

Research Paper

Enzymatic and mechanical disruption before successive photodynamic therapy targets the extracellular matrix of *Candida albicans*Amanda Bellini^a, Luana Mendonça Dias^a, César Augusto Abreu-Pereira^a,
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ABSTRACT

Objective: To evaluate whether pretreatment strategies targeting the extracellular matrix (ECM), such as DNase I and low-frequency ultrasound, enhance the efficacy of successive antimicrobial photodynamic therapy (aPDT) against *Candida albicans* biofilms and to assess the effects on biofilm components.**Methods:** Forty-eight-hour *C. albicans* (ATCC 90028) biofilms were treated under four conditions: (I) aPDT [Photodithazine (PDZ) (25 mg/L) for 20 min + Light-Emitting Diode (LED) (660 nm, 18 J/cm²)], (II) DNase + aPDT [5 min with 20 U/mL DNase (Dn) I before aPDT], (III) sonication + aPDT [So, 7 W, 170–190 J before aPDT], (IV) Dn + So + aPDT. Ten successive aPDT applications were performed. Colony-forming units per milliliter (CFU/mL) was assessed after each application. After applications 1, 5, and 10, total and insoluble dry weight, soluble/insoluble proteins, water-soluble polysaccharides (WSP), alkali-soluble polysaccharides (ASP), and extracellular DNA (eDNA) were quantified. Biofilm architecture was analyzed by scanning electron microscopy (SEM).**Results:** All groups showed significant CFU/mL reduction after ten aPDT applications compared to untreated control: aPDT (5.7 log₁₀), Dn + aPDT (5.5 log₁₀), So + aPDT (5.3 log₁₀), Dn + So + aPDT (5.3 log₁₀) ($p < 0.05$). Insoluble proteins decreased after the first application (46%), and soluble proteins after the fifth and tenth (45%; $p \leq 0.032$). WSP and eDNA were significantly reduced ($p \leq 0.047$). No significant differences were observed for total/insoluble dry weight, proteins, and ASP. SEM revealed wall deformities and fewer clusters.**Conclusion:** Successive aPDT applications reduced CFU/mL, WSP, and eDNA in *C. albicans* biofilms regardless of pretreatment strategy.

1. Introduction

The incidence of bacterial and fungal infections has been increasing in recent years [1–3]. Many fatal fungal diseases are caused by species of the *Candida* genus [4–7], and the risk of immunocompromised patients contracting a fungal disease is higher and more severe [8]. During the COVID-19 pandemic, there was a significant increase in the number of patients with fungal diseases, and patients with acute COVID-19 infection were found to be particularly susceptible to pathogenic fungi. The situation worsens due to the potential risk of oral *Candida* infection transmission in patients requiring mechanical ventilation during COVID-19 treatment [9].

Candida albicans is the most prevalent and pathogenic species of the

Candida genus that colonizes the oral cavity and causes superficial mucocutaneous infections, such as Oropharyngeal Candidiasis (OPC), or disseminated forms of infection. Polyene, azole, and echinocandin antifungals have been used to treat OPC [10]. However, due to the increase in drug resistance and the high recurrence rates after antifungal treatment, investigation of new therapeutic strategies for *C. albicans* biofilm inactivation remains necessary [11,12].

Antimicrobial photodynamic therapy (aPDT) is a promising alternative treatment for superficial infections caused by *C. albicans* [13–15]. Photodynamic treatment requires the use of a photosensitizer (PS), which, when activated by a light source in the presence of oxygen, generates reactive oxygen species (ROS), such as singlet oxygen, that cause irreversible cellular damage to microorganisms [16]. The most

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used PSs in aPDT belong to the porphyrin and phenothiazine families [17–19]. Photodithazine® (PDZ), a water-soluble chlorin e6, has been successfully employed in aPDT for microbial inactivation due to its high capacity for singlet oxygen generation in the red light presence [20].

Photodynamic treatment promotes complete inactivation of planktonic cultures of *C. albicans*; however, biofilms exhibit reduced susceptibility to aPDT [21–24]. The tolerance of biofilm-embedded microorganisms to aPDT can be attributed to the structural organization of biofilm, which offers protection and tolerance to therapeutic agents [25]. The extracellular matrix (ECM) of the biofilm is formed by the accumulation of substances derived from cell lysis or produced and secreted by cells; it contributes to the preservation of biofilms and the maintenance of stable interactions between cells, surface, and environment, hindering the action of conventional drugs [26,27]. The ECM comprises polysaccharides, proteins, lipids, and nucleic acids [28]. Extracellular DNA (eDNA) plays a decisive role in promoting biofilm adhesion to the substrate and, together with polysaccharides, contributes to the structural integrity of the ECM [29]. Once biofilm is established, approaches that reduce extracellular polymeric substances can disrupt the ECM, weaken the biofilm structure, and enhance microbial inactivation [30].

Treatment with DNase or ultrasound can be an adjuvant to anti-biofilm therapies by disrupting biofilm components that impede drug penetration [30–33]. Treatment of biofilms with 50 µg/mL of DNase I resulted in a 30 % reduction in biofilm biomass [31]. In addition, DNase I enhanced the susceptibility of *C. albicans* biofilm to amphotericin B [30,32]. Fluconazole-susceptible *C. albicans* biofilm incubated with DNase I (5 min) before aPDT (PDZ combined with light-emitting diode (LED) reduced viable colony counts (CFU/mL) and eDNA levels in the ECM [30]. In fluconazole-resistant *C. albicans* biofilms, this treatment resulted in an approximately 1.62 log₁₀ reduction and decreased water-soluble polysaccharides by 36.3 % and eDNA by 72.3 %. DNase I disrupted the ECM of fluconazole-susceptible and -resistant *C. albicans* biofilm and allowed the diffusion of PDZ and light through the biofilm ECM, increasing the treatment's effectiveness [30]. Furthermore, mechanical methods can also disturb the ECM [34]. The combination of ultrasound and aPDT resulted in an approximately 98 % reduction of *Enterococcus faecalis* biofilms [35].

Although DNase and ultrasound have each been investigated as adjuvant to improving the effects against *C. albicans* biofilms, their behaviour in combination with aPDT successive applications remain unexplored. Prior studies have assessed single-session treatments, remaining unclear whether these biofilm weakening strategies could potentiate the cumulative efficacy of repeated aPDT applications. To address this gap, the present study evaluated enzymatic (DNase) and mechanical (low-frequency ultrasound) disruption, applied alone or in combination, before successive aPDT against *C. albicans* biofilms, with a particular focus on ECM degradation and structural alterations.

2. Material and methods

2.1. Biofilm formation

The *C. albicans* strain (ATCC 90028) was thawed and plated on Petri dishes containing Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol (0.05 g/L) and incubated at 37 °C for 48 h. After this period, 20 colonies were inoculated into 20 mL of Yeast Nitrogen Base (YNB) medium supplemented with glucose (100 mM) and incubated at 37 °C for 16 h. Then, 16.1 mL of the starter culture was added to flasks containing 305.9 mL of fresh YNB medium (1:20 dilution). The inoculum was incubated at 37 °C until it reached the mid-log growth phase (approximately 8 h), corresponding to an optical density (OD at 540 nm) of 0.55 ± 0.08 . The *C. albicans* suspension was adjusted to reach a concentration of 10^7 CFU/mL [31].

Aliquots of 1 mL of the inoculum were transferred to the wells of a 24-well plate. Then, 1 mL of fresh YNB medium supplemented with 100

mM glucose was added to each well. The plates were incubated for 90 min at 37 °C under orbital shaking (75 rpm), corresponding to the adhesion phase [23]. Subsequently, the liquid culture and non-adhered cells were removed, and the adhered cells were washed twice with sterilized phosphate-buffered saline PBS solution (PBS; pH 7.4), and 2 mL of RPMI 1640 medium buffered with 3-(N-morpholino) propane-sulfonic acid (MOPS) at pH 7.8 were added for biofilm formation, followed by incubation at 37 °C (24 h). After 24 h, 1 mL of the culture medium was removed, and 1 mL of fresh medium (RPMI + MOPS) was added. The plates were then incubated for an additional 24 h. After incubation, the 48-hour-old mature biofilms were washed twice with PBS solution, and treatments were applied. These procedures were performed in quadruplicate on three different occasions.

2.2. Preparation of the photosensitizer and irradiation parameters

Photodithazine® (PDZ; Russia, VETAGRAN, Co., Moscow, Russia) at 500 mg/L (stock solution) was diluted using phosphate-buffered saline (PBS, pH 7.2) to achieve a 25 mg/L working solution. A red LED emitter (Biotable 3.4, São Carlos, Brazil) with a wavelength of 660 nm was used to excite the PDZ. The LED dose evaluated was 18 J/cm² (11 min of exposure) at 27 mW/cm² of irradiance, measured at the sample plane after device pre-calibration to ensure uniform delivery to each well bottom. Irradiation was delivered from below through the transparent bottoms of 24-well, flat-bottom plates positioned directly on the LED aperture (effective source-sample distance ≈ 1 mm), and the illuminated area per well corresponded to the well's inner diameter (≈ 15.6 mm; area ≈ 1.9 cm²). A 20-minute pre-irradiation time was used. The parameters for aPDT were selected based on previous studies, where complete inactivation of *C. albicans* was not achieved (sub-lethal dose) [14]. The photodynamic treatment consisted of 10 successive applications, performed immediately after applying ECM disruption strategies: DNase application for eDNA degradation and low-frequency ultrasound for mechanical destabilization.

