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The effectiveness of curcumin-mediated antimicrobial photodynamic therapy depends on pre-irradiation and biofilm growth times

Daniela Alejandra Cusicanqui Méndez[a], Eliezer Gutierrez[a], Giuliana Campos Chaves Lamarque[a], Veridiana Lopes Rizzato[a], Marília Afonso Rabelo Buzalaf[b], Maria Aparecida Andrade Moreira Machado[a], Thiago Cruvinel[a,*]

[a] Department of Pediatric Dentistry, Orthodontics and Public Health, Bauru School of Dentistry, University of São Paulo, Alameda Dr. Octávio Pinheiro Brisolla, 9-75, Vila Universitária, Bauru, SP 17012-901, Brazil
[b] Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil

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ABSTRACT

Background: The aim of this study was to determine the influence of distinct pre-irradiation times (PIT) of curcumin on the effectiveness of antimicrobial photodynamic therapy (aPDT) against intact dentin caries biofilms grown for 3 or 5 days.
Methods: The microcosm biofilms grew on non-fluorescent glass blocks immersed in McBain medium with 1% sucrose, using microaerophilic conditions at 37 °C for 3 or 5 days. The biofilms were treated by the association of 600 μmol.L^{-1} curcumin using different pre-irradiation times (1, 2 or 5 min) combined with 0 or 75 J.cm^{-2} blue LED. Then, the vitality of biofilms was determined by confocal scanning laser microscopy (CSLM), after being stained with the mixture of ethidium bromide and fluorescein diacetate. Statistical analysis was performed by two-way ANOVA and post-hoc Tukey tests, after arcsine transformation ($P < 0,05$).
Results: In comparison to control, curcumin alone (PIT = 5 min) and all combinations of curcumin and LED reduced significantly the vitality of 3-day biofilms. Distinctly, only curcumin plus LED using PITs of 2 or 5 min were effective in reducing the vitality of 5-day biofilms.
Conclusion: Curcumin-mediated aPDT significantly decreased the vitality of intact dentin caries microcosms grown during 3 or 5 days, although successful treatments of 5-day biofilms required longer PITs in comparison to their counterparts.

1. Introduction

Curcumin is a phytotherapic compound extracted from the root of *Curcuma longa*, commonly used as a traditional Asian spice, and also as an antitumor, anti-oxidant and anti-inflammatory agent [1,2]. It is increasingly being employed as a photosensitizing agent (Ps) in antimicrobial photodynamic therapy (aPDT), to reduce the viability of oral microorganisms [3–8].

The action of aPDT depends on the combination of three distinct elements: a photosensitizing agent (Ps), a light source with a complimentary wavelength, and endogenous oxygen [9]. Initially, Ps must be applied on target cells for a specific pre-irradiation time (PIT) [10]. Then, the irradiation of sensitized cells promotes the excitation of Ps molecules and stimulates the transference of electrons to nearby structures [11], generating two possible types of photodynamic reactions, type I (involving free radicals) and type II (involving singlet oxygen) [9,12]. These reactions can be detected simultaneously, damaging or killing microbial cells by the oxidation of cellular organelles, DNA and/or destruction of cellular membrane [9,13]. It is noteworthy that higher intensities of oxidative stress are needed to kill mature biofilms in comparison to immature ones or planktonic cells, since greater volumes of extracellular matrix increase the chemical complexity and tridimensional structure of biofilms [9].

Although a previous study demonstrated that curcumin-mediated aPDT was able to reduce the vitality of dentin caries microcosms [14], the effect of changes in PIT should also be investigated to elucidate their possible influences on therapeutic results, regarding complex biofilms with different maturation. It is crucial consider that the antimicrobial resistance of these biofilms increases over time, due to the continuous acquisition of nutrients, and the interaction, co-aggregation and metabolic cooperation of several species of microorganisms [15,16]. However, there is a lack of evidence about these parameters to

* Corresponding author.
E-mail address: thiagocruvinel@fob.usp.br (T. Cruvinel).

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Table 1
Vitality of intact microcosm biofilms (%) grown during 3 days, considering whole biofilms, and their outer, middle and inner layers. *P* values are presented to compare a specific group with its control (L-C-).

