absorptions. P-values below 0.05 were considered statistically significant.

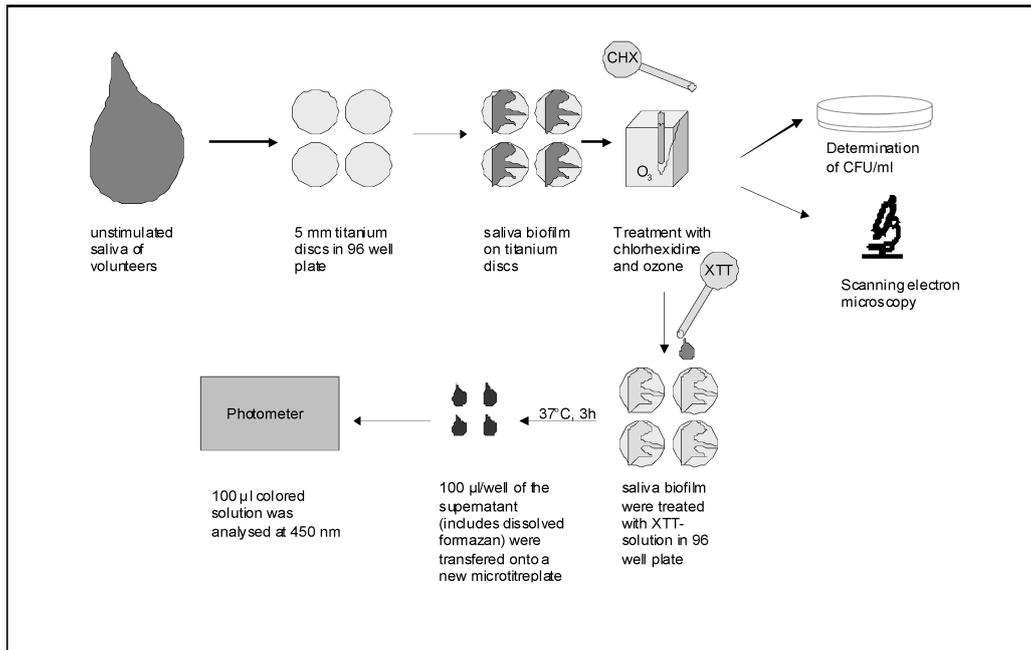


Figure 1: Schematic view of the experiment

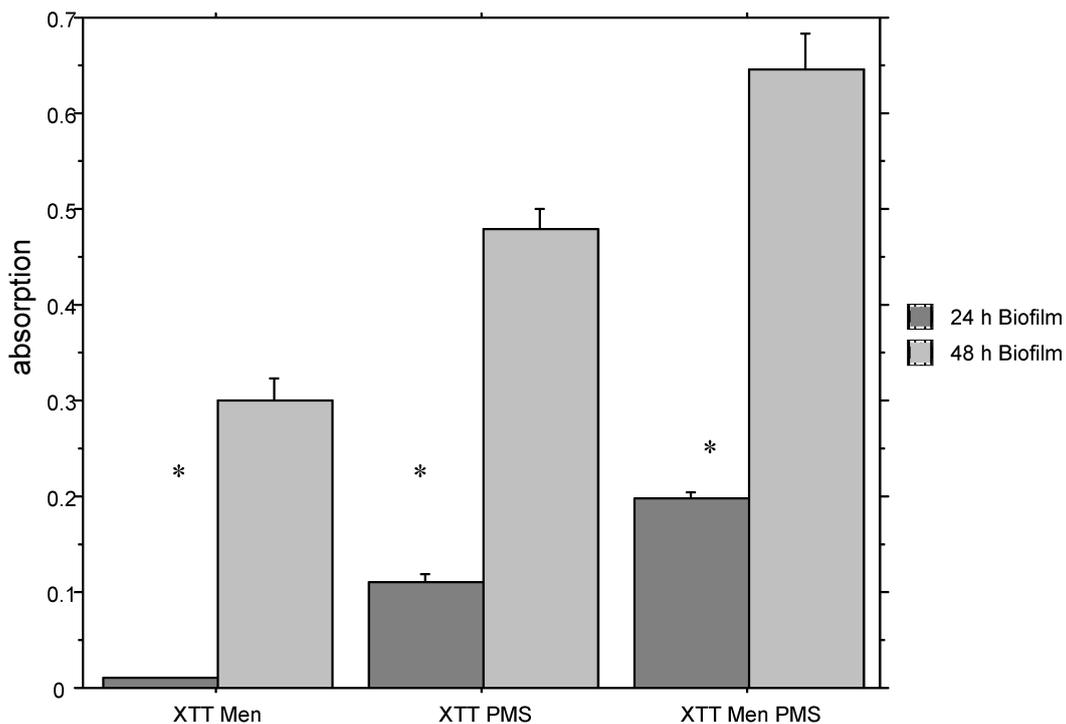


Figure 2: Diagram of the absorption at 450 nm (reference wavelength 620 nm) of XTT metabolized by saliva biofilm (XTT+menadione, XTT+ phenazine methosulfate, XTT+ menadione+ N- methyl-phenazinium methylsulfate). Mean cell numbers \pm standard deviations, *($p < 0.01$)

Results

Cultivation of biofilms

The cultivation procedure was constantly checked for cultures by determining the CFU and for reproducibility by microscopy. Cell densities of 10^8 CFU/ml were regularly be removed from the test objects by ultrasound scaling.