2.3. Strategies for biofilm disruption and treatments used

C. albicans biofilms were subjected to the treatments outlined in Table 1. The samples were subjected to treatments with DNase I and/or sonication to achieve a disorganization of the ECM.

For chemical disorganization, DNase I (AMPD1; Sigma-Aldrich) was prepared in 0.1 M sodium acetate buffer (pH 5.5) at a concentration of 20 U/mL. 48-hour biofilms were incubated with DNase I (Dn) for 5 min at 37 °C [32].

For mechanical disorganization, the 48-hour biofilms were mechanically disrupted by sonication (So) at 7 W for 30 s, delivering approximately 170–190 J of energy [36,37].

Following ECM disorganization, ten successive applications of aPDT were performed (Apl#1, Apl#2, Apl#3, Apl#4, Apl#5, Apl#6, Apl#7, Apl#8, Apl#9, and Apl#10), followed by culture dilution and plating. Sixteen experimental groups were evaluated: P+L+ (PDZ + red LED/aPDT group); P–L+ (only red LED); P+L– (only PDZ); P–L– (experimental control/absence of PS and light application); Dn+P+L+ (DNase followed by aPDT); Dn+P–L+ (DNase followed by red LED); Dn+P+L– (DNase followed by PDZ); Dn+P–L– (only DNase I); So+P+L+ (sonication followed by aPDT); So+P–L+ (sonication followed by red LED); So+P+L– (sonication followed by PDZ); So+P–L– (only sonication); Dn+So+P+L+ (DNase followed by sonication and aPDT); Dn+So+P–L+ (DNase followed by sonication and red LED); Dn+So+P+L– (DNase followed by sonication and PDZ); Dn+So+P–L– (DNase followed by sonication). The experiments were carried out in quadruplicate on three occasions (n=19/group).

2.4. Assessment of the efficacy of applied treatments

After the treatments, the biofilms were washed three times with NaCl

Table 1

Evaluated conditions and treatment groups. Positive signs (+) indicate the presence of a specific treatment, while negative signs (-) indicate its absence. P = PDZ (25 mg/L for 20 min); LED (660 nm for 18 J/cm²); Dn = Dnase I (20 U/mL for 5 min); So = Sonication (7 W; 170 to 190 J; 30 s).

Treatment conditions	Groups	PDZ cr (25 mg/L for 20 min)	LED (660 nm for 18 J/cm ² 11 min)	DNase I (20 U/mL for 5 min)	Sonication (7 W; 170 to 190 J; 30 s)
aPDT	P+L+	+	+	-	-
	P+L-	+	-	-	-
	P-L+	-	+	-	-
	P-L-	-	-	-	-
Dn+aPDT	Dn+P+L+	+	+	+	-
	Dn+P+L-	+	-	+	-
	Dn+P-L+	-	+	+	-
	Dn+P-L-	-	-	+	-
So+aPDT	So+P+L+	+	+	-	+
	So+P+L-	+	-	-	+
	So+P-L+	-	+	-	+
	So+P-L-	-	-	-	+
Dn+So+aPDT	Dn+So+P+L+	+	+	+	+
	Dn+So+P+L-	+	-	+	+
	Dn+So+P-L+	-	+	+	+
	Dn+So+P-L-	-	-	+	+

(0.89 %). Subsequently, 2 mL of NaCl was added to each well, and the biofilms were carefully detached from the bottom of the plates with a pipette tip, transferred to sterilized microtubes, and submitted to sonication (30 s; 7 w; 190 J). After sonication, an aliquot (0.1 mL) of the suspension was plated in SDA for colony-forming units (CFU/mL) determination and another for total biomass determination (0.1 mL) [38]. The remaining volume (1.8 mL) was centrifuged (5.500 × g; 10 min; 4 °C), and the supernatant (1.8 mL) was separated into three aliquots for the quantification of soluble components of the matrix: water-soluble polysaccharides (WSP) (1 mL), eDNA (0.650 mL) and proteins (0.150 mL) [38,39]. The resultant pellet (insoluble components of extracellular matrix plus fungal cells) was resuspended in Mili-Q water and divided into different aliquots for the investigation of insoluble components of the matrix: insoluble biomass (0.8 mL), insoluble proteins (0.05 mL), and alkali-soluble polysaccharides (ASP) (0.95 mL) [40].

Colony-forming units (CFU/mL) were quantified after each aPDT application (Apl#1 until Apl#10). Biofilm components were quantified after applications Apl#1, Apl#5, and Apl#10 of aPDT.

Scanning Electron Microscopy (SEM) was performed to compare the treatment groups with the control groups under each experimental condition after applications Apl#1 and Apl#10 of aPDT.

2.5. SEM images acquisition

Biofilms were cultivated on circular polystyrene disks obtained from the bottom of a 24-well plate. The disks were sterilized under ultraviolet light (UV) at 650 W for 3 min, then exposed under a laminar flow hood for 30 min on each side. Subsequently, they were placed into a 24-well plate for biofilm formation, followed by treatment application (as previously described) [41]. Then, the biofilms were fixed with 2.5 % glutaraldehyde for 60 min at room temperature, washed twice with PBS (pH 7.4) and dehydrated. Dehydration was carried out with 70 % and 90 % alcohol washes for 60 min each, followed by five washes of 30 min with absolute alcohol (100 %). Samples were stored in a desiccator with silica gel for at least 7 days. Prior to imaging, specimens were sputter-coated with gold (Au, ~10 nm) using a Denton Vacuum Desk V coater (0.05 Torr, 20 mA, 120 s). SEM imaging was performed on a JEOL JSM-6610LV using secondary electrons, accelerating voltage 10 kV, and working distance 10 mm.

2.6. Statistical analysis

For the CFU/mL results, the Shapiro-Wilk test was applied,

indicating a lack of normal distribution of the data. The Levene test was used, and the data demonstrated heteroscedasticity in their variance. Therefore, the non-parametric two-way ANOVA test implemented via the Aligned Rank Transform (ART), with factors Treatment and Application (Apl#1-#10) as factors, and results are reported as mean estimates with 95 % CIs. Because each application level was assessed on independent wells, observations were independent (i.e., not repeated-measures). Post-hoc comparisons were conducted using the ART model with ART-C/emmeans and Tukey adjustment ($\alpha = 0.05$).

For the results concerning the biofilm matrix, the amount were expressed per well (i.e., per single biofilm in one 24-well plate well); µg/well for protein, ng/well for eDNA, and µg/well for ASP or WSP. The normality and homoscedasticity of the data for each biofilm component were assessed using the same tests as the previous variable (CFU/mL). Thus, a two-way ANOVA variance test (Treatment × Application) was performed, followed by Tukey's HSD post hoc test for homoscedastic data and the Games-Howell test for heteroscedastic data, to verify the interaction between groups (control and experimental) and treatment conditions (aPDT; Dn+aPDT; So+aPDT; Dn+So+aPDT). The significance level adopted was 5 % ($\alpha=0.05$), and the analyses were performed using R (ARTool) and SPSS software (IBM SPSS® Statistics, version 21, Chicago, IL, USA).

3. Results

3.1. Viable colony count (CFU/mL)

Comparing the applications under the condition in which the biofilms were subjected only to photodynamic treatment (group P+L+), it can be observed that there was a reduction in cell viability of 3.2 log₁₀ after the Apl#1, 3.5 log₁₀ after the Apl#5, and 5.9 log₁₀ after the Apl#10 of aPDT (P+L+ group). In P+L+ condition, the Apl#10 was statistically lower than Apl#1-#5 (Fig. 1). The results of CFU/mL demonstrate that, for all experimental conditions, the control groups (P-L-, P-L+, and P+L-) did not show statistical differences between applications, remaining consistent from Apl#1 to Apl#10 of aPDT [42].

Upon examining the aPDT applications under the Dn+P+L+ condition, a significant reduction in cell viability of 3.5 log₁₀ was observed after the Apl#1, 4.1 log₁₀ after the Apl#6, and 5.5 log₁₀ after the Apl#10 of aPDT. In Dn+P+L+, the Apl#10 demonstrated statistical similarity to Apl#1-#4 (Fig. 2). Furthermore, it was noticed that the control groups (Dn+P-L-, Dn+P-L+, and Dn+P+L-) showed no statistical differences between applications, remaining consistent from the Apl#1 to Apl#10 (Fig. 2).

