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ORIGINAL ARTICLE

Exposure of *Streptococcus mutans* and *Streptococcus sanguinis* to blue light in an oral biofilm model

Maayan Vaknin[1,2] · Doron Steinberg[1] · John D. Featherstone[3] · Osnat Feuerstein[2]

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Abstract

The potential anti-cariogenic effect of blue light was evaluated using an oral biofilm model. Two species, *Streptococcus mutans* and *Streptococcus sanguinis,* were cultivated *ex vivo* on bovine enamel blocks for 24 h, either separately or mixed together, then exposed to blue light (wavelengths 400–500 nm) using 112 J/cm^2. Twenty four or 48 h after exposure to light the biofilm structure and biomass were characterized and quantified using SEM and qPCR, respectively. Bacterial viability was analyzed by CLSM using live/dead bacterial staining. Gene expression was examined by RT-qPCR. After exposure to light, *S. mutans* biomass in mono-species biofilm was increased mainly by dead bacteria, relative to control. However, the bacterial biomass of *S. mutans* when grown in mixed biofilm and of *S. sanguinis* in mono-species biofilm was reduced after light exposure, with no significant change in viability when compared to control. Furthermore, when grown separately, an upregulation of gene expression related to biofilm formation of *S. mutans*, and downregulation of similar genes of *S. sanguinis*, were measured 24 h after exposure to blue light. However, in mixed biofilm, a downregulation of those genes in both species was observed, although not significant in *S. mutans*. In conclusion, blue light seems to effectively alter the bacterial biomass by reducing the viability and virulence characteristics in both bacterial species and may promote the anti-cariogenic balance between them, when grown in a mixed biofilm. Therefore, exposure of oral biofilm to blue light has the potential to serve as a complementary approach in preventive dentistry.

Keywords *Streptococcus mutans* · *Streptococcus sanguinis* · Mixed biofilm · Blue light · Dental caries · Ecological balance

Introduction

Biofilms have been described as a diverse community of microorganisms embedded in a highly organized extracellular matrix, involved in a wide range of metabolic, physical, and molecular interactions [1, 2]. The biofilm structure serves as a physical barrier which can protect the penetration and movement of antimicrobial agents within the biofilm [3]. The oral biofilm in the mouths of humans is composed of various microbial communities including approximately 600 species or phylotypes [4]. Among them, is the highly cariogenic bacterial species associated with dental caries, *Streptococcus mutans*, which has the ability to synthesize extracellular polysaccharides such as glucans or fructans via extracellular enzymes that include glucosyltransferase and fructosyltransferase [4].

Streptococcus sanguinis is one of the most abundant species in the early dental biofilm, recognized as an etiological agent of several extra-oral diseases such as infective endocarditis [5]. The oral health status can be influenced by the relative levels of *S. mutans* and *S. sanguinis*. While a predominance of *S. mutans* is associated with individuals experiencing high caries levels, a predominance of *S. sanguinis* is associated with individuals without carious lesions [6]. Both species coexist in the human oral biofilm; however, they apparently have an antagonistic connection [7]. The oral streptococci produce diverse antimicrobial compounds to compete for adhesion-binding sites on the tooth surface and to modulate the growth of other species. These compounds include bacteriocins, peptide toxins formed by *S. mutans*, and hydrogen peroxide (H_2O_2), produced by *S. sanguinis* [8]. The ecological balance in the oral environment

✉ Osnat Feuerstein
osnatf@ekmd.huji.ac.il

[1] Biofilm Research Laboratory, Institute of Dental Sciences, Faculty of Dental Medicine, Hebrew University-Hadassah, Jerusalem, Israel

[2] Dental Materials Laboratory, Department of Prosthodontics, Faculty of Dental Medicine, Hebrew University-Hadassah, P.O.B. 12272, 91120 Jerusalem, Israel

[3] Department of Preventive and Restorative Dental Sciences, School of Dentistry, University of California San Francisco, San Francisco, CA, USA

may change through less or more cariogenic bacteria, resulting in pH differences that could affect the dental caries progression [7].

Blue light (wavelengths, 400–500 nm), commonly used in restorative dentistry for the polymerization of composite resin materials, has been proposed as an alternative antimicrobial treatment, against oral diseases and oral malodor [9]. Additionally, exposure of S. mutans biofilm to blue light revealed a delayed bacterial death phenomenon with an increase in dead bacteria even when re-grown into a new biofilm [9, 10]. The aim of the present study was to evaluate the potential anti-cariogenic effects of blue light on S. mutans and S. sanguinis, when they were cultivated either separately or mixed together, grown in an oral biofilm model.

Materials and methods

Preparation of the oral biofilm model

Enamel blocks were prepared from extracted bovine teeth (BUA Approval #003909-03, UCSF) and were kept in 0.1% thymol solution at 4 °C until used. After a primary polishing of the enamel surface with sandpaper of 800 grit up to 1200 grit, the enamel was cut into cubic blocks measuring 2 mm × 5 mm using diamond discs mounted on a low-speed sectioning machine (Buehler, Dusseldorf, Germany) [11]. Nail varnish was used to seal the tooth blocks leaving a window measuring 2 mm × 2 mm on the enamel surface. The blocks were fastened to a plastic stand with epoxy glue. Each block was autoclaved in distilled water.

