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**Original Research**

# Interaction between XTT Assay and *Candida Albicans* or *Streptococcus Mutans* Viability

***Eduardo Buozi Moffa[1,2], Fernanda E Izumida[3], Maria Carolina M Mussi[4], Walter L Siqueira[5], Janaina Habib Jorge[6], Eunice T Giampaolo[7]***

***Contributors:***

[1]Postdoctoral Fellow, Department of Pediatric Dentistry, Orthodontics and Public Health, Bauru Dental School, University of São Paulo, Bauru, Brazil; [2]Professor, Department of Prosthodontics, Ceuma University, São Luís, Maranhão, Brazil; [3]Post-graduate Student, Department of Dental Materials and Prosthodontics, School of Araraquara Dental, UNESP-Universidade Estadual Paulista, São Paulo, Brazil; [4]Postdoctoral Fellow, Department of Oral Pathology, School of Dentistry, University of Sao Paulo, Sao Paulo, SP, Brazil; [5]Associate Professor, Department of Dentistry and Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, ON, Canada; [6]Assistant Professor, Department of Dental Materials and Prosthodontics, School of Araraquara Dental, UNESP-Universidade Estadual Paulista, São Paulo, Brazil; [7]Professor, Department of Dental Materials and Prosthodontics, School of Araraquara Dental, UNESP-Universidade Estadual Paulista, São Paulo, Brazil.

***Correspondence:***

Moffa EB. Universidade Estadual Paulista, School of Araraquara Dental, Department of Dental Materials and Prosthodontics, Rua Humaitá 1680, CEP: 14801-903, Araraquara, São Paulo, Brasil. Tel: +551633016410, Fax: +551633016406. Email: du_moffa@yahoo.com.br

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***Abstract:***

**Background:** Methods based on metabolic activity and cell viability, such as XTT reduction assay and counting colony-forming units (CFU), have been frequently used for biofilm evaluation. The association between the metabolic activities experiments with counting CFU is labor-intensive that need to be done separately. Thus, the aim of this study was to evaluate the effects of XTT salt, and other compounds used to XTT reduction assay on the cell viability of *Candida albicans* and *Streptococcus mutans* biofilms.

**Material and Methods:** *C. albicans* or *S. mutans* was formed on a microtiter plate. After 48 h, the mature biofilm, to evaluate the effect of these different compounds of XTT on cell viability, the biofilms were divided into five experimental groups ($n = 5$). The plates were incubated for 3 h in dark conditions at 37°C. The cell viability was evaluated by counting the number CFU $mL^{-1}$.

**Results:** The results obtained for *C. albicans* and *S. mutans* showed differences between the incubation solutions for both microorganisms, however, the differences remained $>10^6$ CFU $mL^1$.

**Conclusion:** These findings indicate that the XTT salt and the compounds used for the XTT reduction assay did not exhibit any effect on cell viability of the microorganisms tested.

***Key Words***: Biofilm, *Candida albicans*, *Streptococcus mutans*, XTT

## Introduction

Fungal and bacterial biofilms are structured microbial communities attached to surfaces and embedded within a self-produced extracellular polymeric substance (EPS)[1] which provide a significant proportion of human infections since acting as reservoirs for persistent of infection.[2] Among the medically relevant fungi are *Candida albicans* that have been isolated from biofilms on indwelling medical devices such as endotracheal tubes, various types of catheters, neurosurgical shunts, and others.[3] In the oral environment, *Candida* biofilms are also critical in the development of denture stomatitis. It is estimated that among denture wearers, 67% are affected by this fungal infection.[4] These biofilms are formed into cracks and imperfections of the unpolished surface of the dentures which is in contact mainly with the palatal mucosa.[5] Denture stomatitis is normally asymptomatic, however, when signs and symptoms are present they may display mucosal bleeding, swelling, burning or other painful sensations, halitosis, unpleasant taste and dryness in the mouth.[6] Moreover, in patients' disability, diabetes, immunosuppression, or the use of broad-spectrum antibiotics, the opportunistic nature of *C. albicans* gives rise to the invasive hyphal form. Despite antifungal therapy to treat denture stomatitis, infection is reestablished soon after treatment ceases, suggesting that denture biofilm may serve as a protected reservoir of *C. albicans*.[4] The resistance to antimicrobial agents and protection from host defenses is the main reasons why biofilm-associated infections are frequently refractory to conventional therapy.[7] Several studies have investigated biofilm phases and their susceptibility to antimicrobial agents.[2,8,9] According to Chandra *et al.*, 2001[7] these clinical observations emphasize the importance of biofilm formation to both superficial and systemic candidiasis and the inability of current antifungal therapy to cure such diseases.