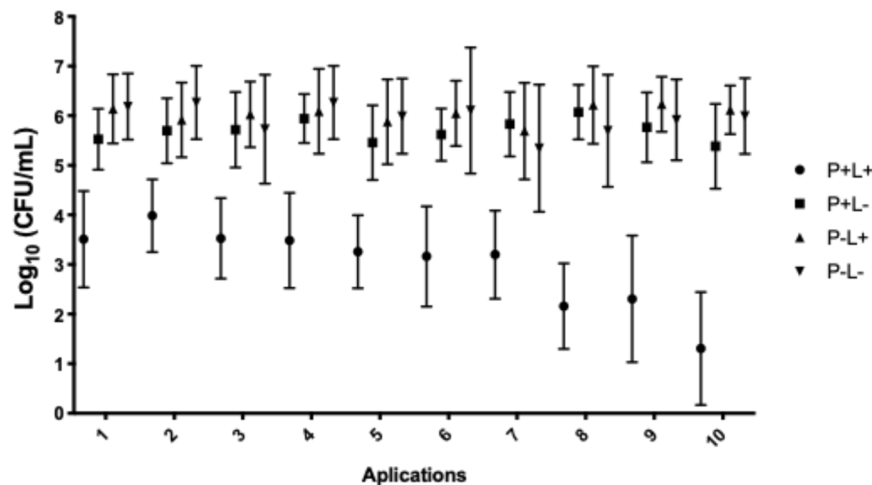


Fig. 1. Graph depicting the mean $\pm$ 95 % confidence interval of Log_{10} (CFU/mL) values of *C. albicans* biofilms after successive applications of aPDT. Biofilms subjected to aPDT (P+L+ group), biofilms treated only with Photodithazine (P+L- group), biofilms subjected only to LED (P-L+ group), and untreated biofilms (P-L- group). The minimum and maximum values of the log_{10} (CFU/mL) error bar represent the lower and upper limits of the confidence interval (95 %) after applications Apl#1 until Apl#10 ($n = 19/\text{group}$) ($p > 0.05$).

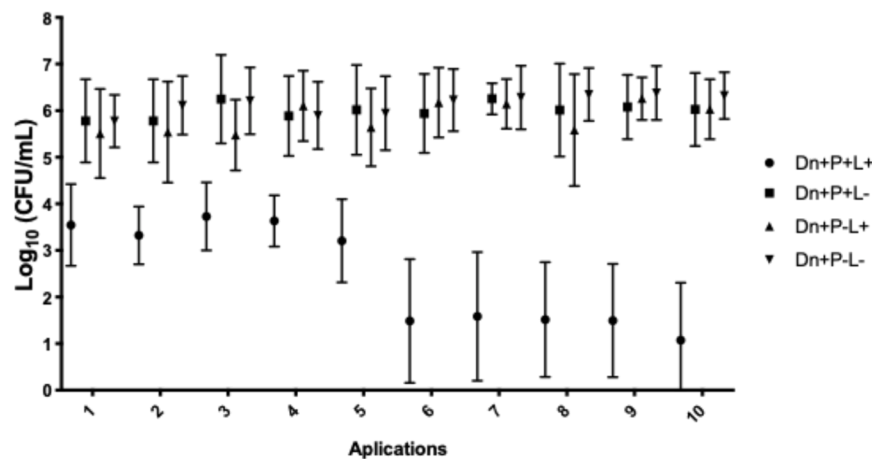


Fig. 2. Graph depicting the mean $\pm$ 95 % confidence interval of Log_{10} (CFU/mL) values of *C. albicans* (ATCC 90,028) biofilms from the Dn+aPDT condition after successive applications of aPDT. *C. albicans* biofilms subjected to Dn+aPDT (Dn+P+L+ group), biofilms treated with Dn+PDZ (Dn+P+L- group), biofilms subjected to Dn+red LED (Dn+P-L+), and biofilms treated only with DNase (Dn+P-L-). The minimum and maximum values of the log_{10} (CFU/mL) error bar represent the lower and upper limits of the confidence interval after applications Apl#1 until Apl#10 ($n = 19/\text{group}$) ($p > 0.05$).

Upon examining the outcomes under the sonication followed by aPDT condition, the So+P+L+ group exhibited a decrease in CFU/mL of 3.4 log_{10} after the Apl#1 and 5.2 log_{10} after the Apl#10 of aPDT, with these differences demonstrating statistical significance. The control groups (So+P+L-, So+P-L+, and So+P-L-) showed no significant differences across applications, remaining consistent from Apl#1 to Apl#10 (Fig. 3).

Upon analyzing the applications within the Dn+So+P+L+ group, a reduction in cellular viability of 3.5 log_{10} after the Apl#1 and 5.3 log_{10} after the Apl#10 of aPDT was observed. Within this same condition, the Apl#1 and Apl#2 were statistically lower compared to Apl#10. Furthermore, it was noted that the control groups (Dn+So+P-L-, Dn+So+P-L+, and Dn+So+P+L-) did not exhibit statistical differences between applications, remaining consistent from Apl#1 to Apl#10 (Fig. 4).

3.2. Total dry weight

In Apl#1, under the So+aPDT, Dn+aPDT, and Dn+So+aPDT treatment conditions, the total dry weight values of the experimental groups

were reduced ($p \leq 0.006$) compared to their respective controls, and statistically similar to each other ($p \geq 0.463$) (Table 2). In the aPDT condition, the total dry weight values were statistically identical to their respective control groups ($p = 0.093$). In Apl#5 and Apl#10, all treatment conditions (aPDT, So+aPDT, Dn+aPDT, and Dn+So+aPDT) showed a statistically significant reduction ($p \leq 0.003$) in the total dry weight compared to their respective control groups and were similar to each other ($p \geq 0.076$) (Table 2).

3.3. Insoluble dry weight

Remaining insoluble dry weight, Apl#1 showed no statistically significant reductions compared to the respective control groups under evaluated treatment conditions ($p \geq 0.276$; Table 3). In Apl#5, a statistically significant decrease ($p \leq 0.032$) in the insoluble dry weight was observed under the So+Dn+aPDT treatment condition compared to its respective control, with the other conditions being similar to their respective control group ($p \geq 0.434$; Table 3). In Apl#10, a significant reduction ($p \leq 0.004$) in the experimental group compared to its respective control was observed for the So+aPDT and So+Dn+aPDT

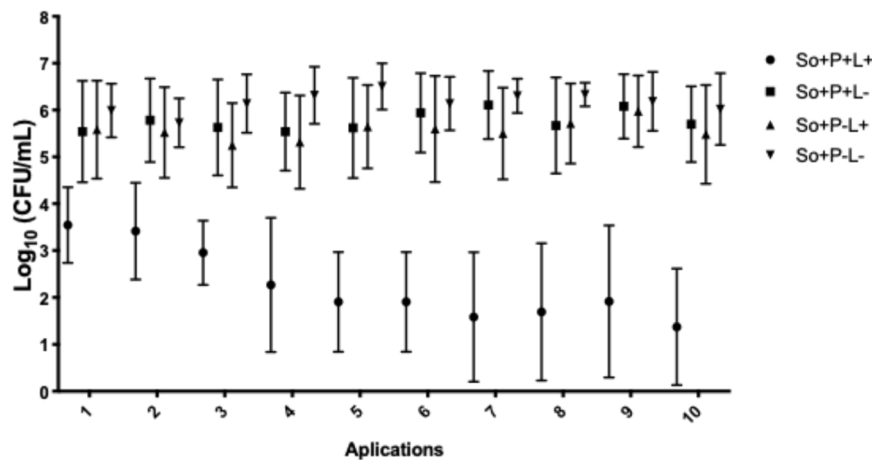


Fig. 3. Graph depicting the mean $\pm$ 95 % confidence interval of Log_{10} (CFU/mL) values of *C. albicans* (ATCC 90,028) biofilms from the So+aPDT condition after successive applications of aPDT. *C. albicans* biofilms subjected to So+aPDT (So+P+L+), biofilms treated with So+PDZ (So+P+L-), biofilms subjected to So+red LED (So+P-L+), and biofilms treated only with So (So+P-L-). The minimum and maximum values of the log_{10} (CFU/mL) error bar represent the lower and upper limits of the confidence interval after applications Apl#1 until Apl#10 ($n = 19/\text{group}$) ($p > 0.05$).

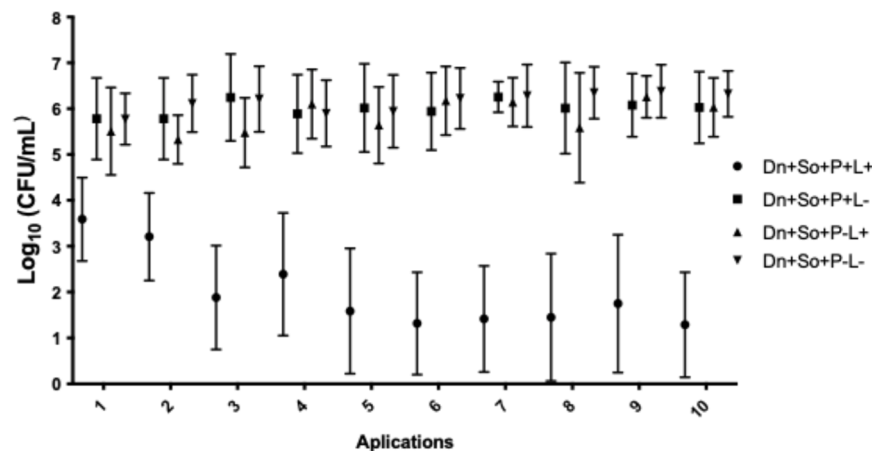


Fig. 4. Graph depicting the mean $\pm$ 95 % confidence interval of Log_{10} (CFU/mL) values of *C. albicans* (ATCC 90,028) biofilms from the Dn+So+aPDT condition after successive applications of aPDT. *C. albicans* biofilms subjected to Dn+So+aPDT (Dn+So+P+L+ group), biofilms treated with Dn+So+PDZ (Dn+So+P+L- group), biofilms subjected to Dn+So+red LED (Dn+So+P-L+), and biofilms treated only with Dn+So (Dn+So+P-L-). The minimum and maximum values of the log_{10} (CFU/mL) error bar represent the lower and upper limits of the confidence interval after applications Apl#1 until Apl#10. The non-intersection of the error bars denotes statistical difference ($n = 19/\text{group}$) ($p > 0.05$).

conditions, and statistical similarity was observed in the other treatment conditions ($p \geq 0.221$; Table 3).