Biofilm formation

Planktonic *S. mutans* UA159 and planktonic *S. sanguinis* 10556 were grown in brain heart infusion broth (BHI, Difco Labs, Detroit, USA) at 37 °C in 95% air/5% CO_2 for 24 h [3, 12]. The overnight culture was diluted 1:50 in BHI containing 2% sucrose (final concentration). Biofilm of either *S. mutans* or *S. sanguinis* or mixed were seeded on the surface of enamel blocks fixed inside the wells of a 12-well TCP (Corning Incorporated, NY, USA) The plates were incubated at 37 °C in 95% air/5% CO_2 for 24 h. Following 24 h, the medium was removed and the blocks were washed with 200 μl phosphate buffer saline (PBS). Before exposure of the samples to blue light, 50 μl of PBS was added to the biofilm [10].

Exposure to blue light

A LED device emitting non-coherent blue light (wavelengths, 400–500 nm) (ART-L5 lamp, BonART Medical Technology, New Taipei City, Taiwan) was used. The distance between the light source tip and the biofilm surface of the exposed sample immobilized on the enamel blocks was set at 1 cm with a power density of 0.623 W/cm^2, measured by a power meter (Ophir, Jerusalem, Israel) [10, 12]. Under these light conditions, a minimal increase in the temperature of the cell media was measured [13]. Bacterial biofilm was grown on enamel immersed in PBS (50 μl) and was exposed to blue light continuously during 3 min, equivalent to accumulated fluences of 112 J/cm^2. Non-exposed biofilm served as the control. The fluence was calculated according to the following formula:

$$\text{Fluence or energy density}\left(\frac{J}{\text{cm2}}\right) = \text{power density}\left(\frac{W}{\text{cm2}}\right) \times \text{exposure time (second)}$$

Following blue light exposure, all samples were cultivated again in fresh BHI medium containing 2% sucrose solution, incubated at 37 °C in 95% air/5% CO_2 for 24 or 48 h, for continued growth of the biofilms. A schematic representation of the experimental stages is shown by a flowchart in Fig. 1.

Table 1 Genes tested, their biological functions and primers sequence based on the NCBI of *S. mutans* or *S. sanguinis* genome database

Gene	Description	Primer sequence (5′-3′)	
		Forward	Reverse
gtfB	Glucan production	AGCAATGCAGCCAATCTACAAAT	ACGAACTTTGCCGTTATTGTCA
gtfC		GGTTTAACGTCAAAATTAGCTGTATT	CTCAACCAACCGCCACTGTT
gtfP		GGACAGCCGGTTCAAGTACA	TTTGCTTCTTCAGCAACGGC
brpA	Biofilm-regulation	GGAGGAGCTGCATCAGGATTC	AACTCCAGCACATCCAGCAAG
Ftf	Fructans production	AAATATGAAGGCGGCTACAACG	CTTCACCAGTCTTAGCATCCTGAA
gbpB	Glucan-binding proteins	AGGGCAATGTACTTGGGGTG	TTTGGCCACCTTGAACACCT
sspC	Biofilm-surface proteins	GCTACTGATCCACGTAGGGC	CTTCGGTGTTGGTGCAGTTG
sspD		GGAAGGCTCTCGCTTTGAGT	GACAGGTGACTGGGGTTCAG
16S *S.m*	Normalizing internal standard	CCTACGGGAGGCAGCAGTAG	CAACAGAGCTTTACGATCCGAAA
16S *S.s*		GTGAGGTAACGGCTCACCAA	CCACTCTCACACCCGTTCTT

S.m, *Streptococcus mutans*; *S.s*, *Streptococcus sanguinis*

Fig. 1 A schematic representation of study design of biofilm exposure to blue light, using the ex vivo model of biofilm growth on bovine enamel blocks from *S. mutans* or *S. sanguinis* or when mixed together

Scanning electron microscopy

Biofilm structure immobilized on enamel surfaces was characterized by SEM, 24 h and 48 h after light exposure. The samples were washed carefully using PBS solution, and then immersed in 400 μl formaldehyde 4% (Daejung, Ltd Gyeonggi-do, Korea) for 30 min to fixate the cells. Following fixation, the enamel samples were rinsed twice using ddW and incubated at room temperature for 2 h. Next, samples were mounted on a metal stub and coated with Au/Pd prior to SEM analysis. The biofilms were visualized using a high-resolution scanning electron microscope (HR-SEM) Magellan 400 L, FEI (Eindhoven, The Netherlands) [14]. SEM experiments were repeated three times.

qPCR quantification

DNA was extracted from biofilm grown on enamel surfaces and from their supernatant-fluid using a modified alkaline lysis protocol as previously described in Periasamy and Kolenbrander [15]. The obtained DNA was stored at − 20 °C and subsequently used as template for qPCR analysis. *S. mutans* UA159 16S rRNA and *S. sanguinis* 10556 16S rRNA specific primers were used (IDT Integrated DNA Technologies, Inc. Skokie, Illinois, USA). qPCR reaction was carried out in a BIO-RAD CFX96 Real-Time System (Bio-Rad CFX manager 3.1 program). A specific standard curve was constructed using DNA extracted with a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, Darmstadt, Germany) from an overnight culture of *S. mutans or S. sanguinis* according to the manufacturer's instructions. Each group of biofilms exposed to light, consisted of 2 enamel blocks. All experiments were performed in triplicate and repeated three times.