	Groups	Mean ± SD			*p* values	Median	CI (95%)	
							Lower	Upper
Whole Biofilm	L-C-[a]	95.56	±	5.31		97.14	87.11	104.01
	L-C1min[b]	77.51	±	1219	< 0.001	81.07	58.11	96.90
	L-C2min[b]	80.76	±	3.17	0.01	81.42	75.71	85.81
	L-C5min[c]	54.72	±	6.25	< 0.001	52.68	44.78	64.66
	L75C-[b]	84.27	±	2.85	0.04	84.11	79.74	88.80
	L75C1min[c]	38.07	±	0.45	< 0.001	38.16	37.35	38.78
	L75C2min[c]	46.13	±	0.28	< 0.001	46.20	45.68	46.58
	L75C5min[c]	38.64	±	20.78	< 0.001	37.68	5.58	71.70
Outer Layer	L-C-[a]	92.14	±	8.50		94.26	78.60	105.67
	L-C1min[ac]	74.35	±	17.59	0.014	77.75	46.37	102.34
	L-C2min[ac]	74.50	±	15.63	0.14	77.27	49.63	99.37
	L-C5min[bc]	50.17	±	1.88	< 0.001	49.56	47.18	53.17
	L75C-[ac]	70.45	±	6.19	0.04	69.47	60.60	80.29
	L75C1min[b]	19.51	±	5.14	< 0.001	17.96	11.33	27.69
	L75C2min[bc]	45.08	±	16.60	< 0.001	45.22	18.67	71.49
	L75C5min[b]	27.84	±	18.19	< 0.001	28.41	−1.11	56.79
Middle Layer	L-C-[a]	96.16	±	3.40		96.52	90.75	101.58
	L-C1min[bc]	79.31	±	13.57	< 0.001	84.07	57.72	100.91
	L-C2min[b]	84.19	±	6.12	0.04	85.10	74.46	93.93
	L-C5min[ce]	62.22	±	5.72	< 0.001	63.25	53.13	71.32
	L75C-[bc]	83.15	±	5.17	< 0.001	81.71	74.92	91.38
	L75C1min[d]	30.20	±	5.47	< 0.001	28.49	21.50	38.90
	L75C2min[de]	52.60	±	11.22	< 0.001	51.63	34.75	70.45
	L75C5min[d]	34.31	±	15.52	< 0.001	31.86	9.60	59.01
Inner Layer	L-C-[a]	97.58	±	2.45		98.09	93.68	101.49
	L-C1min[b]	79.72	±	13.66	< 0.001	83.04	57.98	101.45
	L-C2min[b]	84.20	±	8.36	0.02	86.71	70.90	97.51
	L-C5min[c]	55.31	±	6.50	< 0.001	56.23	44.98	65.65
	L75C-[b]	82.88	±	6.16	< 0.001	81.70	73.08	92.68
	L75C1min[c]	39.58	±	5.29	< 0.001	39.19	31.17	47.99
	L75C2min[c]	50.69	±	10.79	< 0.001	49.45	33.51	6787
	L75C5min[c]	38.24	±	14.35	< 0.001	38.52	15.41	61.08

Distinct superscript letters represent significant statistical differences between groups ($P < 0.05$). SD means standard deviation. CI means confidence interval.

support the development of further *in vitro* studies, and to contribute with clinical decisions [17–19].

Therefore, this study aimed to determine the influence of three curcumin pre-irradiation times on the effectiveness of aPDT to control the vitality of intact dentin caries biofilms grown during 3 and 5 days. The null hypotheses were that pre-irradiation time would have no effect on the vitality of intact dentin caries microcosms (H0'), independently of the time of biofilm growth (H0").

2. Materials and methods

2.1. Ethical standards

This study was approved by the Committee for Ethics in Human Research of the Bauru School of Dentistry (CAAE: 34559314.6.0000.5417), following the ethical standards of Declaration of Helsinki.

2.2. Collection of infected dentin and biofilm growth

These methods were previously described by Mendéz et al. [18,19]. Three children who attended the Clinics of Pediatric Dentistry of the Bauru School of Dentistry were selected as donors of infected dentin. The inclusion criteria were children authorized by their parents, aged between 7 and 11 years old, with the diagnosis of at least one dentin caries lesion in primary molars. The exclusion criteria were the diagnosis of syndromes and/or systemic diseases, the presence of dental caries lesions with pulp exposure, and the use of antibiotics within 90 days prior to dentin collection.