Optimization of the staining solution

The absorption of the colored formazan derivate of XTT converted by the microbes is a measure of the cellular vitality.

A high absorption value indicates high metabolic activity. Figure 2 shows the absorption values (at 450 nm) of the different staining solutions (XTT+Men, XTT+PMS and XTT+Men+PMS) on 24-h- and 48-h-old biofilms. For all

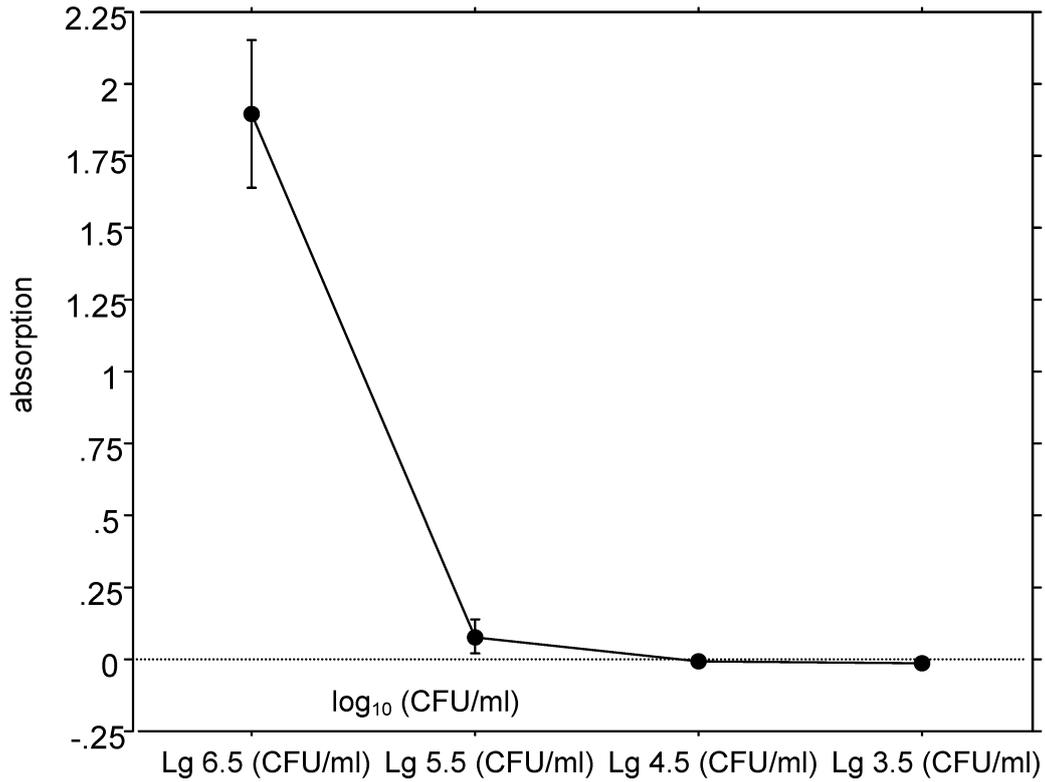


Figure 3: Diagram of the absorption at 450 nm (reference wavelength 620 nm) of XTT + menadione + phenazine methosulfate metabolized by various saliva dilutions. Mean cell numbers ± standard deviations, *(p<0.01)