Confocal laser scanning microscopy

CLSM was used for visualization of bacterial viability on the deeper layers of the biofilm immobilized on enamel surfaces, 24 h and 48 h after exposure to light. The generated biofilms were washed carefully using PBS and stained with 50 μl of LIVE/DEAD BacLight fluorescent dye (Invitrogen Life Technologies, Carlsbad, CA, USA) (1:100) for 20 min in the dark, and at room temperature. Following staining, the samples were rinsed with PBS and were visualized by an Olympus Fluoview 300, Olympus, Japan with a UPLSA × 10/0.4 lenses. The biofilm depth was examined by generating the optical sections of 10 μm [12, 16]. The viable and dead cells were calculated as green and red fluorescence intensity, respectively using ImageJ program. CLSM experiments were repeated twice and one representative images of each experiment is presented.

RNA extraction

Gene expression was examined by extracting RNA from blue light exposed and non-exposed biofilm, previously grown on Hydroxyapatite powder (Marco-Perp Ceramic Hydroxyapatite type 80 μm, BIO-RAD) in 6-well TCP (Corning Incorporated, NY, USA), under the conditions described above. Following 24 h after exposure to light, total RNA was extracted from the biofilm using Tri-Reagent (Sigma-Aldrich, St. Louis, MO, USA) by a previously described method [17–19]. Purity and concentration of the RNA were determined using a Nanodrop ND-1000 Instrument (Wilmington, DE, UAS). The integrity and purity of the RNA were assessed using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). Only samples with RIN^e values above 8.0 were used for cDNA synthesis. The extracted RNA samples were kept at − 80 °C until used.

Reverse transcription

The RNA template of biofilm samples was reverse-transcribed using qScript cDNA synthesis kit (Quanta Biosciences, Inc. Beverly, MA, USA), as previously reported by Shemesh et al. [20]. The synthesized cDNA later was used for real-time qPCR analysis of biofilm-related genes as described in Table 1. The relative expression level of the target genes was analyzed by BIO-RAD CFX96 Real-Time System (Bio-Rad CFX manager 3.1 program). The RT-PCR reaction was performed as described previously at Shemesh et al. [21]. The expression levels of all genes tested by RT-qPCR were normalized using the 16S rRNA gene of *S. mutans* and *S. sanguinis* as an internal standard. Gene expression was expressed in relative values, setting the expression level of the non-exposure control to 1 for each gene. Control reactions were also performed with RNA that was not reverse-transcribed, to ensure that no residual genomic DNA is amplified [18]. The assays were performed in triplicate and repeated three times.

Statistical analysis

Means and standard deviation were calculated. The statistical analysis was performed using multiple *t* test with a significance level of $P < 0.05$ and 2-way ANOVA with multiple comparisons using GraphPad Prism 6 Software.

Results

Visualization of biofilm structure in an oral biofilm model

Appearance of the mono-species biofilm of *S. mutans* and *S. sanguinis* (Fig. 2a, b) and a combination of mixed biofilm structure (Fig. 2c) grown on enamel can be observed from the SEM images. Under the SEM, dense and complex biofilms of bacteria embedded in the extracellular matrix of polysaccharides cover the enamel surfaces, especially in the control samples (Fig. 2, upper rows). Mixed and mono-species *S. sanguinis* biofilms (Fig. 2b, c) exposed to blue light (lower rows) appeared under the SEM with less massive biofilms when compared to the controls (upper rows). However, *S. mutans* mono-species biofilm exposed to blue light (Fig. 2a, lower rows) appeared under the SEM with higher massive biofilms when compared to the controls (upper rows). These findings were observed also 24 h after biofilms exposure to blue light (data not shown).

qPCR analysis of the dental biofilms grown on enamel surfaces

Significant differences in bacterial amount, measured by DNA concentration, were found 24 h after blue light exposure of mono-species biofilms when compared to their appropriate controls, however, with opposite trends: an increase by 78% in *S. mutans* biofilm (Fig. 3a) and a decrease by 85% in *S. sanguinis* biofilm (Fig. 3b). This effect was conserved also 48 h after biofilms exposure to light when compared to the control samples. An increase by 47% in *S. mutans* biofilm (Fig. 3a) and a decrease by 51% in *S. sanguinis* biofilm (Fig. 3b) was observed, however, not statistically significant.

Interestingly, when the two species were grown into mixed biofilm, a significant reduction in *S. mutans* by 57%, with no change in *S. sanguinis* were found 48 h after blue light exposure, when compared to the control (Fig. 3c). A similar reduction, by 60% for *S. mutans* and 64% for *S. sanguinis*, was observed in the mixed biofilms 24 h after light exposure, although it was not statistically significant.