The children were anesthetized and isolated with rubber dam for dentin collection. Then, a sterile dentin curette was employed to collect infected dentin samples from carious lesions. These samples were stored in 2 mL of Brain Heart Infusion [BHI, 37 g of Brain Heart Infusion, deionized water/L, pH 7.2] with 30% glycerol at −80 °C until the moment of use. The teeth were subsequently restored with glass ionomer cement (Ketac Fil Plus®, 3MEspe, Minnesota, USA).

Then, samples were defrosted at room temperature and sonicated vigorously at 40 mW, 1 pulse/s for 15 s (Single Ultra-Sonic Cell Disruptor, Merse, Campinas, Brazil). Volumes of 1.5 mL of samples collected from each one of three children were mixed to prepare a pool of microorganisms. An aliquot of 400 µL of this pool was mixed with 10 mL of modified McBain medium [2.5 $g.L^{-1}$ mucin, 2.0 $g.L^{-1}$ peptone, casein peptone 2.0 $g.L^{-1}$, 1.0 $g.L^{-1}$ yeast extract, 0.35 $g.L^{-1}$ NaCl, 0.2 $g.L^{-1}$ KCl, 0.2 $g.L^{-1}$ $CaCl_2$, 0.001 $g.L^{-1}$ hemin, 0.0002 $g.L^{-1}$ vitamin K1, 0.2% sucrose and 50 $mmol.L^{-1}$ PIPES, deionized water, pH 7.0], and incubated in microaerophilic conditions at 37 °C overnight. Following, microbial suspensions were diluted in 60% glycerol (proportion of 1:1), and stored at −80 °C until the moment of use.

Subsequently, sterile non-fluorescent glass slabs (4 × 4 x 1 mm) with similar dental surface roughness (1200 grit) (Menzel, Braunschweig, Germany) were positioned into each well of a 24-well microtiter plate, and aliquots of 1 mL of pool were equally distributed into the wells. This set was transferred to microaerophilic conditions (5% CO_2) at 37 °C for 24 h. McBain medium was refreshed after each 24 h, until 3 or 5 days of biofilm growth.

2.3. Experimental groups

Glass slabs containing biofilms were randomly distributed in 8 different groups: (a) no treatment (L-C-, control), (b) 600 $\mu mol.L^{-1}$