3.4. Total proteins

After Apl#1 in all evaluated treatment conditions, the experimental groups were similar to their respective controls ($p \geq 0.077$; Table 4). For Apl#5 and Apl#10, all treatment conditions showed a significant reduction ($p \leq 0.001$) in the total protein levels, compared to their respective controls, with these conditions being statistically similar to each other ($p \geq 0.133$; Table 4).

3.5. Insoluble proteins

For Apl#1, a significant reduction ($p \leq 0.001$) in insoluble proteins of the experimental groups compared to their controls was observed for the aPDT, So+aPDT, and So+Dn+aPDT conditions, which were similar to each other ($p = 0.069$; Table 5). After Apl#5, only the aPDT group showed a statistically significant reduction, and the other conditions were similar to their respective controls ($p \geq 0.091$; Table 5). In Apl#10,

for all conditions evaluated (aPDT, So+aPDT, Dn+aPDT, and So+Dn+aPDT), reductions ($p = 0.009$) in protein levels compared to their respective controls were observed, which were similar ($p \geq 0.368$) to each other (Table 5).

3.6. Water-soluble polysaccharides (WSP)

For Apl#1, the aPDT and So+aPDT conditions showed a statistically significant reduction ($p = 0.001$) in the WSP levels of the experimental groups compared to their respective controls (Table 6). In Apl#5, the treatment conditions, aPDT, So+aPDT, and So+Dn+aPDT showed a significant reduction ($p \leq 0.047$) in the WSP levels of the experimental groups compared to their respective controls (Table 6). After Apl#10, the aPDT and So+aPDT conditions showed a significant reduction ($p \leq 0.001$) in WSP and were similar to each other ($p = 0.481$; Table 6).

3.7. Alkali-soluble polysaccharides (ASP)

After Apl#1, only the aPDT treatment condition showed a significant reduction ($p < 0.001$) in ASP values in the experimental group

Table 2

Means and standard deviations of the total dry weight (mg/well) of biofilms evaluated after Apl#1, Apl#5, and Apl# 10. aPDT with biofilm disruption: the biofilms were treated with biofilm disruption strategies followed by aPDT (Dn+aPDT; So+aPDT and So+aPDT group). Biofilm disruption only: the biofilms were treated only with the strategies (Dn+P-L-, So+P-L- and Dn+So+P-L-). Capital letters correspond to the comparison between groups (rows), and lowercase letters correspond to the comparison between treatment conditions (columns). Unequal letters indicate a significant statistical difference ($p < 0.05$) in Tukey or Games-Howell post-test ($\alpha = 0.05$).

Application	Treatment Condition	Group	
		aPDT with biofilm disruption	Biofilm disruption only
Apl#1	aPDT	21.733 $\pm$ 4.083 ^{Aa}	29.833 $\pm$ 3.943 ^{Aa}
	Dn + aPDT	24.133 $\pm$ 2.115 ^{Aa}	33.233 $\pm$ 7.130 ^{Ba}
	So + aPDT	20.000 $\pm$ 2.909 ^{Aa}	33.166 $\pm$ 6.224 ^{Ba}
	Dn + So + aPDT	25.866 $\pm$ 5.736 ^{Aa}	37.900 $\pm$ 6.404 ^{Ba}
Apl#5	aPDT	21.100 $\pm$ 8.931 ^{Aa}	32.933 $\pm$ 3.024 ^{Ba}
	Dn + aPDT	22.733 $\pm$ 2.342 ^{Aa}	34.966 $\pm$ 5.931 ^{Ba}
	So + aPDT	20.072 $\pm$ 3.728 ^{Aa}	30.400 $\pm$ 5.745 ^{Ba}
	So + aPDT	24.733 $\pm$ 2.100 ^{Aa}	37.500 $\pm$ 6.572 ^{Ba}
Apl#10	aPDT	19.766 $\pm$ 1.869 ^{Aa}	30.766 $\pm$ 8.125 ^{Ba}
	Dn + aPDT	22.600 $\pm$ 2.417 ^{Aa}	35.633 $\pm$ 4.944 ^{Ba}
	So + aPDT	20.067 $\pm$ 2.822 ^{Aa}	34.466 $\pm$ 7.025 ^{Ba}
	Dn + So + aPDT	23.366 $\pm$ 2.930 ^{Aa}	36.866 $\pm$ 4.497 ^{Ba}

Table 3

Means and standard deviations of insoluble weight (mg/well) of biofilms evaluated after Apl#1, Apl#5, and Apl#10. aPDT with biofilm disruption: the biofilms were treated with biofilm disruption strategies followed by aPDT (Dn+aPDT; So+aPDT and So+aPDT group). Biofilm disruption only: the biofilms were treated only with the strategies (Dn+P-L-, So+P-L- and Dn+So+P-L-). Capital letters correspond to the comparison between groups (rows), and lowercase letters correspond to the comparison between treatment conditions (columns). Unequal letters indicate a significant statistical difference ($p < 0.05$) in the Tukey or Games-Howell post-test ($\alpha = 0.05$).

Application	Treatment Conditions	Group	
		aPDT with biofilm disruption	Biofilm disruption only
Apl#1	aPDT	1.350 $\pm$ 0.978 ^{Ab}	1.382 $\pm$ 0.217 ^{Aa}
	Dn + aPDT	1.484 $\pm$ 0.350 ^{Ab}	1.515 $\pm$ 0.258 ^{Aa}
	So + aPDT	0.900 $\pm$ 0.702 ^{Aa}	1.038 $\pm$ 0.290 ^{Aa}
	Dn + So + aPDT	1.529 $\pm$ 0.715 ^{Ab}	1.588 $\pm$ 0.511 ^{Aa}
Apl#5	aPDT	1.312 $\pm$ 0.495 ^{Aa}	1.284 $\pm$ 0.477 ^{Aa}
	Dn + aPDT	1.347 $\pm$ 0.470 ^{Aa}	1.415 $\pm$ 1.101 ^{Aa}
	So + aPDT	1.136 $\pm$ 0.762 ^{Aa}	1.055 $\pm$ 0.605 ^{Aa}
	Dn + So + aPDT	1.170 $\pm$ 0.715 ^{Aa}	1.550 $\pm$ 0.521 ^{Ba}
Apl#10	aPDT	1.144 $\pm$ 0.456 ^{Aa}	1.268 $\pm$ 0.339 ^{Aa}
	Dn + aPDT	1.433 $\pm$ 0.223 ^{Aa}	1.440 $\pm$ 0.164 ^{Aa}
	So + aPDT	1.092 $\pm$ 0.159 ^{Aa}	1.366 $\pm$ 0.178 ^{Ba}
	Dn + So + aPDT	1.080 $\pm$ 0.333 ^{Aa}	1.590 $\pm$ 0.579 ^{Ba}

compared to its respective control. After Apl#5, So+aPDT, and So+Dn+aPDT conditions showed a significant reduction ($p \leq 0.002$) in ASP values in the experimental groups compared to their respective control groups, with no significant differences among them ($p \geq 0.066$; Table 7). For Apl#10, all treatment conditions showed a significant reduction ($p \leq 0.003$) in ASP levels in the experimental groups compared to their respective controls (Table 7).

3.8. Extracellular DNA (eDNA)

After Apl#1, Apl#5, and Apl#10, in all treatment conditions evaluated (aPDT, So+aPDT, Dn+aPDT, and So+Dn+aPDT), a statistically significant reduction ($p \leq 0.001$) in eDNA levels was observed in the experimental groups compared to their respective controls (Table 8).

Table 4

Means and standard deviations of total protein levels ($\mu\text{g/well}$) of biofilms evaluated after Apl#1, Apl#5, and Apl#10. aPDT with biofilm disruption: the biofilms were treated with biofilm disruption strategies followed by aPDT (Dn+aPDT; So+aPDT and So+aPDT group). Biofilm disruption only: the biofilms were treated only with the strategies (Dn+P-L-, So+P-L- and Dn+So+P-L-). Capital letters correspond to the comparison between groups (rows), and lowercase letters correspond to the comparison between treatment conditions (columns). Unequal letters indicate a significant statistical difference ($p < 0.05$) in Tukey or Games-Howell post-test ($\alpha = 0.05$).