A dominance of *S. mutans* bacteria as the main pathogenic agent in the oral biofilm was observed, compared to the bacterial amount of *S. sanguinis*, in both treated and control samples. In the mixed control group, the DNA concentration of *S. mutans* biofilm was 42% greater than *S. sanguinis* in biofilm grown for 24 h, and this difference increased to 92% after 48 h, indicating a dominance of *S. mutans* bacteria in the oral biofilm model. This ratio between *S. mutans* and *S. sanguinis*

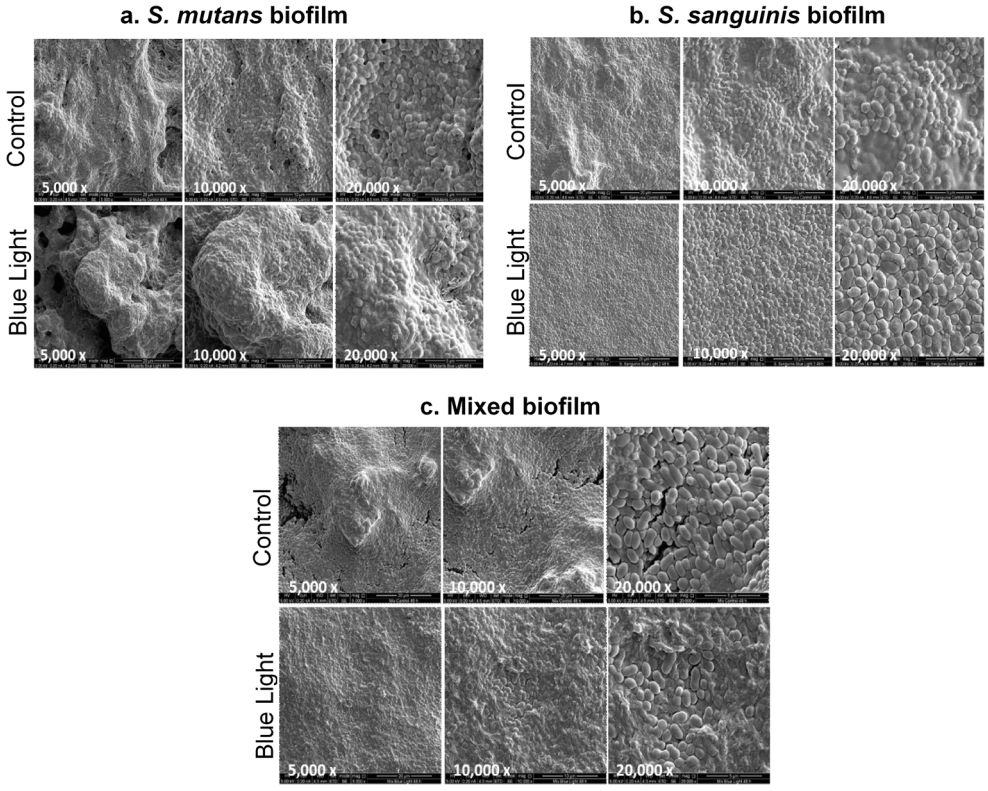

Fig. 2 Scanning electron microscopy of *S. mutans* (**a**) or *S. sanguinis* (**b**) or mixed (**c**) biofilms, grown on enamel surface for 48 h after exposure to blue light. Biofilm structure on enamel surfaces was observed at different magnifications (× 5000, × 10,000, and × 20,000). Images in the upper row represent the control; images in the lower row represent the samples exposed to blue light using 112 J/cm^2 ($n = 3$ for each experimental group)