Denture stomatitis is now recognized as a complex process involving the formation of biofilm on the denture. As mentioned previously oral biofilm with have multiple organisms, including also *S. mutans*, which is one of the principal etiological agent in human dental caries.[10] These organisms have ability to form biofilms on the dental surface that lead to fluctuations in pH due to metabolic activity of cells. The result of these fluctuations may be the dissolution of the dental hard tissues and, consequently, the formation of a caries lesion.[11] Studies have been shown that oral bacterial

populations may interact with *C. albicans* in a complex manner to modulate the nature of the bacterial/fungal biofilm matrix.[12,13] *S. mutans* biofilm formation increases *Candida* and the interaction of *Candida* with other microorganisms can determine its virulence.[14] Pereira Cenci *et al.*,[15] suggested that besides the mutual stimulus for the growth of these microorganisms, there is also an aggregation of one another, which improves the adhesion process. The more complex the composition of the biofilms, the more resistant they are to antimicrobial agents. The potential of drugs to treat a variety of infections may be evaluated by some techniques. Currently, methods based on metabolic activity and cell viability, such as XTT reduction assay and counting colony-forming units (CFU), respectively, have been frequently used for biofilm evaluation.[12,16-19] XTT reduction assay is a semi-quantitative colorimetric method that measures the mitochondrial activity of metabolically active cells. It is based on the capacity of mitochondrial succinate dehydrogenase to reduce the yellow XTT salt (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) to an orange water-soluble formazan.[20] The colorimetric alterations resulting of the reaction between cells and XTT salt can be measured spectrophotometrically and is proportional to the number of metabolically active microbial cells. Despite to be a rapid and convenient method, problems regarding intra- and inter-species variability have been reported.[21]

Another method commonly used for biofilm evaluation is counting CFU. While the XTT assay is a method that assesses the metabolic activity, counting CFU is unlikely to be significantly affected by the metabolic state of the cells.[13] Based on complementary data obtained by two methods mentioned above, the many studies have associated the evaluation of metabolic activity (through XTT reduction assay) with counting CFU, which can become the process labor-intensive and slow.[1] One possibility would be to perform XTT reduction assay and counting CFU in the same samples. In that way, the metabolic activity of intact biofilm could be firstly evaluated, followed by quantification of viable microorganisms in biofilm by counting CFU. However, to the best knowledge of the authors to date no study has compared the potential effect of XTT salt on the viability of cells submitted to XTT reduction assay.

Thus, the aim of this study was to evaluate the effects of XTT salt and other compounds used to XTT reduction assay on the cell viability of *C. albicans* and *S. mutans* biofilms.

## Materials and Methods

### *Microorganisms and growing conditions*

*C. albicans* (ATCC 90028) and *S. mutans* (ATCC 25175) were used. Stock cultures were maintained at −70°C. After recovery, *C. albicans* and *S. mutans* were maintained on sabouraud dextrose agar (SDA) and Mitis Salivarius agar base, respectively, stored at 4-6°C during the experimental period. To prepare the yeast inoculum, a loopful of the stock culture of *C. albicans* was streaked onto SDA and incubated aerobically at 37°C for 48 h. One loopful of this young culture was transferred to 20 mL of RPMI-1640 medium and incubated at 37°C for 21 h. To prepare the bacterial inoculum, a loopful of the stock culture of *S. mutans* was streaked onto Mitis Salivarius and incubated at 37°C for 48 h anaerobically. One loopful of this culture was then transferred to 20 mL brain heart infusion (BHI) medium and incubated anaerobically at 37°C during 18 h. Cells of the resultant cultures (*C. albicans* and *S. mutans*) were harvested, washed twice with phosphate-buffered saline (PBS; pH 6.8) at 3220 g for 5 min and resuspended in the RPMI-1640 medium for *C. albicans* and BHI medium for *S. mutans*. *C. albicans* and *S. mutans* suspensions were spectrophotometrically standardized to $10^7$ cells $mL^{-1}$ and $10^8$ cells $mL^{-1}$, respectively.[7,14]

### *Biofilm development*

Aliquots of 200 µl of the standardized cell suspensions (*C. albicans* or *S. mutans*) were transferred into each well of polystyrene 96-well microtiter plates. The plates were the first incubated for 90 min at 37°C, in an orbital shaker, at 75 rev $min^{-1}$ for the adhesion phase. After this period, the wells were washed with 200 µl of PBS to remove loosely adhered cells. Then, the wells were filled with 200 µl of RPMI-1640 medium (*C. albicans*) or BHI medium (*S. mutans*), and the plates were incubated at 37°C, in an orbital shaker, at 75 rev $min^{-1}$, for 48 h for biofilm development (mature biofilm). Thereafter, the wells were carefully washed with 200 µl of PBS to remove nonadherent cells. Biofilm development was performed in aerobic or anaerobic conditions to *C. albicans* or *S. mutans*, respectively.