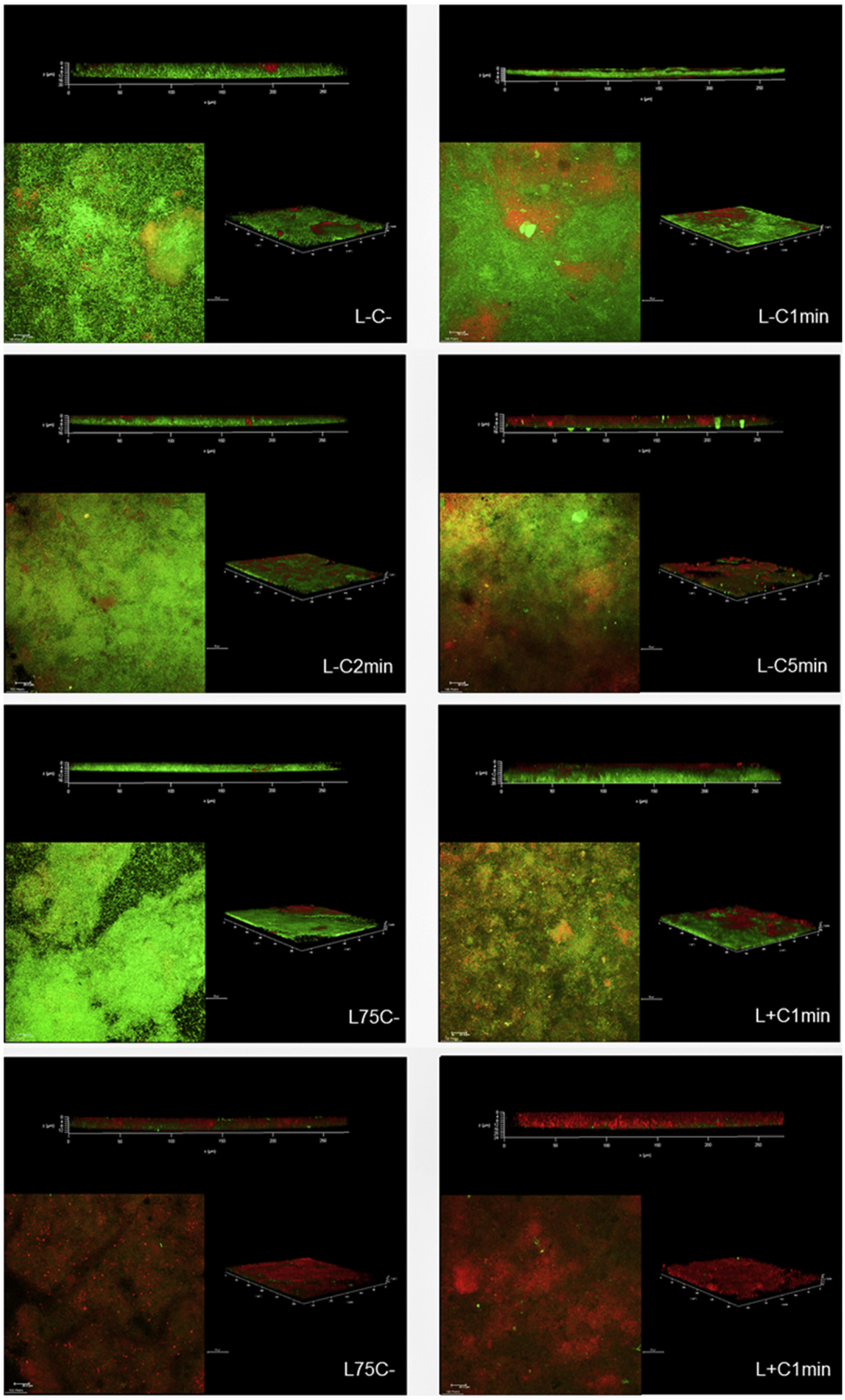

Fig. 1. Bi and tridimensional CSLM images of intact whole biofilms grown during 3 days. The groups are represented as follows: L-C- (control, no treatment), L-C1min (600 μmol.L^{-1} curcumin with 1 min of pre-irradiation), L-C2min (600 μmol.L^{-1} curcumin with 2 min of pre-irradiation), L-C5 min (600 μmol.L^{-1} curcumin with 5 min of pre-irradiation), L75C- (75 J.cm^{-2} LED alone), L75C1min (600 μmol.L^{-1} curcumin with 1 min of pre-irradiation + 75 J.cm^{-2} LED), L75C2min (600 μmol.L^{-1} curcumin with 2 min of pre-irradiation + 75 J.cm^{-2} LED), and L75C5min (600 μmol.L^{-1} curcumin with 5 min of pre-irradiation + 75 J.cm^{-2} LED). Note a significant reduction of the vitality of biofilms observed after the combination of curcumin and LED.

curcumin with 1 min of pre-irradiation (L-C1min), (c) 600 μmol.L^{-1} curcumin with 2 min of pre-irradiation (L-C2min), (d) 600 μmol.L^{-1} curcumin with 5 min of pre-irradiation (L-C5 min), (e) 75 J.cm^{-2} LED (L75C-), (f) 600 μmol.L^{-1} curcumin with 1 min of pre-irradiation + 75 J.cm^{-2} LED (L75C1min), (g) 600 μmol.L^{-1} curcumin with 2 min of pre-irradiation + 75 J.cm^{-2} LED (L75C2min), and (h) 600 μmol.L^{-1} curcumin with 5 min of pre-irradiation + 75 J.cm^{-2} LED (L75C5min). These analyses were made in duplicate and the experiments were repeated once (n = 4/group). All these conditions were applied on biofilms grown for 3 and 5 days.

2.4. Photodynamic antimicrobial therapy (aPDT)

Curcumin (Sigma-Aldrich, S. Louis, USA) and Biotable® RGB

Table 2
Vitality of intact microcosm biofilms (%) grown during 5 days, considering whole biofilms, and their outer, middle and inner layers. *P* values are presented to compare a specific group with its control (L-C-).