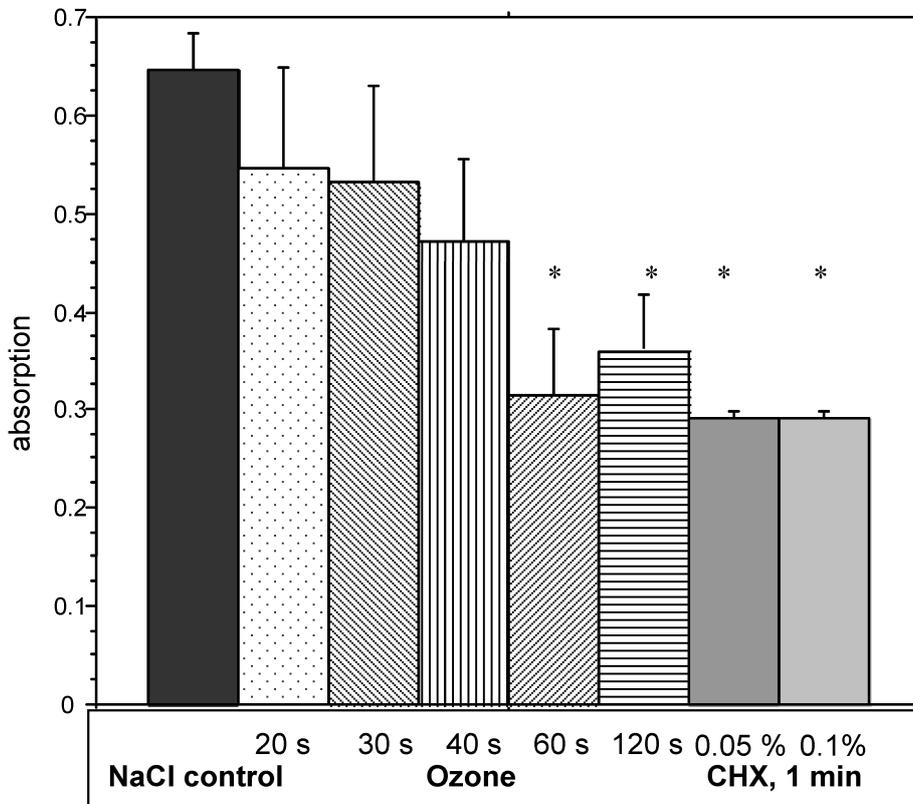


Figure 4: Diagram of the absorption at 450 nm (reference wavelength 620 nm) of XTT+ menadione+ phenazine methosulfate, metabolized by saliva biofilms for 48 h after treatment with 0.9% NaCl solution (control), gaseous ozone and chlorhexidine. Mean cell numbers ± standard deviations, *(p<0.02) vs. 0.9% NaCl control

staining solutions, significant ($p < 0.01$) differences were visible between the 24-h and 48-h biofilms.

In the case of XTT+Men, the absorption at 450 nm increased to a value an average of 30 times higher (from 0.01 to 0.30) after 48 h than after 24 h. Within these 24 h, the absorption value of XTT+PMS increased to five times (from 0.10 to 0.50) the original value, while the absorption of XTT+Men+PMS was increased three times from 0.20 to 0.65.

The absorption values of XTT+Men+PMS were always significantly ($p < 0.01$) higher than those of the other straining solutions.

Determination of the measuring range

Figure 3 shows the absorption values at 450 nm determined by dilution of the saliva and subsequent staining with XTT+Men+PMS. There are significant differences ($p < 0.01$) between the absorption values of the biofilms up to increments of $4.5 \log_{10}$. Absorption was no longer measurable in concentrations from $3.5 \log_{10}$ (CFU/ml), except for negative controls.

