Advances in Microbiology, 2014, 4, 945-954
Published Online October 2014 in SciRes. http://www.scirp.org/journal/aim
http://dx.doi.org/10.4236/aim.2014.413105

Scientific Research

Influence of Sub-Lethal and Lethal Concentrations of Chlorhexidine on Morphology and Glucosyltransferase Genes Expression in *Streptococcus mutans* UA159

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Received 18 August 2014; revised 12 September 2014; accepted 2 October 2014

Abstract

Chlorhexidine (CHX) is regarded as one of the most successful antiplaque agents in controlling the formation of dental biofilm. Nevertheless, molecular mechanisms of their effects in *Streptococcus mutans* are largely unknown. In this work, the effects of sub-lethal and lethal concentrations of chlorhexidine (CHX) on planktonic or biofilm-organized *Streptococcus mutans* cells were investigated in dose- and time-dependent manner. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) for planktonic cells and biofilm conditions were determined by standard methods. Quantitative PCR (qPCR) was used to quantify the relative levels of *glucosyltransferase B* (*gtfB*), *gtfC* and *gtfD* transcription of *S. mutans* in the presence of CHX. The CHX activity in the initial biofilm structure and morphological alterations in planktonic cells were examined by Scanning Electron Microscopy (SEM). The results indicate that CHX increased expression of *gtfC* and *gtfD* in planktonic *S. mutans* cells and CHX reduced the expression of *gtfB*, *gtfC*, and *gtfD* in biofilms. High concentrations of CHX resulted in several wilted *S. mutans* planktonic cells with spilled intracellular material, while decreased cells' chain length and matrix was found when the initial biofilm was exposed to increasing concentrations of CHX. CHX's effects against

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How to cite this paper: da Silva, A.C.B., Stipp, R.N., Mattos-Graner, R. de O., Sampaio, F.C. and de Araújo, D.A.M. (2014) Influence of Sub-Lethal and Lethal Concentrations of Chlorhexidine on Morphology and Glucosyltransferase Genes Expression in *Streptococcus mutans* UA159. *Advances in Microbiology*, **4**, 945-954. http://dx.doi.org/10.4236/aim.2014.413105

bacteria depend on the type of growth organization and the concentration and time of exposure to the drug. At sub-lethal concentrations, CHX affects the expression of glucosyltransferases, which may have anticariogenic effect.

Keywords

Planktonic Cells, Biofilm, Microorganism, Scanning Electron Microscopy

1. Introduction

Chlorhexidine (CHX) is a successful chemical plaque control used routinely in general dental practice for controlling the formation of dental biofilms [1]. Considering its clinical application in Cariology, this drug can be used as "shock treatment" in patients with high risk and caries activity [2]. In this case, 0.12% CHX is generally used in association with a prophylactic regimen containing oral hygiene instructions, dietary advice, professional dental prophylaxis and topical fluoride application [3].

S. mutans is the main causative agent of human dental caries, mainly as a result of its ability to adhere to teeth surfaces and its acidogenicity [4] [5]. Such bacteria produce three glucosyltransferases, GtfB, GtfC, and GtfD, which synthesize soluble and insoluble glucan polymers from sucrose [6]. These polysaccharides enhance the colonization of cariogenic bacteria and promote the formation of dental plaque on tooth surfaces [7].

Despite studies having already proved that CHX has been efficient on plaque control [8]-[11], few investigations have focused on structural or molecular changes that can occur in cells, the biofilm matrix, or both [12] [13], when exposed at sub MIC and MIC concentrations of this drug.

The aim of this study was to evaluate the *in vitro* antibacterial effects of CHX digluconate on *S. mutans* planktonic or biofilm cells in a dose- and time-dependent manner.