	Groups	Mean ± SD			*p* values	Median	CI (95%)	
							Lower	Upper
Whole Biofilm	L-C-^{a}	99.13	±	0.74		99.30	97.96	100.30
	L-C1min^{ab}	93.46	±	1.92	0.31	93.27	90.40	96.51
	L-C2min^{bc}	77.20	±	4.28	< 0.001	77.19	70.39	84.01
	L-C5min^{ab}	92.38	±	4.47	0.24	91.93	85.26	99.49
	L75C-^{bc}	81.68	±	7.26	< 0.001	83.91	70.12	93.23
	L75C1min^{bc}	83.86	±	12.79	0.01	84.47	63.50	104.21
	L75C2min^{d}	36.34	±	15.84	< 0.001	34.36	11.13	61.55
	L75C5min^{d}	49.61	±	9.03	< 0.001	48.43	35.24	63.97
Outer Layer	L-C-^{a}	97.84	±	2.51		98.60	93.85	101.83
	L-C1min^{ab}	87.83	±	6.40	0.20	89.71	77.65	98.02
	L-C2min^{b}	70.54	±	5.08	< 0.001	69.16	62.45	78.63
	L-C5min^{ab}	85.38	±	2.32	0.07	86.05	81.69	89.08
	L75C-^{b}	77.94	±	15.47	0.01	80.37	53.34	102.55
	L75C1min^{b}	75.17	±	18.57	< 0.001	76.19	45.62	104.71
	L75C2min^{c}	26.10	±	11.82	< 0.001	24.56	7.30	44.91
	L75C5min^{c}	34.58	±	3.93	< 0.001	36.21	28.33	40.83
Middle Layer	L-C-^{a}	98.33	±	1.72		98.82	95.59	101.07
	L-C1min^{ac}	93.78	±	3.52	0.40	94.98	88.18	99.38
	L-C2min^{b}	74.08	±	2.24	< 0.001	74.61	70.53	77.64
	L-C5min^{ac}	91.85	±	5.16	0.16	92.57	83.64	100.07
	L75C-^{bc}	89.34	±	2.92	0.03	89.12	84.69	93.98
	L75C1min^{bc}	83.90	±	11.35	< 0.001	84.29	65.84	101.95
	L75C2min^{d}	23.53	±	9.16	< 0.001	20.42	8.96	38.10
	L75C5min^{d}	37.02	±	6.40	< 0.001	37.49	26.83	47.21
Inner Layer	L-C-^{a}	99.41	±	0.62		99.62	98.42	100.41
	L-C1min^{ac}	90.91	±	5.29	0.10	91.84	82.50	99.32
	L-C2min^{b}	77.94	±	8.09	< 0.001	80.28	65.07	90.82
	L-C5min^{ac}	86.32	±	9.99	0.02	86.30	70.43	102.22
	L75C-^{bc}	82.52	±	1.38	< 0.001	82.18	80.31	84.72
	L75C1min^{bc}	86.85	±	9.79	0.03	86.93	71.27	102.43
	L75C2min^{d}	36.21	±	16.78	< 0.001	34.06	9.51	62.91
	L75C5min^{d}	40.06	±	7.26	< 0.001	38.05	28.50	51.62

Distinct superscript letters represent significant statistical differences between groups ($P < 0.05$). SD means standard deviation. CI means confidence interval.

(Institute of Physics of São Carlos, São Carlos, Brazil) were used as a photosensitizing agent and a light source, respectively.

The solution of curcumin was prepared immediately prior to its use, with dilution in sterile deionized water to 0.8% DMSO (Merck KGaA, Frankfurter, Germany) (600 μmol.L^{-1} curcumin). This diluent was also used to treat control group. Aliquots of 1 mL of curcumin or diluent were transferred into the wells of 24-well microtiter plates and maintained in dark conditions until the moment of use.

After biofilm growth, specimens were washed twice in CPW medium [5 g of yeast extract, 1 g of peptone, 8.5 g of NaCl, and 0.5 g L-cysteine − HCl, deionized water per liter, pH 7.3], to remove unbounded cells. Subsequently, the specimens were incubated in wells containing curcumin for different pre-irradiation times (1, 2 or 5 min), or in wells containing the diluent for 2 min, both in dark conditions. The parameters for irradiation are described as follows: visible blue light wavelength (455 ± 30 nm), distance of ≈ 25 mm, irradiance of 40 mW.cm^{-2}, and one irradiation time (1870 s), corresponding to a energy density of 75 J.cm^{-2} (total energy = 7.5 J). The output power of Biotable® RGB was controlled by an optical power meter (1916-C Optical Power Meter, Newport, Irvine, USA) throughout the study.

2.5. Vitality of intact biofilms

Glass specimens were collected and stained with 10 μL of the mixture containing 0.25 g.L^{-1} ethidium bromide (Sigma Chemicals Co., St. Louis, USA) and 2.5 g.L^{-1} fluorescein diacetate (Sigma Chemicals Co., St. Louis, USA) under dark conditions [20]. Then, the samples were washed in CPW medium to remove the excess of reagents.