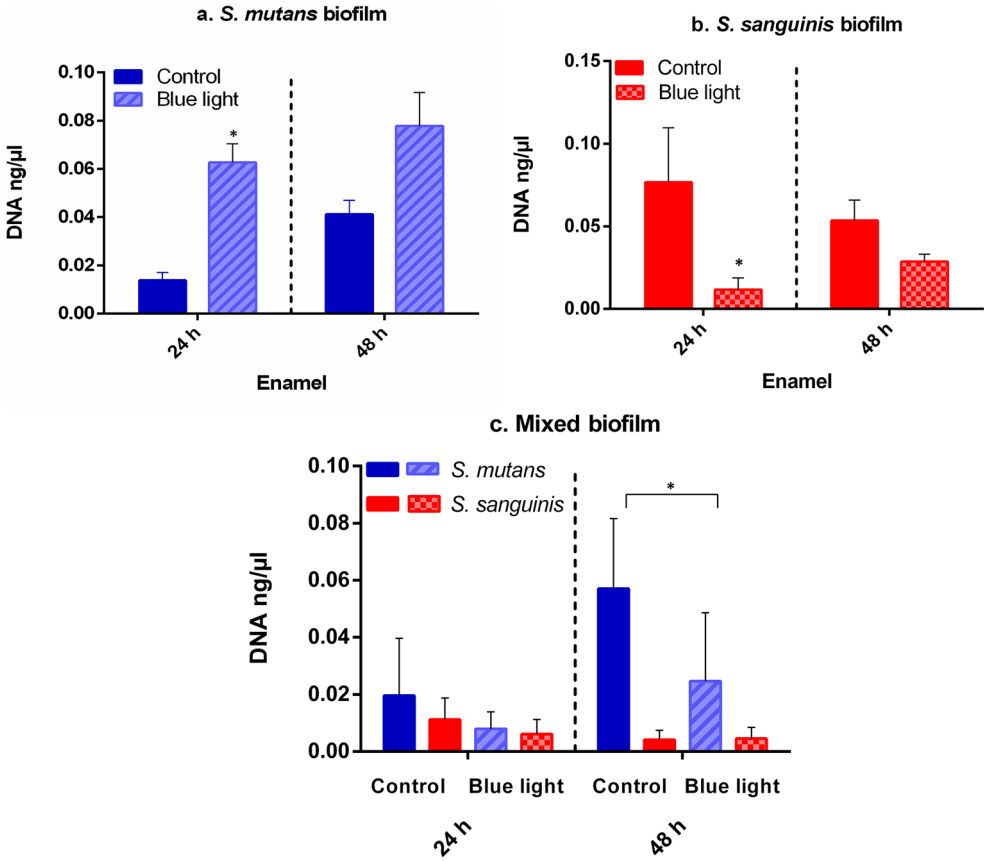

Fig. 3 qPCR analysis of *S. mutans* (**a**) or *S. sanguinis* (**b**) or mixed together (**c**) biofilms grown on enamel blocks from control and treated samples 24 h or 48 h after exposure to blue light using 112 J/cm^2 (blue, *S. mutans*; red, *S. sanguinis*; **P* < *0.05*, *t* test, compared to control, *n* = 6 for each experimental group)

was also different in treated samples, with dominance of *S. mutans* biomass by 50% greater than *S. sanguinis* biomass grown for 24 h. This difference increased to 75% after 48 h (Fig. 3c).

Biofilm viability under the CLSM

An increase in the number of dead bacteria 48 h after light exposure of the mono-species *S. mutans* biofilms was observed (Fig. 4b) when compared to the control (Fig. 4a). Interestingly, under similar treatment conditions, a minimal effect on bacterial viability was shown in the mono-species *S. sanguinis* (Fig. 4e) and in mixed biofilms (Fig. 4 h) when compared to the controls (Fig. 4d, g). According to the signal intensity of live and dead bacteria in each biofilm sample, the calculated live/dead bacterial ratio of *S. mutans* mono-species biofilm 48 h after exposure to light was significantly decreased when compared to control (data not shown).

The analysis of CLSM scans for live and dead bacteria from the outer layer (0 μm) to the deepest layers of the biofilms showed that the bacterial viability of either *S. mutans* or *S. sanguinis* or in mixed biofilms was reduced 48 h after blue light exposure (Fig. 4c, f, i). It was evidenced mainly in the *S. mutans* mono-species treated samples vs. control biofilms (Fig. 4c), by a significant increase in dead bacteria, through all the layers, even in the deepest layers, with slight increase in the live bacteria. An increase in dead bacteria and a decrease in live bacteria in *S. sanguinis* (Fig. 4f) and mixed (Fig. 4i) biofilms was observed as well, 48 h after exposure to light. However, these differences were not statistically significant.

Furthermore, differences were found in the biofilm thickness between the control and the treated samples 48 h after exposure to light. Biofilm thickness of *S. sanguinis* was lower by 50 μm in the treated samples when compared to the control (Fig. 4f). Interestingly, although the increase in dead bacteria, treated samples of *S. mutans* and mixed biofilms were thicker by 40 and 30 μm, respectively, in comparison to the control (Fig. 4c, i).

Expression level of *S. mutans* and *S. sanguinis* genes in separate and in mixed biofilm

Exposure of biofilms to blue light resulted in several bacterial gene expression changes, associated with biofilm formation, when analyzed 24 h after treatment, and compared to the appropriate control. In the mono-species biofilms, results in *S. mutans* showed upregulation of *gtf*B, *gtfC*, and *ftf* genes, which mediated glucan and fructans production, thus, contributing to bacterial adhesion in biofilm (Fig. 5a, blue column). On the other hand, the genes of *S. sanguinis* biofilm, *SspC* and *SspD*, that mediated glucan-binding proteins involved in