2. Materials and Methods

2.1. Bacteria, Growth Conditions and Chemicals

S. mutans UA159 was acquired from ATCC (Cat. #700610), which had its genome sequenced [14]. This microorganism was grown in Brain Heart Infusion (BHI) (Difco, MD, USA) at 37˚C and atmosphere enriched with 10% CO_2. Chlorhexidine digluconate (20% aqueous solution, Cat. #C9394) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. CHX MIC and MBC Assay for Planktonic Cells

The MIC and MBC for planktonic cells were determined by overnight standard 96-well plate microdilution method and agar plating [15]. Two-fold dilutions of the drug were prepared in each test well containing Brain Heart Infusion broth (BHI), with CHX concentrations ranging from 0.56 µg/ml to 72 µg/ml. Aliquots of bacterial suspension (20 µL), adjusted spectrophotometrically to match the optical density of A_{550nm} = 0.5, were added to each 180 µL of drug-containing culture medium. Control wells included culture medium only, culture medium and CHX, and wells without CHX. Plates were closed and incubated for 18 h at 37˚C and atmosphere enriched with 10% CO_2. After incubation, MIC was determined visually and defined as the lowest drug concentration in which growth-turbidity was not observed. Assays were repeated three times in duplicate. Viable counting from control wells and from test wells was performed on BHI agar plates to determine MBC. MBC was defined as the lowest drug concentration in which no colony-forming units were observed on the plate.

2.3. CHX MBC Assay for Biofilm Cells (MBC-b)

Biofilm analyses were formed in 20 mm diameter, 15 mm deep polystyrene multidishes (six wells plates), by incubation of 20 µL of bacterial suspension (A_{550nm} = 0.5) and 5 mL BHI supplemented with 0.1% sucrose. After 18 h of growth, CHX was added directly to media-containing wells at final concentrations of 50 µg/ml, 100 µg/ml, 500 µg/ml, 800 µg/ml or 1000 µg/ml. Wells which did not receive CHX were set as control. Whole plate was closed and homogenized on an orbital mixer (80 rpm) for 60 s, and incubated for additional 5 min at 37˚C and atmosphere enriched with 10% CO_2. Afterwards, the medium was aspirated and biofilm was washed three

times with 5 mL of 0.9% NaCl solution (saline) to remove non-adherent cells. Whole biofilm layer was dislodged with the aid of a cell disrupter (Lifter Cell/3008; Costar, Corning, NY) in presence of 2 mL saline. Part of dislodged biofilm suspension was subjected to expression analysis (below) and part (50 µL) subjected to dilution for viable counting onto BHI agar plates aiming at quantifying MBC in the biofilm (MBC-b). MBC was defined as the lowest drug concentration after exposure that prevented colony forming units growing on agar medium. Assays were performed in duplicate and repeated three times.

2.4. Analysis of *gtf* Expression in Planktonic Cells under CHX

The expression levels of all genes were normalized by amplification of 16*S rRNA* of *S. mutans* as an internal standard. Overnight *S. mutans* broth culture was inoculated into 300 mL of pre-warmed BHI and let growth until reaches A_{550nm} = 0.3. Homogenized culture was then distributed into 20 mL aliquots in new tubes, and exposed to sub-lethal doses of CHX at 1.1 µg/ml, 2.2 µg/ml and 4.5 µg/ml each. Tubes which received the same volume of saline instead of CHX were set as control. All tubes were incubated for 2, 4 and 6 h at 37˚C, 10% CO_2. Aliquots of cultures (50 µL) at each time point were subjected to viable counting onto BHI agar plates. Suspensions were quickly cooled on ice and centrifuged at 5500 g at 4˚C for 4 min. Cells were washed in cold saline and stored with 220 µL of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE) at −80˚C until use.

2.5. Analysis of *gtf* Expression in Biofilm Cells under CHX

Biofilms exposed to CHX at 100 µg/ml, 500 µg/ml and 800 µg/ml were selected to analysis of *gtf* expression. Dislodged cell suspensions were centrifuged, washed in cold saline and stored with 220 µl of TE at −80˚C, until use.