Antimicrobial treatment

A reduced XTT conversion was observed in saliva biofilms which had been subjected to antimicrobial treatment with gaseous ozone and chlorhexidine. This reduction was significant after a 60-s or 120-s ozone treatment ($p < 0.02$) and a one-minute chlorhexidine treatment ($p < 0.01$) (see Figure 4). There was no difference in the conversion of XTT using a 0.05% or a 0.1% chlorhexidine solution. No difference could be seen in the scanning electron micrographs between the untreated biofilm and the biofilm treated with ozone. In both samples, the cells appear plump and the biofilm has a loosely bound structure. The bacteria in the biofilm treated with chlorhexidine are damaged and the overall structure appears to be tighter (Figure 5). The CLSM images confirmed these observations (Figure 6). After treatment with ozone, parts of the biofilm were dyed red (cells with damaged membranes). After CHX treatment, no green colored areas (cells with intact membranes) were identified.

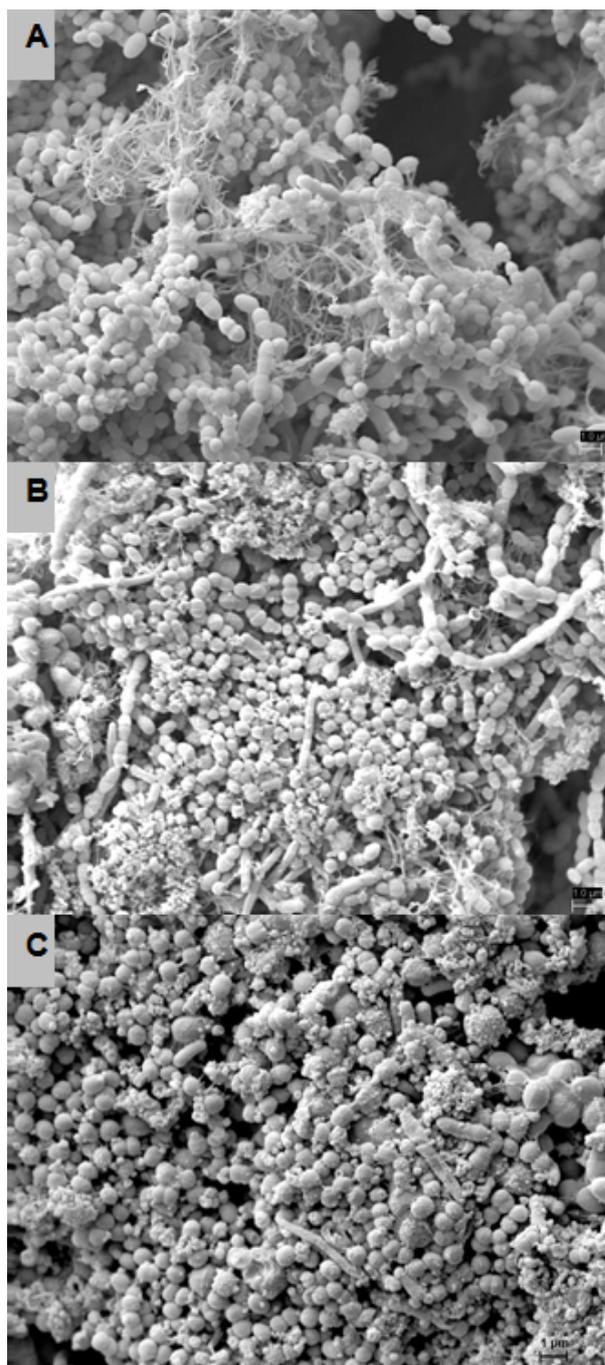


Figure 5: SEM micrograph: 48-h mature saliva biofilm: (A) untreated, (B) after ozone treatment, (C) after chlorhexidine treatment. Magnification 10,000 x