Application	Treatment Condition	Group	
		aPDT with biofilm disruption	Biofilm disruption only
Apl#1	aPDT	0.030 $\pm$ 0.007 ^{Aa}	0.045 $\pm$ 0.006 ^{Aa}
	Dn + aPDT	0.024 $\pm$ 0.023 ^{Aa}	0.044 $\pm$ 0.006 ^{Aa}
	So + aPDT	0.020 $\pm$ 0.003 ^{Aa}	0.040 $\pm$ 0.034 ^{Aa}
	Dn + So + aPDT	0.022 $\pm$ 0.021 ^{Aa}	0.051 $\pm$ 0.036 ^{Aa}
Apl#5	aPDT	0.028 $\pm$ 0.005 ^{Aa}	0.046 $\pm$ 0.007 ^{Bab}
	Dn + aPDT	0.020 $\pm$ 0.004 ^{Aa}	0.039 $\pm$ 0.007 ^{Ba}
	So + aPDT	0.021 $\pm$ 0.004 ^{Aa}	0.042 $\pm$ 0.009 ^{Bab}
	Dn + So + aPDT	0.029 $\pm$ 0.005 ^{Aa}	0.049 $\pm$ 0.006 ^{Bb}
Apl#10	aPDT	0.023 $\pm$ 0.004 ^{Aa}	0.055 $\pm$ 0.009 ^{Ba}
	Dn + aPDT	0.021 $\pm$ 0.006 ^{Aa}	0.050 $\pm$ 0.008 ^{Bab}
	So + aPDT	0.022 $\pm$ 0.004 ^{Aa}	0.045 $\pm$ 0.004 ^{Bb}
	Dn + So + aPDT	0.024 $\pm$ 0.003 ^{Aa}	0.032 $\pm$ 0.004 ^{Bc}

Table 5

Means and standard deviations of insoluble protein levels ($\mu\text{g/well}$) of biofilms evaluated after Apl#1, Apl#5, and Apl#10. aPDT with biofilm disruption: the biofilms were treated with biofilm disruption strategies followed by aPDT (Dn+aPDT; So+aPDT and So+aPDT group). Biofilm disruption only: the biofilms were treated only with the strategies (Dn+P-L-, So+P-L- and Dn+So+P-L-). Capital letters correspond to the comparison between groups (rows), and lowercase letters correspond to the comparison between treatment conditions (columns). Unequal letters indicate a significant statistical difference ($p < 0.05$) in Tukey or Games-Howell post-test ($\alpha = 0.05$).

Application	Treatment Condition	Group	
		aPDT with biofilm disruption	Biofilm disruption only
Apl#1	aPDT	0.020 $\pm$ 0.003 ^{Ab}	0.043 $\pm$ 0.005 ^{Ba}
	Dn + aPDT	0.026 $\pm$ 0.004 ^{Aa}	0.031 $\pm$ 0.006 ^{Ab}
	So + aPDT	0.027 $\pm$ 0.004 ^{Aa}	0.033 $\pm$ 0.008 ^{Bb}
	Dn + So + aPDT	0.024 $\pm$ 0.005 ^{Ab}	0.042 $\pm$ 0.008 ^{Ba}
Apl#5	aPDT	0.019 $\pm$ 0.008 ^{Aa}	0.030 $\pm$ 0.007 ^{Ba}
	Dn + aPDT	0.023 $\pm$ 0.003 ^{Ab}	0.027 $\pm$ 0.004 ^{Aa}
	So + aPDT	0.028 $\pm$ 0.006 ^{Ab}	0.033 $\pm$ 0.008 ^{Aa}
	Dn + So + aPDT	0.024 $\pm$ 0.005 ^{Ab}	0.028 $\pm$ 0.005 ^{Aa}
Apl#10	aPDT	0.020 $\pm$ 0.004 ^{Aa}	0.031 $\pm$ 0.006 ^{Ba}
	Dn + aPDT	0.023 $\pm$ 0.005 ^{Aa}	0.028 $\pm$ 0.002 ^{Ba}
	So + aPDT	0.022 $\pm$ 0.005 ^{Aa}	0.031 $\pm$ 0.006 ^{Ba}
	Dn + So + aPDT	0.022 $\pm$ 0.006 ^{Aa}	0.029 $\pm$ 0.004 ^{Ba}

3.9. Scanning electron microscopy (SEM)

At 500 $\times$ magnification (a, d, g, j) and 1000 $\times$ magnification (b, e, h, k), a higher prevalence of *C. albicans* cells can be observed in the group without treatment (P-L- group) (d, e, j, k) compared to the group submitted to the photodynamic treatments (P+L+ groups) (a, b, g, h). At 4000 $\times$ magnification (c, f, i, l), the presence of deformities in the cell walls is noted in the P+L+ groups (c, i; red arrows), which is not observed in the P-L- groups (f, l) (Fig. 5). In P-L- group (f), it is possible to observe ECM surrounding the cells.

The groups subjected to Apl#10 of aPDT (g, h, i) show lower amounts of *C. albicans* cells, as well as a higher prevalence of cell membrane deformities (red arrows) when compared to the groups subjected to Apl#1 of aPDT (a, b, c) (Fig. 5).

At 500 $\times$ magnification (a, d, g, j) and 1000 $\times$ magnification (b, e, h, k), a higher prevalence of *C. albicans* can be observed in the Dn+P-L-

Table 6

Means and standard deviations of WSP levels ($\mu\text{g}/\text{well}$) of biofilms evaluated after Apl#1, Apl#5, and Apl#10. aPDT with biofilm disruption: the biofilms were treated with biofilm disruption strategies followed by aPDT (Dn+aPDT; So+aPDT and So+aPDT group). Biofilm disruption only: the biofilms were treated only with the strategies (Dn+P-L-, So+P-L- and Dn+So+P-L-). Capital letters correspond to the comparison between groups (rows), and lowercase letters correspond to the comparison between treatment conditions (columns). Unequal letters indicate a significant statistical difference ($p < 0.05$) in the Tukey or Games-Howell post-test ($\alpha = 0.05$).

Application	Treatment Condition	Group	
		aPDT with biofilm disruption	Biofilm disruption only
Apl#1	aPDT	0.030 $\pm$ 0.005 ^{Aa}	0.077 $\pm$ 0.005 ^{Ba}
	Dn + aPDT	0.015 $\pm$ 0.003 ^{Ab}	0.021 $\pm$ 0.002 ^{Ac}
	So + aPDT	0.018 $\pm$ 0.004 ^{Ab}	0.060 $\pm$ 0.006 ^{Bb}
	Dn + So + aPDT	0.015 $\pm$ 0.003 ^{Ab}	0.019 $\pm$ 0.002 ^{Ac}
Apl#5	aPDT	0.026 $\pm$ 0.003 ^{Aa}	0.073 $\pm$ 0.006 ^{Ba}
	Dn + aPDT	0.018 $\pm$ 0.004 ^{Aab}	0.024 $\pm$ 0.007 ^{Ab}
	So + aPDT	0.016 $\pm$ 0.003 ^{Ab}	0.025 $\pm$ 0.010 ^{Bb}
	Dn + So + aPDT	0.016 $\pm$ 0.002 ^{Ab}	0.026 $\pm$ 0.002 ^{Bb}
Apl#10	aPDT	0.025 $\pm$ 0.002 ^{Aa}	0.074 $\pm$ 0.004 ^{Ba}
	Dn + aPDT	0.019 $\pm$ 0.003 ^{Aa}	0.027 $\pm$ 0.003 ^{Ab}
	So + aPDT	0.018 $\pm$ 0.006 ^{Aa}	0.032 $\pm$ 0.004 ^{Bb}
	Dn + So + aPDT	0.020 $\pm$ 0.003 ^{Aa}	0.028 $\pm$ 0.004 ^{Ab}

Table 7

Means and standard deviations of ASP levels ($\mu\text{g}/\text{well}$) of biofilms evaluated after Apl#1, Apl#5, and Apl#10. aPDT with biofilm disruption: the biofilms were treated with biofilm disruption strategies followed by aPDT (Dn+aPDT; So+aPDT and So+aPDT group). Biofilm disruption only: the biofilms were treated only with the strategies (Dn+P-L-, So+P-L- and Dn+So+P-L-). Capital letters correspond to the comparison between groups (rows), and lowercase letters correspond to the comparison between treatment conditions (columns). Unequal letters indicate a significant statistical difference ($p < 0.05$) in the Tukey or Games-Howell post-test ($\alpha = 0.05$).

Application	Treatment Condition	Group	
		aPDT with biofilm disruption	Biofilm disruption only
Apl#1	aPDT	0.059 $\pm$ 0.005 ^{Aa}	0.079 $\pm$ 0.006 ^{Ba}
	Dn + aPDT	0.056 $\pm$ 0.008 ^{Aa}	0.061 $\pm$ 0.007 ^{Ab}
	So + aPDT	0.056 $\pm$ 0.003 ^{Aa}	0.061 $\pm$ 0.009 ^{Ab}
	Dn + So + aPDT	0.058 $\pm$ 0.005 ^{Aa}	0.061 $\pm$ 0.003 ^{Ab}
Apl#5	aPDT	0.061 $\pm$ 0.001 ^{Aa}	0.077 $\pm$ 0.004 ^{Ba}
	Dn + aPDT	0.058 $\pm$ 0.003 ^{Aa}	0.063 $\pm$ 0.003 ^{Ab}
	So + aPDT	0.056 $\pm$ 0.004 ^{Aa}	0.066 $\pm$ 0.003 ^{Bb}
	Dn + So + aPDT	0.054 $\pm$ 0.003 ^{Aa}	0.074 $\pm$ 0.006 ^{Ba}
Apl#10	aPDT	0.060 $\pm$ 0.003 ^{Aa}	0.078 $\pm$ 0.002 ^{Ba}
	Dn + aPDT	0.054 $\pm$ 0.003 ^{Aab}	0.065 $\pm$ 0.005 ^{Bb}
	So + aPDT	0.058 $\pm$ 0.004 ^{Aab}	0.075 $\pm$ 0.004 ^{Ba}
	Dn + So + aPDT	0.053 $\pm$ 0.002 ^{Ab}	0.061 $\pm$ 0.002 ^{Bb}

groups (d, e, j, k) compared to the Dn+P+L+ groups (a, b, g, h). At 4000 $\times$ magnification (c, f, i, l), the presence of deformities in the cell walls is noted after the Apl#1 in the Dn+P+L+ group (c, red arrows), which is not observed in the Dn+P-L- groups (f, l). A lower presence of cell clusters is also observed after the Apl#10 of the Dn+P+L+ group (i, yellow arrows), compared to the Dn+P-L- group (l, yellow arrow) (Fig. 6).