Three representative fields of each sample were chosen for visualization in the confocal scanning laser microscope (CSLM) model TCS-SPE (Leica, Wetzlar, Germany) (488 nm excitation, double detection BP520 for fluorescein and D650 for ethidium bromide; 40x, NA 0.65). To compare the images, the power and aperture of the laser were kept constant. At each site, the entire depth extension was analyzed and recorded, based on the biofilm thickness. The images were corrected and synthesized using the Multicolor Analysis software (Leica, Wetzlar, Germany). The entire extensions of fields were analyzed in three different layers (inner, middle and outer), using the Leica QWIN Image Analysis software (Leica, Wetzlar, Germany) to detect green and red signals for live and dead cells, respectively [21]. The intensities of green and red fluorescence were used to calculate the percentage of vitality of biofilms, according to the following formula:

$$\textbf{Vitality of intact biofilm}\ (\%) = \frac{\text{Green intensity}}{\text{Red intensity} + \text{green intensity}} \text{x}\,100$$

2.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM® SPSS® Statistics, New York, USA). The differences between groups were detected by two-way ANOVA and post-hoc Tukey tests, after arcsine transformation. *P* values < 0.05 were considered significant.

3. Results

In comparison to control (L-C-), curcumin alone (PIT = 5 min) and all combinations of curcumin and LED (PITs 1 min, 2 min and 5 min) reduced significantly the vitality of 3-day whole biofilms (Table 1, Fig. 1). Distinctly, only aPDT treatments with PITs of 2 and 5 min were