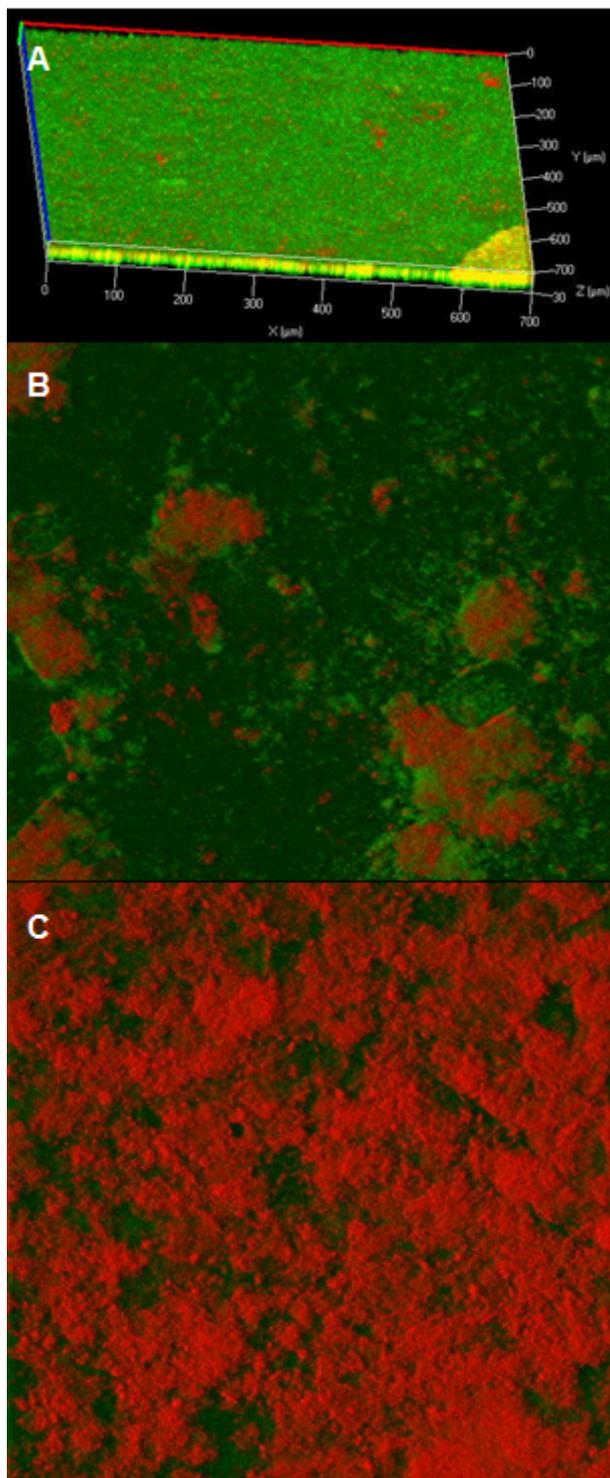


Figure 6: CLSM micrographs: 48-h mature saliva biofilm: (A) untreated, (B) after ozone treatment, (C) after chlorhexidine treatment. Magnification 100 x

Discussion

Causing typical dental diseases, such as caries and periodontitis, biofilms complicate the elimination of microbes responsible for forming the biofilms by antimicrobial substances. The objective of this study was to develop a biofilm model suitable for testing the efficacy of antimicrobial substances with non-culture-based detection of

the vitality of saliva biofilms using XTT, and to prepare a suitable XTT assay.

Our study had several limitations. We used saliva of volunteers to create a practically relevant biofilm model. For better understanding of the interactions between bacteria and XTT, we performed our experiments on machined titanium discs to exclude material hydrophobicity, retention niches such as cavities and porosities into which the biofilm could adhere. Titanium implants have been successfully used in dentistry and biofilms on titanium are the central problem in peri-implantitis. Peri-implantitis of osseointegrated oral implants is not a mono-infection by single pathogens; rather, they show the characteristics of mixed infections. Whereas the single species of a mixture of bacteria could not induce experimental abscesses, the combination of these species could do it [24]. Plaque biofilms containing multiple species of appropriate bacteria should be more relevant for studying dental diseases and antimicrobial efficacies [25]. The first step was the use of only aerobic cultivation methods. Most XTT assays are carried out aerobically. In the next step we will use a subgingival plaque biofilm under anaerobic conditions for XTT assays. But studies also showed that the same microbiota that can be found around implants (under anaerobic conditions) also can be found around teeth (under aerobic conditions) [26], [27].