When compared to the groups subjected to Apl#1 of aPDT (a, b, c, d, e, f), the groups subjected to Apl#10 (g, h, i, j, k, l) show lower amounts of *C. albicans* cells, as well as a lower presence of cell clusters (yellow arrows) (Fig. 6).

At 500 $\times$ magnification (a, d, g, j) and 1000 $\times$ magnification (b, e, h, k), a higher prevalence of *C. albicans* can be observed in the So+P-L- groups (d, e, j, k) compared to the So+P+L+ groups (a, b, g, h). At 4000 $\times$ magnification (c, f, i, l), the presence of deformities in the cell walls is noted in the Apl#1 of the So+P+L+ group (c; red arrows), along

Table 8

Means and standard deviations of eDNA levels (ng/well) of biofilms evaluated after Apl#1, Apl#5, and Apl#10. aPDT with biofilm disruption: the biofilms were treated with biofilm disruption strategies followed by aPDT (Dn+aPDT; So+aPDT and So+aPDT group). Biofilm disruption only: the biofilms were treated only with the strategies (Dn+P-L-, So+P-L- and Dn+So+P-L-). Capital letters correspond to the comparison between groups (rows), and lowercase letters correspond to the comparison between treatment conditions (columns). Unequal letters indicate a significant statistical difference ($p < 0.05$) in the Tukey or Games-Howell post-test ($\alpha = 0.05$).

Application	Treatment Condition	Group	
		aPDT with biofilm disruption	Biofilm disruption only
Apl#1	aPDT	21.670 $\pm$ 2.098 ^{Aa}	60.521 $\pm$ 1.526 ^{Ba}
	Dn + aPDT	9.250 $\pm$ 2.107 ^{Ab}	51.420 $\pm$ 4.064 ^{Bb}
	So + aPDT	20.083 $\pm$ 2.810 ^{Aa}	54.772 $\pm$ 4.727 ^{Bb}
	Dn + So + aPDT	12.735 $\pm$ 1.698 ^{Ab}	45.763 $\pm$ 3.760 ^{Bc}
Apl#5	aPDT	16.541 $\pm$ 1.990 ^{Aa}	65.442 $\pm$ 5.982 ^{Ba}
	Dn + aPDT	14.732 $\pm$ 0.910 ^{Aa}	52.638 $\pm$ 3.333 ^{Bb}
	So + aPDT	18.345 $\pm$ 4.423 ^{Aa}	60.590 $\pm$ 4.689 ^{Ba}
	Dn + So + aPDT	16.747 $\pm$ 1.540 ^{Aa}	52.365 $\pm$ 3.013 ^{Bb}
Apl#10	aPDT	11.681 $\pm$ 1.256 ^{Aa}	65.168 $\pm$ 3.505 ^{Ba}
	Dn + aPDT	9.563 $\pm$ 1.117 ^{Aa}	53.297 $\pm$ 1.221 ^{Bb}
	So + aPDT	11.468 $\pm$ 4.228 ^{Aa}	57.995 $\pm$ 7.803 ^{Ba}
	Dn + So + aPDT	10.144 $\pm$ 1.851 ^{Aa}	50.070 $\pm$ 2.482 ^{Bb}

with a lower presence of cell clusters (c; yellow arrow), compared to the So+P-L- group (f; yellow arrow). Deformities in the cell walls are also observed in the Apl#10 of the So+P+L+ group (i; red arrow), which are not seen in the So+P-L- group (Fig. 7).

When compared to the groups subjected to ultrasound followed by a single application (Apl#1) of aPDT treatment (So+P+L+ group; a, b, c, d, e, f), the groups that received multiple applications (Apl#10) (So+P+L+ group; g, h, i, j) showed a slight reduction in *C. albicans* cell quantity (Fig. 7).

At 500 $\times$ magnification (a, d, g, j) and 1000 $\times$ magnification (b, e, h, k), a superior prevalence of *C. albicans* can be observed in the Dn+So+P-L- groups (d, e, j, k) compared to the Dn+So+P+L+ groups (a, b, g, h) (Fig. 8). At 4000 $\times$ magnification (c, f, i, l), the presence of deformities in the cell walls is noted in the Dn+So+P+L+ groups (c, i; red arrows), along with a lower presence of cell clusters (c, i; yellow arrows), compared to the Dn+So+P-L- groups (f, l; yellow arrows) (Fig. 8).

When compared to the groups subjected to Dnase+ultrasound followed by a single application of aPDT (Apl#1) (Dn+So+P+L+ group; a, b, c), the groups that received multiple applications (Apl#10) (So+P+L+ group; g, h, i, j) showed a reduction in *C. albicans* cell quantity (Fig. 8).

4. Discussion

Candida infections are intimately linked to biofilm formation, which consists of large numbers of cells arranged in a community within an ECM, attached to surfaces [43]. The ECM protects microbial cells in biofilm by shielding cells from immune responses and reducing anti-fungal agent penetration, which makes infections more difficult to treat [44]. This study is the pioneer to combine and evaluate chemical (DNase), mechanical (sonication), and combined (DNase + sonication) strategies to disrupt *C. albicans* biofilms, focusing on the improvement of the penetration of the PS and light, to enhance the effectiveness of photodynamic treatment. The results showed that these treatments effectively weakened the biofilm structure, as evidenced by reductions in cell viability and its ECM components, with morphological alterations observed by SEM.

According to results of viability by CFU/mL, after successive applications of aPDT, the group of aPDT (P+L+) demonstrated a progressive reduction in *C. albicans* counts, with a decrease of 3.5 log₁₀ after the Apl#5, reaching 5.9 log₁₀ of reduction after the Apl#10. The Apl#10

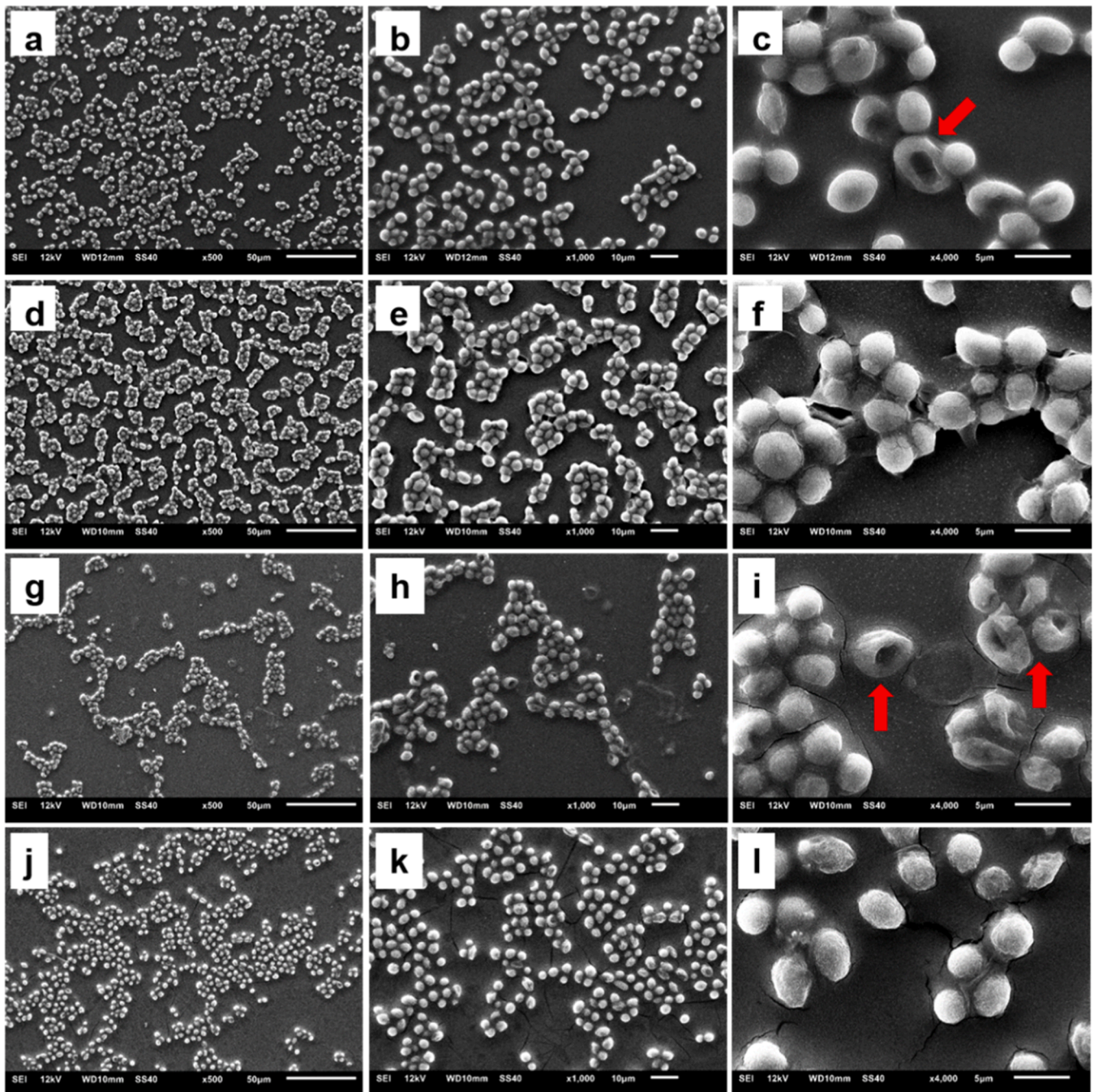


Fig. 5. SEM images of *C. albicans* biofilms referring to aPDT condition after Apl#1 and Apl#10. Biofilms from P+L+ group after Apl#1 (a, b, c), P+L+ group after Apl#10 (g, h, i), P-L- group (d, e, f - Control of Apl#1 biofilms), and P-L- group (j, k, l Control of Apl#10 biofilms). Red arrows indicate deformities in the cell wall.