2.6. RNA Isolation from Planktonic Cells and Biofilms

Total *S. mutans* RNA from either planktonic cells or biofilms was extracted and purified using an RNeasy RNA isolation column (Qiagen-Sciences, MD). Briefly, frozen cells were mechanically disrupted with 0.16 g of 0.1 mm diameter Zirconium Beads (Biospec, OK, USA) on a Mini-bead beater (Biospec), at maximum power (3 cycles of 30 s with 60 s on ice). Further RNA purification, followed by digestion with on-column RNase-free DNase I, was performed as recommended by the manufacturer. To remove small traces of remaining DNA, purified RNA was treated with DNase I (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA sample concentrations were determined at A_{260nm} (NanoDrop, Thermo Scientific, USA) and integrity by 1.2% formaldehyde agarosegels stained with ethidium bromide [17].

2.7. Reverse Transcription and RT-PCR

Reverse transcription of experimental samples together with negative controls was carried out with 1 µg RNA using Superscript III RT (Invitrogen, Life Technologies, Carlsbad, CA, USA) as previously described [16]. After reaction, water was added, so that the product concentration was relative to RNA 10 ng/µl. Controls for cDNA synthesis included samples without RNA template and samples without reverse transcriptase.

Quantification of cDNA was performed using the Step One Plus Real-Time PCR System (Applied Biosystems, Life Technologies, USA) and all primers were constructed by Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA) using nucleotide sequence information provided [16] (**Table 1**). Each reaction mixture (25 µL) contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 0.6 µM of each appropriate forward/reverse primer and 3 µL of cDNA sample (30 ng relative RNA). Cycling conditions included initial denaturation at 95˚C for 10 min, followed by a 50-cycle amplification, consisting of denaturation at 95˚C for 15 s, annealing at 54˚C for 15 s and extension at 72˚C for 30 s each. Controls included reaction mixtures without cDNA template to rule out primer dimers formation or presence of contaminating DNA. A standard DNA amplification curve starting from 300 ng to 0.003 ng (10-fold dilutions) and a melting-point product curve were obtained for each primer set/run. Assays were performed in duplicate with three or more independent RNA samples.

2.8. Scanning Electron Microscopy (SEM)

2.8.1. Planktonic Cells

The CHX effects on cell morphology were investigated by SEM. Planktonic cells were grown in presence of

Table 1. Primer sequences used in this study (listed 5' to 3' end) and amplicon sizes.

Primers (QRT-PCR)	Sequence5'-(Forward/Reverse)	Annealing Temperature	Product Size
16*S rRNA*	CGGCAAGCTAATCTCTGAAA/GCCCCTAAAAGGTTACCTCA	54˚	190 bp
SMU.910 (*gtfD*)	TGATTCGTGGTATCGTCCTAA/GTTGAGACTTTCTTGGCTGCT	54˚	199 bp
SMU.1004 (*gtfB*)	CGAAATCCCAAATTTCTAATGA/TGTTTCCCCAACAGTATAAGGA	54˚	197 bp
SMU.1005 (*gtfC*)	ACCAACCGCCACTGTTACT/AACGGTTTACCGCTTTTGAT	54˚	161 bp

CHX (1.1 µg/ml, 2.2 µg/ml, 4.5 µg/ml) or not (control) for 2, 4 and 6 h. Cultures were centrifuged, washed with saline two times and re-suspended with saline in original's volume. Bacterial suspensions (50 µL) were applied on cover slip surfaces and subjected to fix process with 55˚C (60 min) and 2.5% glutaraldehyde (30 min). Glutaraldehyde excess was removed, and cells were dehydrated within ethanol series (50% to 100%) for 20 min each. Finally, ethanol was removed and cover slips dried at room temperature. Samples were sputter-coated with gold and analyzed under scanning electron microscope as recommended by manufacturer (JEOL, JSM, 5600LV, Japan).