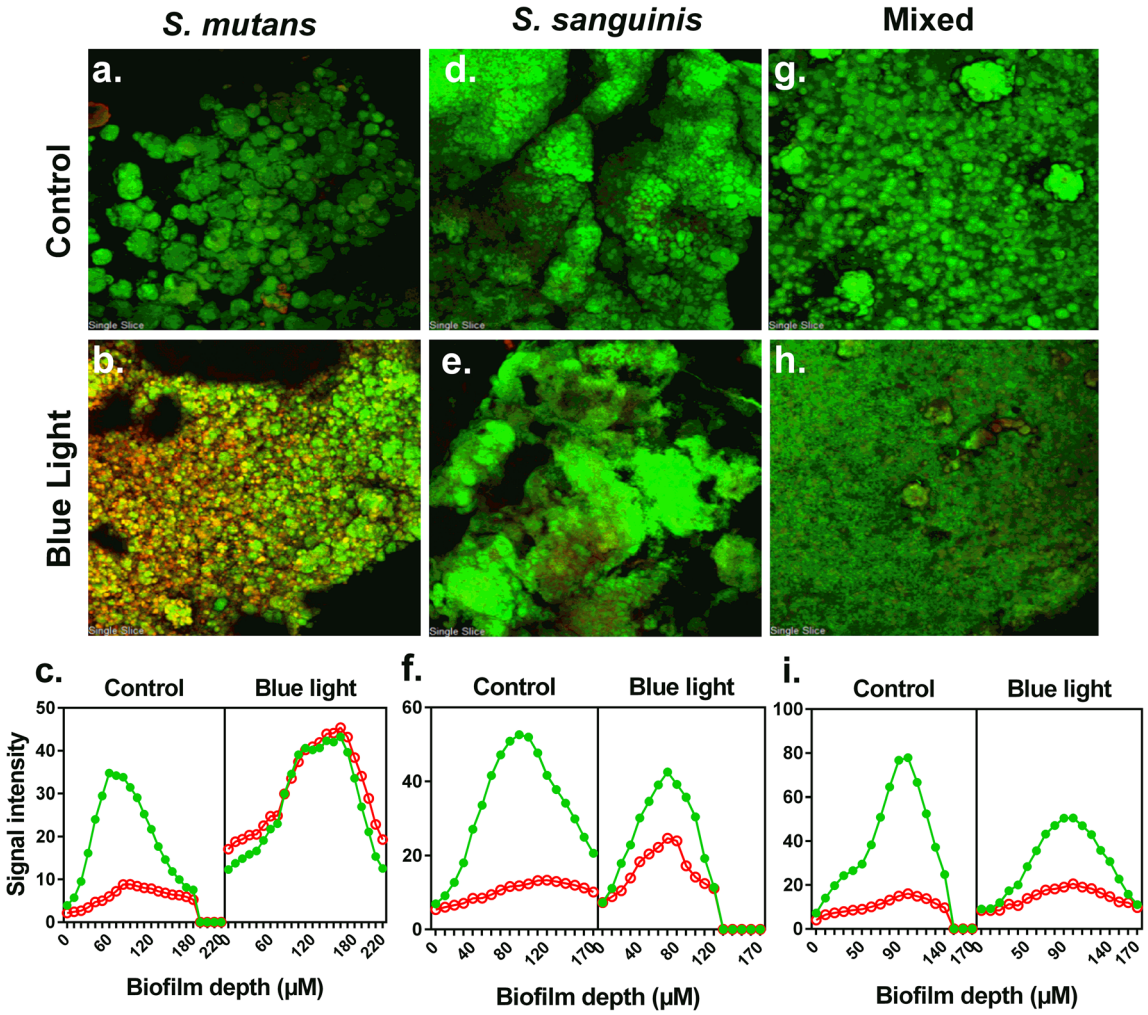

Fig. 4 Confocal laser scanning microscopy scans and analysis of *S. mutans* (**a**–**c**) or *S. sanguinis* (**d**–**f**) or mixed (**g**–**i**) biofilm immobilized on enamel surfaces. Live/dead staining of control and treated biofilms grown for 48 h after blue light exposure using 112 J/cm^2. Green pixels indicate live bacteria, red pixels indicate dead bacteria, and yellow pixels indicate co-localization of both. Control samples (upper row) and light treated samples (lower row). CLSM scans of live and dead bacteria from the outer layer (0 μm), at 10 μm intervals, to the deepest layers of the *S. mutans* (**c**) or *S. sanguinis* (**f**) or mixed (**i**) biofilms were analyzed. Live bacteria in green plot and dead bacteria in red plot

biofilm formation, were downregulated 24 h after exposure to blue light (Fig. 5a, red column). In the mixed biofilm, a similar down-regulation effect on these genes appeared in *S. sanguinis* (Fig. 5b, red column). However, contrary to the upregulation of mono-species *S. mutans* genes related to biofilm adhesion (Fig. 5a, blue column), when grown in mixed biofilm, a downregulation was observed, although not significant (Fig. 5b, blue column).

Discussion

The present study explores the effects of blue light on *S. mutans* and *S. sanguinis* mono-species and mixed oral biofilms in an ex vivo oral biofilm model. The dual biofilm is composed of the highly cariogenic pathogen *S. mutans*, associated with dental caries, and the commensal species *S. sanguinis*, which is strongly correlated with oral health [22]. Using this ex vivo model, we explored the sustained change in the coexistence of those bacterial species grown into biofilms on enamel blocks, 24 h and 48 h after exposure to blue light. Important questions arise. First, how does blue light influence the interspecies interactions of the two bacteria grown in biofilm? Second, if blue light affects the balance between *S. mutans* to *S. sanguinis*, how does blue light treatment for prevention of oral diseases alter the microbial composition toward a less or more cariogenic environment?

Various *in vitro* models have been proposed in the field that explores oral biofilms [23–26]. Most models used homo-species biofilm immobilized on plastic surfaces. Clearly, the