demonstrated statistical differences from the initial five applications, demonstrating that the cumulative effect of multiple aPDT treatments is crucial for achieving greater biofilm reduction. Multiple aPDT sessions weakening the biofilm structure progressively and increasing the exposure of microbial cells to the PS and light; this process increases the cumulative intracellular oxidative stress over time [14]. As a result, the intracellular oxidative stress gradually reduces cell integrity, making *C. albicans* more susceptible to subsequent treatments and potentially avoiding the biofilm reestablishment [14,23].

The combined approach of DNase with aPDT (Dn+P+L+) progressively reduced *C. albicans* viability and demonstrates the additive impact of DNase when combined with aPDT. This effect is attributed to DNase's ability to break down ECM components, allowing the PS to penetrate

and promote cell inactivation upon LED light exposure [45]. These results corroborate with previous studies in which successive applications of aPDT resulted in a progressively enhanced antimicrobial impact [39, 46].

The combination of sonication with aPDT (So+P+L+ group) produced a progressive decline in fungal cell viability in biofilms, which aligns with the results of DNase treatment. The So+P+L+ group significantly reduced colony count, reaching 5.2 log₁₀ after the Apl#10. This result suggests that the mechanical disruption of the biofilm caused by sonication reduced its density and enhanced the permeability to both the PS and light. The possible mechanism behind this effect is the generation of mechanical vibrations, which may disaggregate biofilm clusters and loosen the ECM components [37]. As consequence, drug

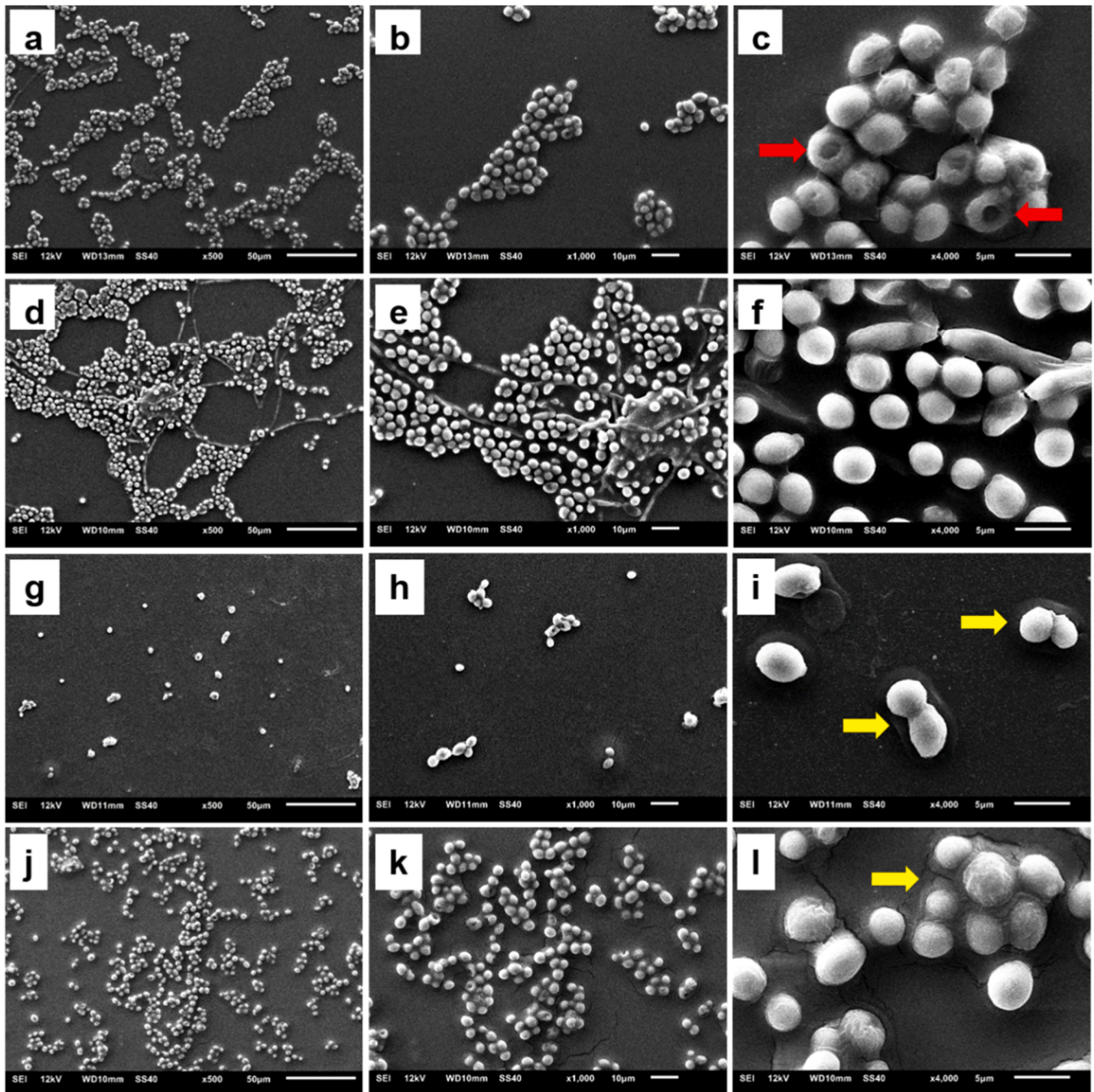


Fig. 6. SEM images of *C. albicans* biofilms referring to Dn+aPDT condition after Apl#1 and Apl#10. Biofilms from Dn+P+L+ group after Apl#1 (a, b, c), DNP+L+ group after Apl#10 (g, h, i), Dn+P-L- group (d, e, f; Control of Apl#1 biofilms), and Dn+P-L- group (j, k, l; Control of Apl#10 biofilms). Red arrows indicate deformities in the cell wall. Yellow arrows indicate cell clusters.

penetration is enhanced, allowing more uniform distribution of the PS throughout the biofilm. Sonication weakens fungal cells by enhancing membrane permeability and increasing *C. albicans* susceptibility to oxidative stress induced by aPDT [34]. Studies demonstrate that mechanical disruption leads to cell wall irregularities, which consequently can increase antimicrobial susceptibility [34]. This effect, along with the increased production of ROS by aPDT, likely contributes to the substantial reduction of *C. albicans* cells' viability in biofilm.

In the Dn+So+P+L+ group, the fungal cell viability in biofilms was progressively reduced, reaching a total of 5.3 log₁₀ after the Apl#10. The repeated applications of aPDT after DNase and sonication, effectively weakened the biofilm structure, making it more porous to facilitate

diffusion and penetration of molecules (PS) and light. Previous studies have already demonstrated that mechanical disruption strategies, such as ultrasound, enhance the effectiveness of antimicrobial agents by promoting reductions in microbial viability across different biofilm cultures [37,47]. The present study differs from previous ones in that it applies sonication to enhance enzymatic activity, where its mechanical impact probably facilitated DNase access to deeper layers of the ECM, resulting in increased biofilm degradation. Here, the generation of microstreaming and shear forces probably disrupted the biofilm architecture, promoting detachment and dispersion of both the biofilm and its matrix, thereby enhancing the susceptibility of fungal cells in biofilms and the biofilm per se to external agents.