2.8.2. Biofilms

The CHX effects on the initial phases of biofilm formation were investigated by SEM. Sterile glass cover slips (Ø = 13 mm, GLASTEC, Brazil) were placed in each well of a 24-well plate. BHI medium (1.5 ml) supplemented with 0.1% sucrose containing CHX (1.1 µg/ml, 4.5 µg/ml, 72 µg/ml, 1200 µg/ml or 2000 µg/ml) or not (control) were transferred to wells. Aliquots of 50 µL bacterial suspension (A_{550nm} = 0.5) were then added, mixed, and incubated at 37˚C, 10% CO_2 for 2 or 4 h. Biofilm grown on slides was washed three times with PBS to remove non-adherent cells and processed for SEM analysis as previously described.

2.9. Statistical Analysis

Relative expression was calculated by Pfaffl mathematical model [17]. Briefly, cycle threshold values of expression levels (C_t) obtained from dilution curves were used to determinate quantitative PCR (qPCR) efficiency for each primer set. Each *gtf* gene of treated cells was normalized to the 16*S rRNA* gene (internal control) [16]. These values were then compared to those from the non-treated, 16S-normalized control, to determine the changes in *gtf* genes expression. Using C_t values, statically significance was obtained at $p < 0.01$ or $p < 0.05$, using ANOVA Dunnett Bilateral test, under BioEstat 5.0 free software.

3. Results

3.1. CHX Antimicrobial Effects

CHX MIC and MBC for planktonic cells were 2.2 µg/ml and 18 µg/ml, respectively, while CHX MBC for biofilm (MBC-b) was 800 µg/ml (data not shown). Thus, sub-micvalues were established in concentrations lower than 2.2 µg/ml and 800 µg/ml for planktonic cells and biofilm, respectively. Planktonic cells exposed to 4.5 µg/ml CHX and 9 µg/ml CHX reduced the bacterial counts by nearly 6 and 20 fold, respectively. In biofilms, a high coefficient of variation (56.4%, $p < 0.01$) of CFU/ml counts was observed only in 500 µg/ml CHX. In 100 µg/ml and control groups, these coefficients were below 20% ($p < 0.01$). In 800 µg/ml, there was no cell growth.

3.2. CHX Effect on 16S Gene Expression

The values of Ct 16*S rRNA* gene did not vary in the experiments with planktonic cells [mean ct 13.24 (±0.48)]. Regarding the CHX exposure, there was 7% variation between exposure and no exposure, whereas taking into account the control and minor concentrations (C1: 1.1 µg/ml and C2: 2.2 µg/ml), this variation was less than 5% (data not shown). Thus, the 16*S rRNA* gene can be successfully used as a reference gene on our conditions. Moreover, Ct values of 16*S rRNA* did not change in response to CHX in biofilm-growing cells [mean Ct 13.24 (±0.48)]. Ct values of 16*S rRNA* showed higher coefficient of variation (6.05%) in the group with the highest CHX concentration (C3: 800 µg/ml) than with other groups (C1: 100 µg/ml and C2: 500 µg/ml; <3.0%).

3.3. CHX Effect on *gtfs* Gene Expression

In planktonic cells, CHX increased *gtfC* ($p < 0.05$) and *gtfD* ($p < 0.01$) expression only in cell cultures with 6 h growth, at C3 (4.5 µg/ml) concentration, with 9- and 20-fold, respectively (**Figure 1**). In the biofilm environment, the expression of *gtfB and gtfD* was reduced in C2 (500 µg/ml) and C3 (800 µg/ml) concentrations, while there was decreased *gtfC* expression only for C2 concentration, when compared with the control ($p < 0.01$) (**Figure 2**).

3.4. SEM Analyses

SEM analyses of planktonic cells and biofilm exposed or not to CHX are shown in **Figure 3** and **Figure 4**.