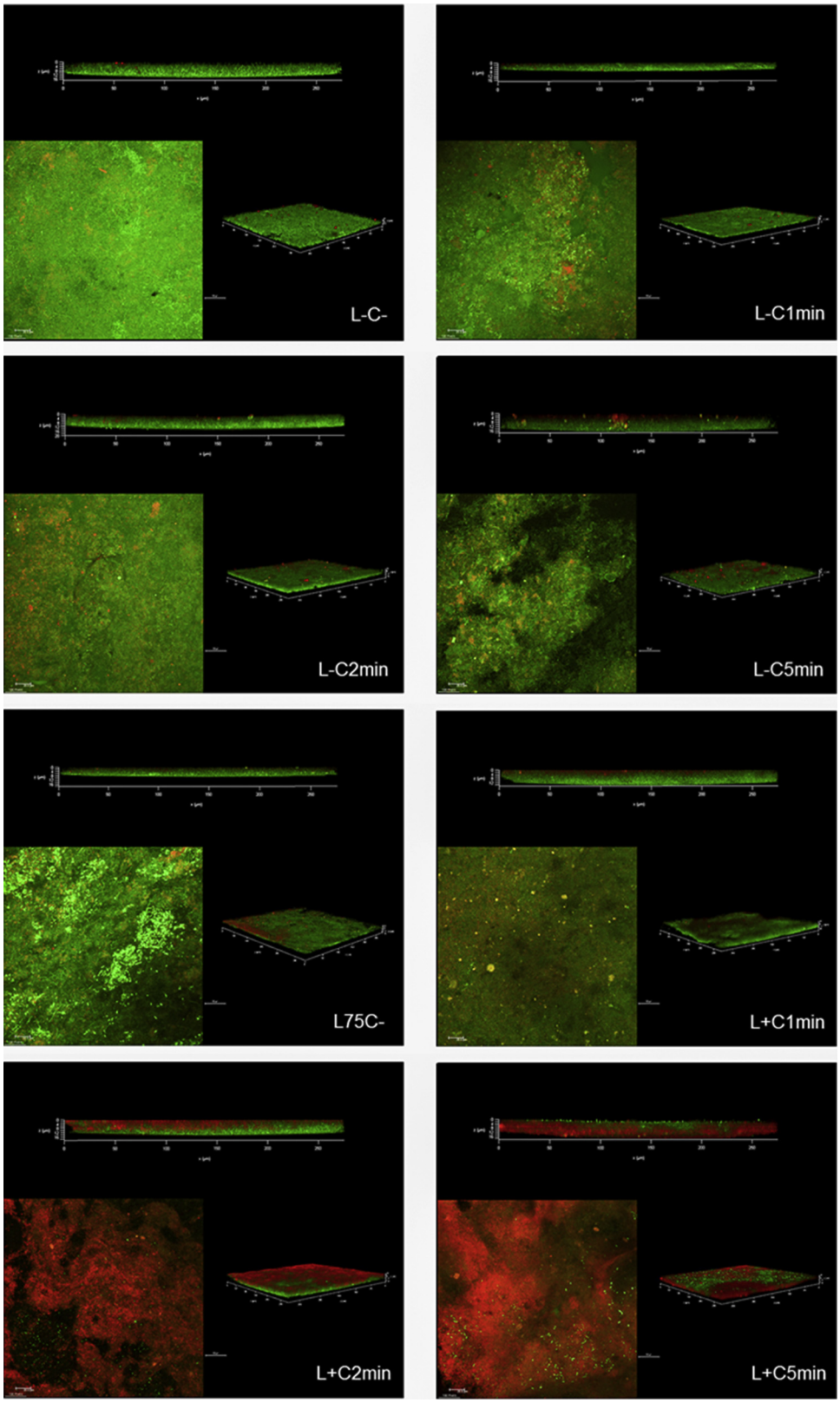

Fig. 2. Bi and tridimensional CSLM images of intact whole biofilms grown during 5 days. The groups are represented as follows: L-C- (control, no treatment), L-C1min (600 μmol.L^{-1} curcumin with 1 min of pre-irradiation), L-C2min (600 μmol.L^{-1} curcumin with 2 min of pre-irradiation), L-C5 min (600 μmol.L^{-1} curcumin with 5 min of pre-irradiation), L75C- (75 J.cm^{-2} LED alone), L75C1min (600 μmol.L^{-1} curcumin with 1 min of pre-irradiation + 75 J.cm^{-2} LED), L75C2min (600 μmol.L^{-1} curcumin with 2 min of pre-irradiation + 75 J.cm^{-2} LED), and L75C5min (600 μmol.L^{-1} curcumin with 5 min of pre-irradiation + 75 J.cm^{-2} LED). Note a significant reduction of the vitality of biofilms observed after the combination of curcumin and LED.

effective in reducing significantly the vitality of 5-day whole biofilms (Table 2, Fig. 2).

More mature biofilms offered greater resistance to aPDT. This result is evident when comparing the antimicrobial effect of 600 μmol.L^{-1} curcumin (PIT = 1 min) plus 75 J.cm^{-2} LED (L75C1min) on 3-day biofilms (reduction of 62% of viable cells) and 5-day biofilms (reduction of 17% of viable cells) (Tables 1 and 2). Also, curcumin alone produced approximately 46% and 7% of mortality on 3- and 5-day biofilms, respectively (Fig. 3).

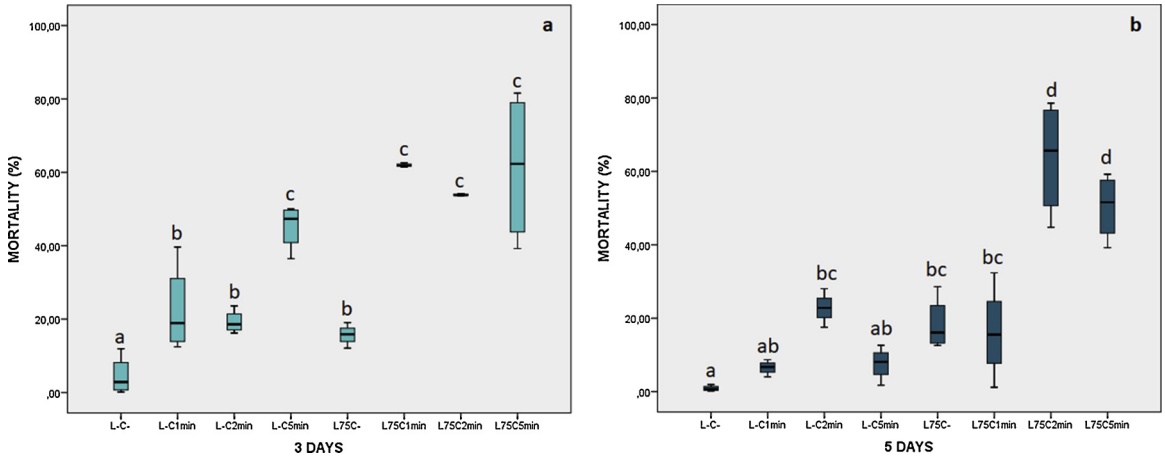

Fig. 3. Percentage of the mortality of whole dentin caries microcosms according to their growth times.