### *Preparation of XTT solution and experimental groups*

The biofilm evaluation was performed initially by XTT reduction assay. XTT salt (Sigma, MO, USA) was prepared in ultrapure water at a final concentration of 1 mg $mL^{-1}$. The solution was filter sterilized and stored at −70°C until use. Menadione (Sigma, MO, USA) solution was prepared in acetone at 0.4 mmol/L immediately before each assay. After biofilm formation (48 h), the metabolic activity of the mature biofilms was evaluated by XTT reduction assay before counting CFU. The compound proportion used to perform XTT solution is: 158 µl of PBS supplemented with 200 mM glucose, 40 µl of XTT salt and 2 µl of menadione solution prepared as described above. In this technique, aliquots of this solution must be added to pre-washed biofilms to allow the XTT reduction by live cells.

To evaluate the effect of these different compounds on cell viability, the mature biofilms were divided into five experimental groups ($n = 5$): G1 - 200 µl aliquots of RPMI-1640 medium for *C. albicans* or BHI medium for *S. mutans* (positive

control); G2 - 200 µl aliquots of PBS for both microorganisms; G3 - 200 µl aliquots of PBS supplemented with 200 mM glucose for both microorganisms; G4 - solution of 198 µl of PBS supplemented with 200 mM glucose and 2 µl of menadione for both microorganisms; and G5 - solution containing 158 µl of PBS supplemented with 200 mM glucose, 40 µl of XTT salt and 2 µl of menadione for both microorganisms. The plates were incubated for 3 h in dark conditions at 37°C. The whole content of each well was transferred to a tube and centrifuged at 5000 g for 1 min.

### *Counting CFU*

After 3 h, the cell viability was evaluated by counting CFU. In order to establish the number of CFU $mL^{-1}$, the biofilms (after exposition to diverse components of XTT) were scraped out of the wells of the 96-well microtiter plate, and suspended in 1000 µl of PBS with vigorous vortex mixing for 1 min. Then, ten-fold serial dilutions from $10^{-1}$ to $10^{-3}$ for *C. albicans* were plated onto SDA (SDA, Acumedia Manufactures, Lansing, MI) and $10^{-1}$-$10^{-5}$ for *S. mutans* onto Mitis Salivarius agar (Difco, Detroit, MI, USA) in triplicate. The plates were then incubated at 37°C for 48 h. After incubation, colonies were quantified using a digital colony counter (CP 600 Plus; Phoenix Ind. Com. Equipamentos Científicos, Araraquara, Brazil) and the number of CFU $mL^{-1}$ was determined and then $\log_{10}$ transformed. All assays were performed in quintuplicate on three different occasions.

### *Statistical analysis*

The results were evaluated by two-way analyses of variance, followed by Tukey's test. A significance level of 0.05 was used for all statistical tests.

## Results

The means and standard deviations obtained by CFU $mL^{-1}$ for both microorganisms and experimental groups are presented in Table 1. The analysis of variance two-way analysis of variance (Table 2) showed significant differences among groups for *C. albicans* ($P < 0.001$) and *S. mutans* ($P < 0.001$). For *C. albicans*, G1 exhibited no significant statistical difference compared to G4 but showed lower value of CFU $mL^{-1}$ when compared with G2, G3, and G5. The results also demonstrated that G5 exhibited a higher value of UFC $mL^{-1}$ compared to G1 and G4 groups, but G5 did not significantly differ to G2 and G3.

When the results of *S. mutans* were analyzed, no statistical difference among G1, G2, and G3 was detected. The highest values of CFU $mL^{-1}$ were observed to G4 and G5, which did not differ each other.