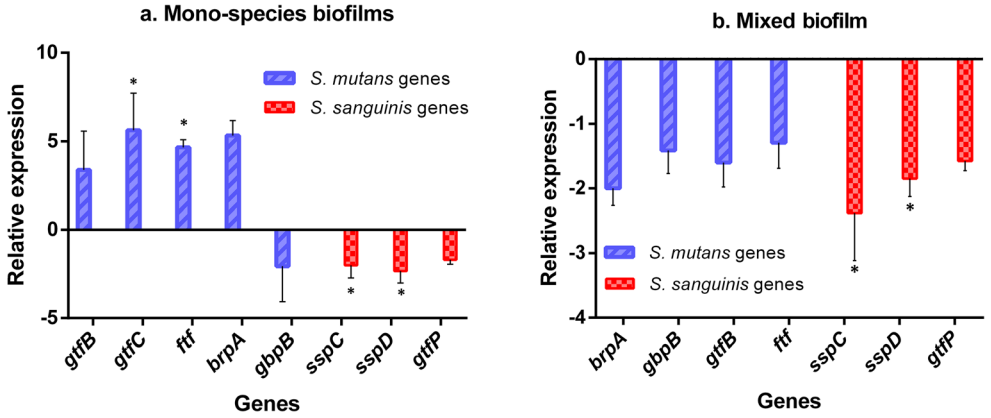

Fig. 5 RT-qPCR analysis of various genes involved in biofilm formation of *S. mutans* and *S. sanguinis* mono-species biofilms (**a**) or when in mixed biofilm (**b**) grown in the oral biofilm model 24 h after exposure to blue light using 112 J/cm^2. (Blue, *S. mutans*; red, *S. sanguinis*; *$P < 0.05$, *t* test, compared to control, $n = 3$ for each experimental group). Gene expression of the exposed group was expressed in relative values, setting the expression level of the non-irradiated control to 1 for each gene. The expression level of all genes tested by RT-qPCR was normalized using the 16S rRNA gene of *S. mutans* and *S. sanguinis* as an internal standard

surface used for adhesion of the bacteria is a crucial factor in the initial biofilm formation process and probably has further implications on the development of the mature biofilm. Our model consisted of mixed biofilms which were grown ex vivo on extracted bovine enamel blocks attached to a plastic stand, thus, partially simulates the oral surface conditions for biofilm growth. Interactions between bacteria may affect the end result of any bacteria growth, and especially after any type of treatment, such as blue light exposure. Clearly, the oral biofilm is comprised of more than two bacterial species; however, our model investigates the dual influence of two bacteria harboring an oral biofilm, associated with caries disease.

Viable biomass is an important property of the biofilm, which may indicate its potential pathogenicity. In our study, differences in biomass were found between the bacteria which were grown separately or mixed together 24 h and 48 h after blue light exposure. Although results showed that after exposure of *S. mutans* biofilm to blue light, the amount of bacteria increased and was significantly higher than that of the control samples (Figs. 2a and 3a). However, CLSM confirmed that this increase in biomass was mainly due to dead bacteria (Fig. 4b, c). These results are in agreement with previous studies that showed delayed phototoxic effects on *S. mutans* grown in biofilms 6 h after blue light exposure for 7–10 min (equivalent to 476–680 J/cm^2) and on *S. mutans* previously exposed to blue light for 3–7 min (equivalent to 112–262 J/cm^2), then regrown into new biofilms [12, 27]. The delayed/sustained effects demonstrated that after blue light exposure the bacterial viability and virulence characteristics of *S. mutans* were impaired [12, 27]. In the present study, we showed that although using a lower fluence of 112 J/cm^2 increased *S. mutans* biomass, the blue light treatment still manifested a delayed death phenomenon that was sustained up to 48 h. Furthermore, this increase in biomass was supported by the upregulation of *S. mutans* biofilm-associated genes (Fig. 5a), a result that was shown also by Steinberg et al. (2008) [3], using similar experimental conditions. Thus, blue light may enhance the ability of *S. mutans* bacteria to form stable biofilm, by promoting the synthesis of extracellular polysaccharides, under the experimental conditions of mono-species growth, without *S. sanguinis*. On the other hand, mono-species biofilm of *S. sanguinis* and also mixed biofilm exposed to blue light resulted in a reduction of the bacterial biomass, shown under the SEM (Fig. 2b, c) and by DNA quantification (Figs. 3b and 2c). Additionally, this decrease in biomass was supported by the downregulation, mainly of *S. sanguinis* biofilm-associated genes (Fig. 5). Our results showed that blue light may have an inhibitory effect on bacterial growth and biofilm pathogenicity of *S. mutans* when it coexists with *S. sanguinis*. This phenomenon is in contrast to the results found in a study using aPDT, green LED light in combination with erythrosine, on biofilms of *S. sanguinis* alone that were more sensitive than mixed biofilms composed of *C. albicans* and *S. sanguinis* [28].

Previous studies used different laser or light sources against oral bacteria, such as CO_2 and diode lasers or UV irradiation in order to reduce the viability of bacteria [29, 30]. Others, using an antimicrobial photodynamic therapy (aPDT) technique, demonstrated an inhibitory effect on cariogenic microorganisms in vitro [31–35], and clinically a reduction in dental plaque formation [36]. As effective as this technology is, it requires the addition of a chemical dye, with the disadvantages of tissue staining and possible toxicity, in order to make the light energy effective. Thus, a direct effect of the light with no intermediates seems to be a preferable approach. More so, those studies using different light treatment techniques demonstrated the phototoxic lethal effect only. In current therapeutic microbiology, the approach has been shifted from killing all bacteria to more holistic means, which change the

pathogenicity of the whole biofilm in a prolonged and beneficial manner [27]. Therefore, in the present study, other aspects related to biofilm virulence were investigated.