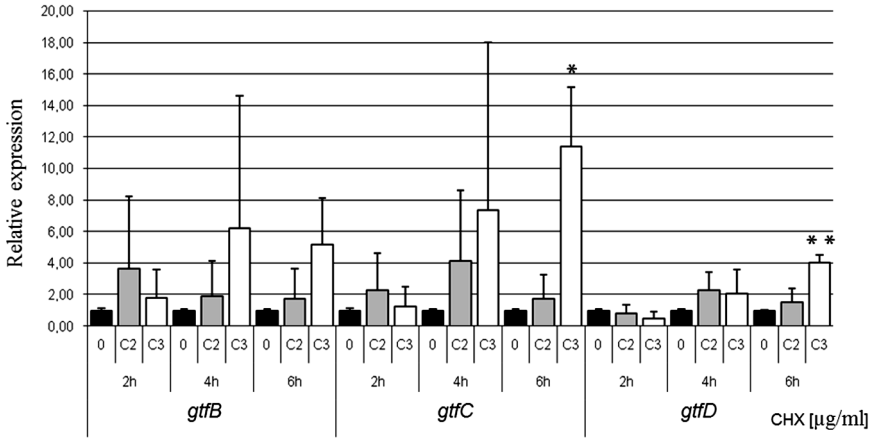

Figure 1. Influence of sub lethal concentrations of chlorhexidine (CHX) on the *gtfs* expression. Planktonic *S. mutans* cells were grown in absence (0, control) or presence (C2, 2.2 µg/ml; C3, 4.5 µg/ml) of CHX and subjected to qPCR analysis. Planktonic cells were exposed to the CHX from the beginning of growth. mRNA levels of *gtfB*, *gtfC* and *gtfD* in each sample were normalized by respective 16*S rRNA*. Then, vehicle-treated controls were set at 100%, and levels for treatments (C2, C3) were expressed relative to this value. The results are expressed as means ± SD of three separate experiments run in duplicate. *$p < 0.05$ and **$p < 0.01$ as compared to control condition.

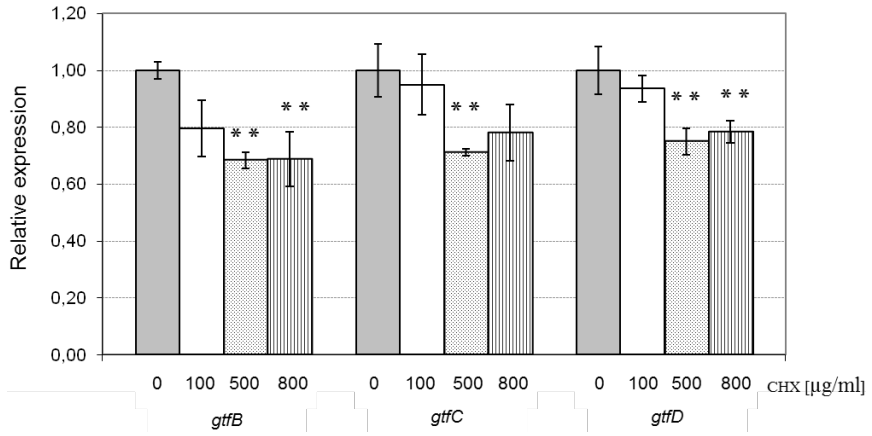

Figure 2. Effects of sub-lethal and lethal chlorhexidine (CHX) pulse-exposition *in vitro S. mutans* biofilms. Biofilms (18 h growth) were exposed to the CHX for a period of 5 min. mRNA levels of *gtfB*, *gtfC* and *gtfD* in each sample was normalized by respective 16*S rRNA*. Vehicle-treated controls were set at 100%, and levels for treatments were expressed relative to this value. Reduction in *gtfs* expression were reached in concentrations ≥ 500 µg/ml. The results are expressed as means ± SD of three separate experiments run in duplicate. **$p < 0.01$ as compared to control condition.