XTT is a colorless tetrazolium salt, which is converted into a colored, water-soluble formazan derivative by dehydrogenases, with succinate dehydrogenase being particularly important [23] as it plays a major role in the energy supply of each individual living cell [28]. Unlike other tetrazolium salts (e.g., CTC and TTC), XTT does not require any insoluble formazans to be extracted. Since the color change in the solution can be directly determined by photometry, the XTT test permits a large number of test objects to be tested for their vitality very quickly. The incubation of bacteria with XTT for 3 h at 37 °C has already been the subject of several publications [23]. What is new is the composition of the XTT staining solution applied. The saliva biofilm contains a large variety of different microbes (Gram-positive, Gram-negative bacteria and fungi). To convert XTT, these microbes require various additives which function as electron carriers. The standard additive for *Candida spp.* is menadione [29], [30], [31], [32], but PMS can be applied as well [33]. For Gram-positive cocci bacteria, PMS is used in most instances [34], but sometimes menadione is also used [35]. In the case of Gram-negative rod-shaped bacteria, mainly menadione is applied [35], [36]. Our own preliminary (unpublished) investigations confirm these results on the suitability of menadione for *Candida albicans* and PMS for *Streptococcus mutans* and *Streptococcus sanguinis* as representatives in dentistry. As for *Pseudomonas aeruginosa*, the combined application of menadione and PMS turned out to be suitable [37]. By means of a culture-based analysis of the saliva, Gram-positive cocci bacteria, i. a., could be isolated. Therefore, the addition of PMS seemed to be indispensable for the colorimetric detection using XTT. McCluskey et al. also exclusively used PMS for the col-

orimetric detection of microbes occurring in activated sludge, and were able to prove that there is a direct correlation between formazan production and oxygen consumption [23].

In a direct comparison, the combined PMS-menadione electron mediators showed the highest XTT conversion in saliva biofilms (see Figure 2). It turned out, however, that a high cell count of at least $4.5 \log_{10}$ (CFU/ml) is required to detect the XTT reduction (see Figure 4). At $5.5 \log_{10}$ (CFU/ml), a very high absorption (1.91) was detected at 450 nm. The higher the number of metabolically active cells, the higher the colorimetric signal. In addition, the higher the metabolism of cells, the higher the signal. Obviously, there is no linear relation between the number of cells and the colorimetric signal [38]. When other dyes (FDA und Syto9) were used, the adsorption even remained constant [39]. After 48 h of biofilm formation, cell densities of ca. $8 \log_{10}$ (CFU/ml) were reached, but following the XTT test, the absorption in the biofilm was 0.65, i.e., less than in planktonic bacteria although the cell density was higher. The amount of retained product may vary between planktonic bacteria and biofilms [38]. Moreover, biofilms are subjected to other conditions than are fresh bacteria suspensions. Many pathogens are persistent and, therefore, exhibit lower metabolic activity [40]. For this reason, a minimum number of pathogens cannot be determined from suspensions and biofilms in exactly the same manner. In addition, planktonic cells can invest more energy in routine metabolism [30]. In our experiments, the absorption was 30 times higher after 48 h than after 24 h, i.e., the conversion of XTT increased due to the growth of the biomass. However, the different metabolic levels do not lead to a logarithmic increase of the XTT reduction. This XTT-related observation was also made by other research groups [39].

Reduced formazan formation was observed due to the antimicrobial treatment of the saliva biofilm. Consequently, the XTT test is suitable for determining the efficacy of antimicrobial substances, especially for screening. The low chlorhexidine concentration used has already been investigated by other researchers, who noted insufficient antimicrobial efficacy in the biofilms [41]. On the other hand, chlorhexidine proved to be slightly superior to ozone. This has also been published by other research groups who applied alternative methods [42]. However, *in vivo* Hauser-Gerspach et al. could not find significant antimicrobial effects of CHX and ozone [43]. The scanning electron micrographs confirmed this result. Following the ozone treatment, the morphology of the cells showed no differences. Although the biofilm treated with chlorhexidine appeared to be damaged compared to the control, only a few cells were morphologically deformed.

In spite of some disadvantages, XTT with the addition of menadione and PMS is a suitable method for determining the vitality in bacterial saliva biofilms and permits assessment of the efficacy of antimicrobial substances.

The assay is easy to perform, and allows a large number of test objects to be tested. It is particularly suited to

screening various factors influencing the biofilm, such as antiseptics or other physical or chemical treatments, for instance, ozone, photodynamic therapy or atmospheric pressure plasma.

Notes

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Competing interests

The authors declare that they have no conflict of interest.

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