4. Discussion

These findings demonstrated that aPDT was able to decrease the vitality of intact dentin caries microcosms grown for 3 or 5 days. However, while the variation of PIT not influenced the results of aPDT on 3-day biofilms, the use of PITs equal to 2 or 5 min was determinant to the effectiveness of aPDT against 5-day biofilms. In addition, curcumin alone significantly affected the vitality of 3-day biofilms (reduction ≅ 45%), by the adoption of a PIT of 5 min. To our knowledge, this is the first *in vitro* study that tested the effect of curcumin-mediated aPDT on complex biofilms, considering distinct PITs and biofilm growth times.

Although several Ps have already been reported in the literature, such as methylene blue [17,22], toluline blue [23], rose bengal [7], photogem® [24], curcumin is the only phytotherapic one. It has a molecular weight of 368.38 $g.mol^{-1}$, presenting low solubility in water and rapid degradation [25]. It is dissolved in solutions containing n-methyl glucamine [3,26], ethanol [5], and dimethylsulfoxide (DMSO) [19], and also being combined with nanoparticles that favor its slow release [27].

It has been suggested that microcosm biofilms are adequate models to test the effectiveness of antimicrobials *in vitro*, because they present more similarities to clinical conditions than planktonic cells or mono/dual species biofilms [14]. In this context, our results demonstrated that shorter times of biofilm growth were associated with slighter resistance to antimicrobial treatments, evidenced by the association of curcumin (PIT = 1 min) and 75 $J.cm^{-2}$ LED. Probably, the thickness and density of biofilms represented a challenge for the penetration of Ps, since the effect of photosensitizing agents depends on their ability in penetrating into the extracellular matrix [19]. In our study, the penetration of curcumin into the biofilm could not be evidenced from these analyses; however, the effectiveness of the association of Ps with LED irradiation can be proven from the significant reduction of the vitality of biofilms from treatment groups compared to control group for both 3- and 5-day biofilms (*P* values varying between < 0.001 and 0.03 for all groups and layers).

In this sense, previous studies showed that *Candida albicans* from 2-day polymicrobial biofilms were more resistant to curcumin-mediated aPDT than those from 1-day biofilms, requiring the application of higher concentrations of Ps to achieve significant antimicrobial effects [28]. Also, the effectiveness of toluidine blue-mediated aPDT on 3-, 7- and 10-day *Streptococcus mutans* biofilms varied in accordance with biofilm growth time, *i.e.*, older biofilms were less susceptible to aPDT treatments [29].

Differently of the evidence that PIT does not interfere in aPDT [17,18], our results suggest that longer PITs can be determinant for obtaining adequate phototoxic effects on more mature complex biofilms. In this sense, when 3-day biofilms were treated, the increment of PIT apparently did not contribute to the reduction of the vitality of intact biofilms; however, the treatment of biofilms with curcumin (PIT = 1 min) was insufficient to increase the mortality of 5-day biofilms significantly (reduction of 16.14%), instead of 63.66% and 51.39% reductions observed after applying PITs of 2 and 5 min, respectively. Three insights emerged from these findings: i) PIT can be modulated to improve the photodynamic effects against complex biofilms, which seemed not be relevant for the treatment of planktonic cells or mono/dual species biofilms; ii) the clinical application of curcumin-mediated aPDT should consider the employment of PITs longer than 2 min, since biofilm-dependent oral diseases are associated with the maintenance of undisturbed biofilms for longer periods; iii) the modulation of PIT could contribute to the application of aPDT for oral treatments using lower concentrations of Ps, reducing the risk of pigmentation of teeth with the minimum loss of its antimicrobial effectiveness.

In conclusion, these results showed that curcumin-mediated aPDT significantly decreased the vitality of intact dentin caries microcosms grown during 3 or 5 days, although the treatment of 5-day biofilms required longer PITs in comparison to 3-day biofilms. Hence, the hypotheses *H0* and *H0'* were rejected. Our findings indicate that curcumin should be applied on dental caries biofilms using pre-irradiation times of at least 2 min, considering longer times of biofilm stagnation for dental demineralization.

Declaration of Competing Interest

The authors declare no conflict of interest.

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