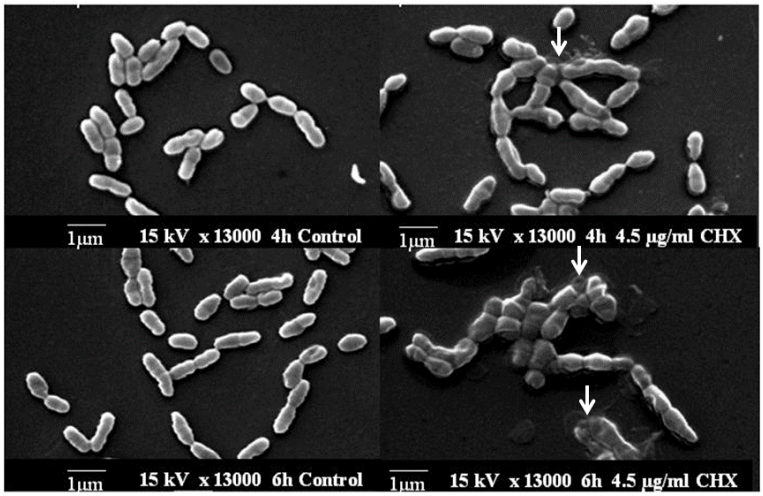

Figure 3. Scanning electron micrograph of planktonic *S. mutans* UA159 cells with and without treatment with sub-lethal and lethal concentrations of chlorhexidine (CHX). Planktonic cells were exposed to the CHX from the beginning of growth. Cells were exposed to CHX at 4.5 µg/ml. Note that after 4 h and 6 h of CHX incubation, several wilted cells (arrows) with spilled intracellular material could be observed (15 kV and 13,000× of magnification).

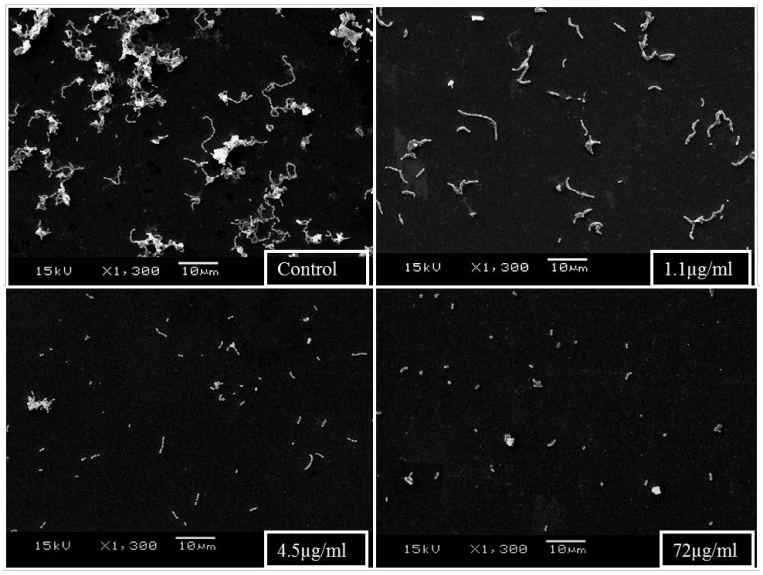

Figure 4. Scanning electron micrograph of *S. mutans* UA159 early biofilm (4 h growth), with and without treatment with sub-lethal and lethal concentrations of chlorhexidine (CHX). Biofilms were exposed to the CHX from the beginning of growth. Biofilms were exposed to CHX which was presented as a gradual decreasing in both the cells' chain length and their matrix when exposed at 1.1, 4.5 or 72 µg/ml concentrations (15 kV and 1300× of magnification).