The phototoxic mechanism of the visible light, when used alone (without dye) involved the activation of endogenous photosensitizers in bacteria, such as flavins and cytochromes, leads to the production of reactive oxygen species (ROS), resulted in cell damage [27, 37, 38]. Chebath-Taub et al. [10] showed that re-growth of new biofilm from *S. mutans* previously exposed to blue light, resulted in a delayed bacterial death effect, mostly in the outer layers of the biofilm. However, our results showed a high number of dead bacteria in the deepest layers of the biofilm 24 h and 48 h after the exposure to blue light (Fig. 4a–c). This phenomenon of the light effect on the biofilm seems to have clinical advantages when compared to chemical antibacterial agents, as not only a successful tool to cope with the physical barrier of biofilms, however, as a possible protective barrier for tissue surfaces by those deep dead layers in the biofilm, i.e., against demineralization of enamel in dental caries. Thus, the increase in biofilm thickness of *S. mutans* that was observed 48 h after exposure to light when compared to control (Fig. 4c) is apparently due to the dead bacteria and created by the delayed phototoxic effect of blue light. Similarly, Steinberg et al. [3], using blue light with lower fluences (34 and 68 J/cm^2) in combination with H_2O_2, on *S. mutans* biofilm, found that bacterial death began in the middle layers of the biofilm and spread through all of the layers of the biofilm over a period of several hours. The differences in the location of the dead bacteria through biofilm layers between those studies may be explained by different experimental conditions.

Interestingly, when *S. mutans* was grown in mixed biofilm, 48 h after exposure to blue light, there was a decrease in bacterial biomass including the live bacteria (Figs. 2c, 3c, and 4i). The opposite effects of blue light on *S. mutans* bacteria when grown in mixed biofilm or separately could be explained by the influence of the H_2O_2 production by the coexisting *S. sanguinis* bacteria. Furthermore, difference in gene expression were observed in our results, compared to the mono-species biofilm, demonstrating that bacteria grown in mixed biofilm behave differently than in separated growth states (Fig. 5). The virulence of *S. mutans* and *S. sanguinis* depends in part on the expression of surface proteins involved in the synthesis and interaction with an extracellular glucan matrix. The adhesion between the tooth surface and the bacteria is largely mediated by adhesive glucan and fructans synthesized from sucrose via three glucosyltransferases *gtf*B, *gtf*C, and *gtfD*, and fructosyltransferase: *ftf* genes and glucan-binding proteins [24]. From our results, blue light treatment upregulated the expression of these polysaccharides synthesis genes in *S. mutans* biofilm (Fig. 5a, blue column), however, downregulated these genes of *S. mutans* bacteria when were grown in a mixed biofilm (Fig. 5b, blue column). These results are supported by the upregulation of *S. mutans* biofilm-associated genes, such as *gtfB*, *brpA*, *smu630*, and *comDE*, when using blue light in combination with H_2O_2 on *S. mutans* mono-species biofilm [3]. In contrast to the upregulation of *S. mutans* biofilm when grown separately, in *S. sanguinis* biofilm, blue light downregulated the expression of these genes in a similar manner, when it was grown separately or in mixed biofilm (Fig. 5, red column).

The mechanism that leads to gene expression changes in bacteria grown in biofilm following exposure to blue light is yet to be determined. The increase in gene expression of *S. mutans* may be a result of an environmental stress, a type of a defense mechanism. Blue light leads to creation of a ROS sensitive state, through the photochemical mechanism which causes irreversible damage to essential biological cell compounds such as lipids, proteins, and nucleic acids [39]. Phototoxic inhibitory effects were found to involve induction of high amounts of reactive oxygen species (ROS) by the bacteria while low amounts of ROS may promote their proliferation [40]. Blue light may initiate the photo-oxidative stress response by regulating the transcription of genes responsible for ROS production in some bacteria [41]. Additionally, studies used molecular genetics and transcriptomics approaches to investigate the blue light response mechanism in different bacteria showed a response at the genome-wide level [42], and an inhibitory effect mediated by the suppression of the genes associated with chromosomal DNA replication and cell division at the transcriptional level [43]. Under stress conditions, the competence process between the dental species decreases, resulting in reduction of antimicrobial compounds production, to focus on maintaining the bacterial essential cellular functions [44]. Furthermore, both streptococcal species share a similar genus and coexist in the same ecological niche, therefore are similar in their metabolic requirements. Previous studies have indicated that a variety of environmental factors such as sodium and potassium concentration, or external pH could influence the GTF activity of *S. sanguinis*. In addition, certain environmental and biological conditions such as cell density, nutritional availability, and competition between bacterial strains may affect the synergistic existence of the mixed bacteria grown together on enamel [45].

Conclusions

In summary, our results indicate that blue light has the potential to influence the pathogenicity of *S. mutans* and *S. sanguinis* species in mixed biofilm, by reducing their virulence characteristics, such as biomass, viability and gene expression. Furthermore, exposure of a dual-species pathogenic biofilm to blue light could affect mechanisms inside the bacteria that result in inhibition of proliferation

and survival functions, and may shift the bacterial composition by changing the microbial equilibrium. Cohen-Berneron et al. (2016) suggested blue light as a unique treatment to reduce pathogenicity of bacteria in oral biofilm using similar fluence that could be obtained by half of the exposure time (about 90 s) when the distance between the tip of the LED and the exposed biofilm is reduced to 2 mm, as is often used in dental applications [12]. Therefore, blue light therapy has a potential to be used in preventive dentistry as an alternative to the conventional antibacterial treatment, due to the absence of side effects and bacterial resistance, and as a complementary tool to the current solutions for supragingival biofilm modification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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