**Table 1: Mean values and SD obtained by counting CFU (CFU $mL^{-1}$) for all experimental groups and microorganisms (*C. albicans and S. mutans*).**

| Groups | *C. albicans* | *S. mutans* |
|---|---|---|
| G1 | 6.44 (0.038)$^{a}$ | 6.62 (0.033)$^{ab}$ |
| G2 | 6.48 (0.048)$^{bc}$ | 6.60 (0.035)$^{a}$ |
| G3 | 6.48 (0.035)$^{bc}$ | 6.61 (0.043)$^{a}$ |
| G4 | 6.46 (0.036)$^{ab}$ | 6.64 (0.020)$^{bc}$ |
| G5 | 6.51 (0.030)$^{c}$ | 6.67 (0.032)$^{c}$ |

Vertically mean values with identical lower letters were not statistically different ($P>0.05$). SD: Standard deviation, CFU: Colony-forming units, *C. albicans: Candida albicans, S. mutans: Streptococcus mutans*

**Table 2: Summary of two-way ANOVA for the logarithmic of CFU $mL^{-1}$.**

| Microorganisms | Effect | SM | DF | *F* value | *P* value |
|---|---|---|---|---|---|
| *C. albicans* | Group | 0.0122 | 4 | 8.48 | <0.001* |
| | Ocasion | 0.0008 | 2 | 0.56 | 0.572 |
| *S. mutans* | Group | 0.0114 | 4 | 11,13 | <0,001* |
| | Ocasion | 0.0038 | 2 | 3,74 | 0,029* |

*: Significant differences among groups, SM: Mean square, DF: Degrees of freedom, CFU: Colony-forming units, ANOVA: Analysis of variance, *C. albicans: Candida albicans, S. mutans: Streptococcus mutans*

## Discussion

The biofilm development on different surfaces involves the following steps: Adhesion of microorganisms to surfaces; organization of cells and colonies formation; self-production of EPS and structural maturation; and finally, the dissemination of biofilm cells.[7] Accordingly Chandra *et al.*,[22] these developmental phases result in biofilms exhibiting a highly complex and heterogeneous architecture in terms of distribution of cells and EPS. This biofilm complexity leads to use of different methods for biofilm evaluations. Among different techniques, the counting CFU and XTT reductions assay have been widely used. Some reports in the literature have been demonstrated that XTT correlates well with CFU/mL. Due to complementary information obtained from these two methods, some studies have associated them, which become the process labor-intensive and slow.[1] One possibility would be to perform XTT reduction assay and counting CFU in the same samples. Therefore, the aim of the present study was to evaluate the effects of XTT salt and other compounds used for XTT reduction assay on the cell viability of *C. albicans* and *S. mutans* biofilms. Since different microorganisms differ in structure, growth and metabolism, it is important to know the assay's reproducibility and uniformity when different microorganisms are studied.

The results obtained for *C. albicans* showed differences between G1 when compared with G2, G3, and G5, and the difference between G4 and G5. Similarly to *C. albicans*, there were significant differences for *S. mutans* when G5 was compared with G1, G2, and G3. In addition, G4 showed significant statistical differences compared to G2 and G3. However, it is important to mention that these differences for both microorganisms are not relevant because all groups remained >$10^{6}$ CFU $mL^{-1}$. These findings indicate that the XTT salt and the compounds used for the XTT reduction assay did not exhibit any effect on cell viability of the microorganisms tested.

The XTT assay has been extensively used for evaluation of metabolic activity in planktonic cultures[23] and biofilms, including bacterial[24,25] and yeast biofilms.[21,24,26] Metabolic estimation using XTT offers several advantages such as quick

measurement time; formation of the colorimetric formazan derivate, aqueous solubility[27] and also it is an inexpensive method that can be performed in most laboratories.[28] Moreover, this method is advantageous to quantify microbial biofilms because it does not require the biofilm disaggregation, as required for counting CFU. However, Moss *et al.*,[27] have reported some disadvantages such as inter-strain comparisons, XTT nonlinearity signal, the disproportion between XTT concentration and colorimetric signal and the XTT assay is sensitive to anabolism. Despite these limitations, the benefits of XTT assay over other methods include simplicity, relative cost, compatibility with high throughput and lack of toxicity.[21,25,29]

Moreover, tetrazolium salts are organic compounds that participate in the biological redox process.[30] Colorimetric formazan are derived from the extracellular reduction of the tetrazolium salt (XTT) by mitochondrial dehydrogenases of metabolic active cells,[27,28] that occurs because NADH, produced through the tricarboxylic acid cycle, transports electrons across the plasma membrane that reduces menadione[31] allowing the colorimetric formazan formation and therefore, metabolic activity of a microbial culture is possible to be quantified using spectrophotometry in the 430-490 nm range.[27] The sensitivity of this assay is menadione-concentration dependent.[28]

**Conclusion**

The results of the present study demonstrate that is possible to use the XTT reduction assay to study the metabolic activity of *C. albicans* and *S. mutans* before counting CFU without any change of the cell viability caused by XTT salt and other compounds used for the XTT reduction assay.

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