Several assays were performed for these morphological analyses and the results are representative of three independent experiments. Cell surfaces did not show any obvious change when cells were exposed to increasing CHX concentrations (1.1, 2.2, or 4.5 µg/ml) for 2 h (data not shown). However, as demonstrated by SEM (**Figure 3**), after 4 or 6 h of exposure at less than-MBC (4.5 µg/ml), several *S. mutans* cells wilted or their membranes ruptured and, as a result, lost a substantial amount of cytoplasm material. SEM analyses of initial control biofilms and those treated with CHX are shown in **Figure 4**, where a decrease in cell chain length and matrix were found when biofilms were exposed to different CHX concentrations (1.1 µg/ml to 4.5 µg/ml), while 1200

µg/ml and 2000 µg/ml of CHX caused extensive precipitation of unknown material on the slide (data not shown).

4. Discussion

It is well recognized that *S. mutans*, when organized in biofilms, has different properties than its planktonic form. Biofilms have a more tolerant phenotype to antimicrobial agents, stress, and host defenses than planktonic cultures do, making them difficult to treat [18]-[20]. Nevertheless, in this study, sub-MIC and MIC concentrations of CHX were selected for the SEM observations and expression analysis in planktonic cells and biofilms to better understand the CHX effect on these cells, since higher concentrations of this drug would cause cell death that could compromise gene expression in this experimental study.

4.1. *gtfs* Genes Expression Analysis

The gene expression of planktonic cells and biofims were observed in the most appropriate experimental growth periods for planktonic cells (2, 4 and 6 h) and biofilms (18 h). Planktonic cells were exposed to the chlorhexidine from the early growth to analyze its growth kinetics. Biofilms were exposed to CHX for 5 min to mimic the oral cavity environment when exposed to CHX during fast mouthwash with the drug.

In this study, methods of RNA extraction from planktonic cells and biofilms were similar, which makes possible the direct comparison of these different conditions of the same bacterial microorganism. The polysaccharides elimination of the biofilm, as a prior step to extraction [21] [22], was not necessary in our *in vitro* conditions due to the shorter time of biofilm formation and low concentration of sucrose.

The 16*S rRNA* gene has been successfully used as a reference gene in studies on *S. mutans* in biofilm and planktonic cells [16] [20] [23]-[26]. Our results indicated that the expression of 16*S rRNA S. mutans* remained stable when exposed to a CHX environment regardless of whether it is in planktonic cells or in biofilms. This suggests that the 16*S rRNA* gene can be used as a reference gene on analysis of expression in the two conditions described above.

The effect of antimicrobial agents on gene expressions that are heavily involved in biofilm formation is of particular interest to dentistry [24] [25]. Different patterns of gene expression between biofilms and planktonic cells were observed [20]. Thus, Li and Burne [27] found that the expression of *gtf* genes was enhanced in bacteria growing as biofilms compared with planktonic growth, implying thata cell-density-dependent intercellular communication system may play a role of regulating thegtfB/C genes expression [28].

According to our findings, CHX inhibited the *gtfs* expression of *S. mutans* organized as biofilm (**Figure 2**) but increased the *gtfs* expression in planktonic cells (**Figure 1**). Koo *et al*. [25] demonstrated that apigenin significantly decreased the expression of *gtfB* and *gtfC and* increased the expression of *gtfD* in *S. mutans* growing in the planktonic state. A similar profile of *gtf* expression was obtained with biofilms.

Tam *et al*. [24] observed that iodine and povidone iodine (PI) in a tetraglycol-carrier cause enhancement of expression of *gtfB* in *S. mutans* in biofilms but not in planktonic bacteria. PI in water induced expression of *gtfB* and *gtfC* in planktonic bacteria. Thus, the vehicle type used together with the active compound or molecule can determine the increased or decreased expression of these genes.

It is probable that planktonic cells express more *gtfs* (**Figure 1**) as a defense mechanism against stress when exposed to CHX. Cells from biofilms are more protected, and they do not need to produce more *gtfs*. In biofilms, CHX inhibited the *gtfs* expression (**Figure 2**). This is a clear indication that CHX at sub-lethal concentrations may have an adverse effect on the adhesion process of these cells on the tooth surface, which is relevant for clinical purposes ofcontrolling oral biofilms' development *in vivo*.

According to Wade [29], high CHX concentrations, as used in clinical practice, kill the cells, and this is not interesting for the balance of microbiota in the biofilm. Successful antimicrobial agents are able to maintain the oral biofilm at levels compatible with oral health but without disrupting the natural and beneficial properties of the resident oral microflora [30].

Thus, in biofilms, low CHX concentrations may have anticariogenic effect by inhibiting the expression of enzymes associated with virulence of *S. mutans*. Furthermore, CHX when used in concentrations of 0.12% and 0.2% for a long time has many side effects such as teeth pigmentation and taste changes [31].

4.2. SEM Analysis

Radford *et al*. [32] demonstrated that biofilm formation *in vivo* can be inhibited using sub-MIC concentrations

of CHX. It is possible that the reduction in the formation of the biofilm matrix exposed to low CHX concentrations (**Figure 4**) may be related to the affinity of CHX with proteins, as well as the ability to reduce the expression of some *gtfs* (**Figure 2** and **Figure 4**).

According to Dunne [33], CHX only kills outer layers of biofilm bacteria, leaving the healthy microorganisms in the inner layers and allowing the growth of these bacteria later when CHX is not present. It is also noteworthy to understand that possible conformational changes in biofilm structure (e.g., water channels) may contribute to the diffusion of CHX into deeper biofilm layers [12]. This variable might explain the unexpected difference in *gtf* expression in planktonic and biofilm forms because viable cells might be protected by the low diffusion rate of the agent in the biofilm matrix.

In this study, planktonic cells, when exposed to higher CHX concentrations, suffered membrane rupture (**Figure 3**). Tattawasart *et al*. [13] found similar results on the CHX effect on *Pseudomonas stutzeri* species sensitive to the drug. Vitkov *et al*. [34], using an *ex vivo* model, showed that some bacterial cells from biofilm sustained ruptured membranes when exposed to CHX. This observation can be explained by the fact that CHX, which is positively charged, binds tightly to negatively charged bacteria membrane, causing its disruption [35]. Under such microbial stress, it is expected that enzyme expression can be altered as observed at concentration 4.5 µg/ml (**Figure 1**).

5. Advances and Limitations

This is the first report to investigate the CHX effects on *gtf* gene expression from planktonic and biofilm-organized *S. mutans*, as well as examining and relating its effect on cell surface and biofilm structure.

A limitation to consider is that, despite the experimental model valuating the gene expression applied in this study has been previously validated (20), certainly a model with a hydroxiapatite surface exposed to saliva would make the model resemble the oral cavity even more than expected. On the other hand, salivary mucins could certainly affect *S. mutans* adherence and interfere with the material for gene expression evaluation. Overall, the two ways to evaluate (gene expression and SEM) complemented each other bring some extra data on biofilms when exposed to CHX.

6. Conclusions

In summary, CHX's effects against bacteria depended on the type of growth organization and the concentration and time of exposure to the drug. CHX may affect cell walls and intervene with biofilm formation mechanisms, and at sub-lethal concentrations, reduces the expression of some *gtfs* in *S. mutans* biofilms, which may exert anticariogenic effect.

From a clinical perspective, the primarily purpose of using mouthwashes containing antimicrobials such as CHX is not the cell death, but rather the control or elimination of these biofilm microorganisms' virulent mechanisms. Hence, frequent low concentrations of these antimicrobials might be enough for controlling the virulent mechanisms of *S. mutans* without great depletion of their population in the oral environment.

Acknowledgements

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (proc. #07/56100-2), Conselho Nacional de Pesquisa, CNPq (proc. #483794/2006-6 and proc. #472988/2009-3) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). RNS and ACBS were supported by FAPESP (#06/55933-8) and CAPES (PNPD/2010), respectively. DAMA and FCS have CNPq fellowships.

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