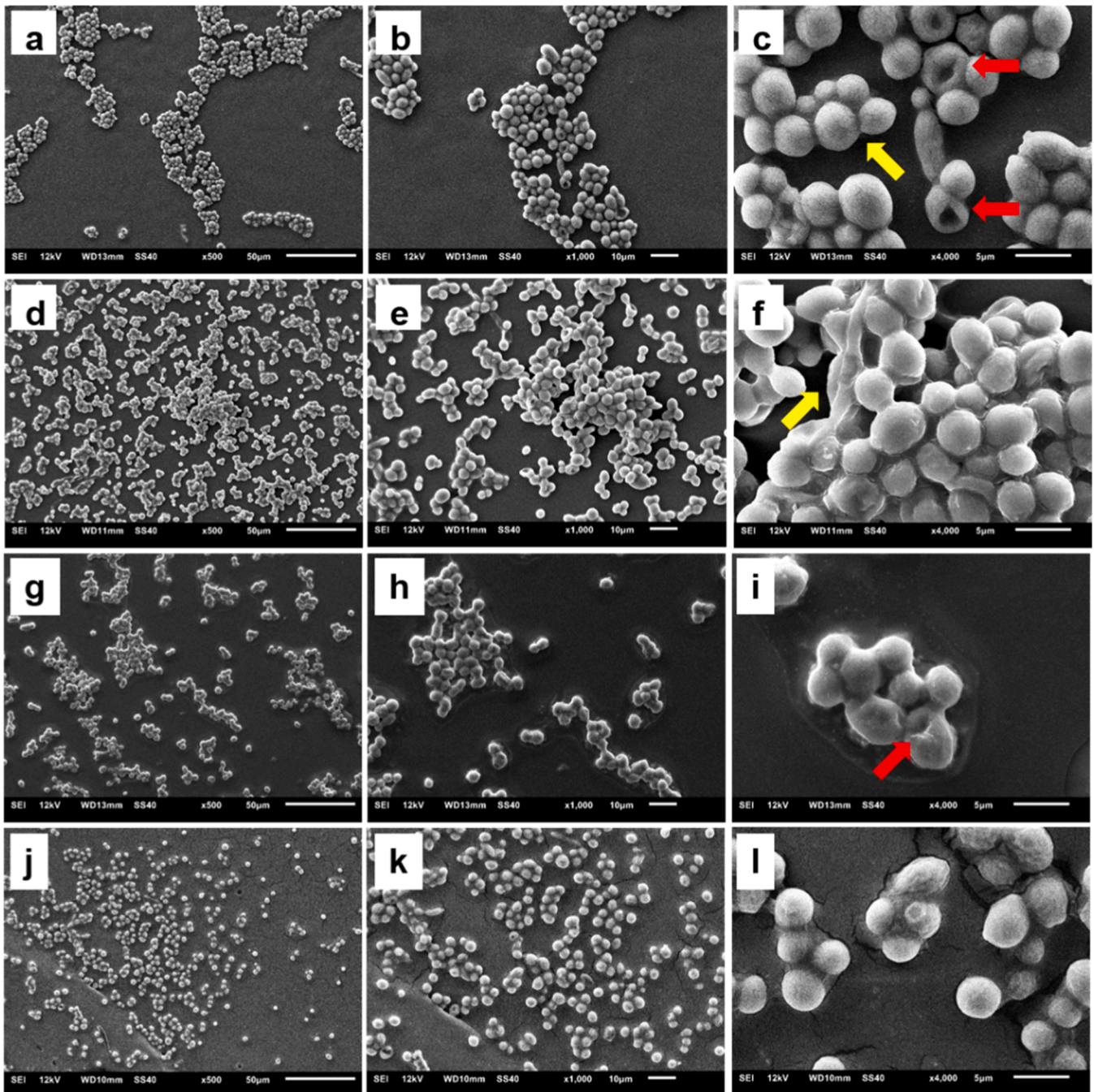


Fig. 7. SEM images of *C. albicans* biofilms referring So+aPDT condition after Apl#1 and Apl#10. Biofilms from So+P+L+ group after Apl#1 1 (a, b, c); So+P+L+ group after Apl#10 (g, h, i), So+P-L- group (d, e, f; Control of Apl#1 biofilms), and So+P-L- group after Apl#10 (j, k, l; Control of Apl#10 biofilms). Red arrows indicate deformities in the cell wall. Yellow arrows indicate cell clusters.

The ECM consists of glycoproteins, carbohydrates, lipids, and nucleic acids, which involve the cells, protecting them from host immunity or environmental factors, and antifungal treatments [48]. The total dry weight in biofilms consists of microbial cells and ECM components [44]. The significant reduction in total dry weight observed in this study across multiple treatment conditions (Apl#1 for So+aPDT, Dn+aPDT, and So+Dn+aPDT; Apl#5 and Apl#10 for all treatments conditions) reinforces the efficacy of DNase and sonication in disrupting biofilm structure. The biofilm disruption likely results from the association of these three treatments working together synergistically. The results of this study suggest that the application of DNase and sonication enhanced microbial cell permeability, thereby increasing the exposure to oxidative

damage from ROS produced during aPDT and making them more susceptible to ECM degradation.

Biofilm's total protein represents the overall protein content comprising soluble (present in the matrix) and insoluble (present in the matrix and from the fungal cells *per se*) fractions. In contrast, soluble protein refers to the structural proteins within the ECM that contribute to biofilm stability and exoenzymes secreted by fungal cells to exert their effect in the extracellular milieu [49]. The lack of a significant reduction in total protein levels after Apl#1 in all evaluated conditions demonstrates that a single application was insufficient to decrease a substantial portion of the biofilm's protein structure, also aligning with a previous study [31]. Due to its protective nature, this outcome can be attributed

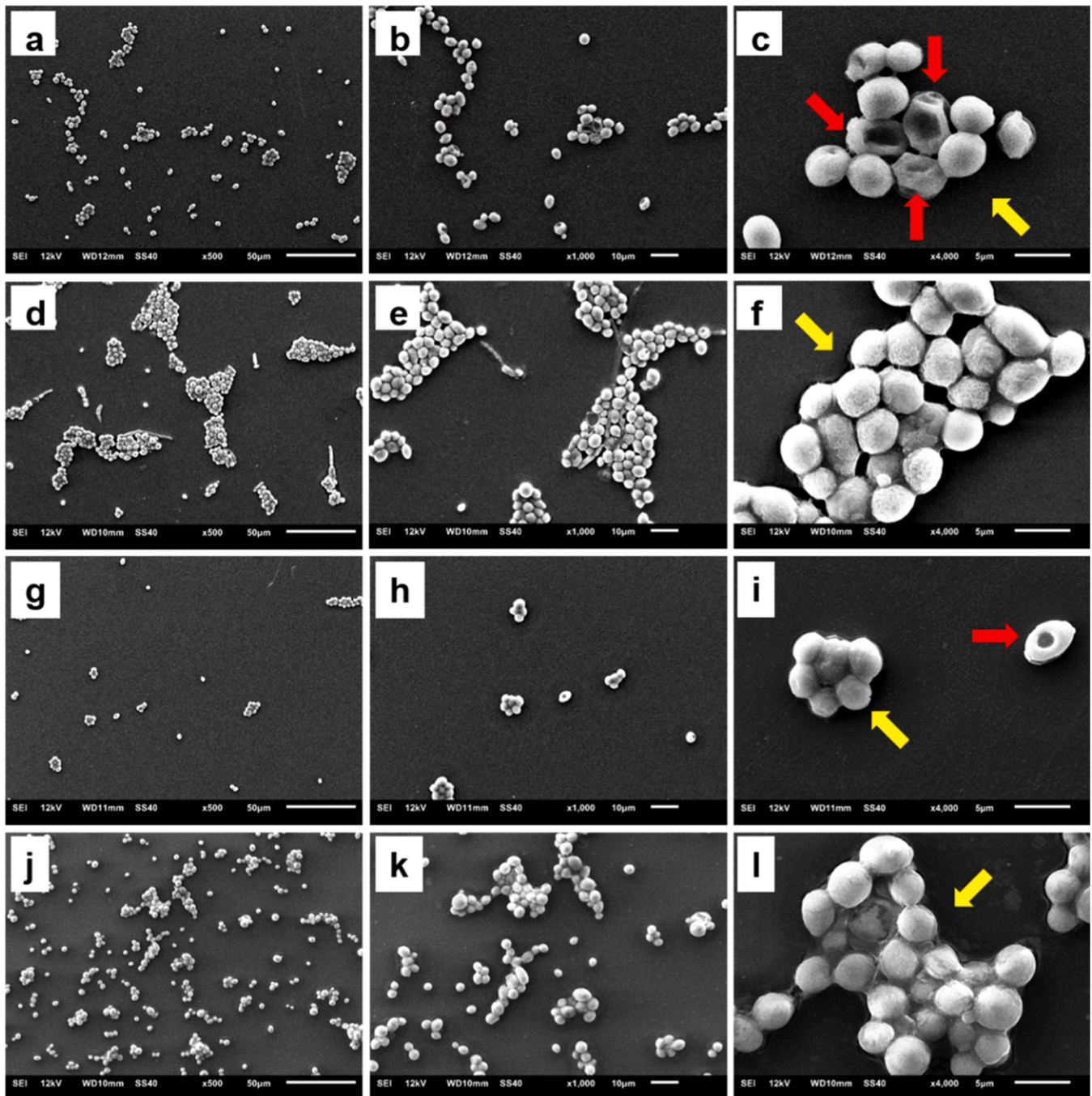


Fig. 8. SEM images of *C. albicans* biofilms referring Dn+So+aPDT condition after Apl#1 and Apl#10. Biofilms from Dn+So+P+L+ group after Apl#1 (a, b, c), Dn+So+P+L+ group after Apl#10 (g, h, i), Dn+So+P-L- group (Control of Apl#1 biofilms), and, Dn+So+P-L- group (Control of Apl#10 biofilms). Red arrows indicate deformities in the cell wall. Yellow arrows indicate cell clusters.

to the ECM's ability to shield proteins from enzymatic and oxidative degradation during the early stages [49]. On the other hand, throughout Apl#1 to Apl#10 cycles, the data revealed significant decreases in total protein levels, indicating that repeated exposures to aPDT, DNase, and sonication synergistically damage the ECM and promote the breakdown of key proteins, besides other structural matrix components. The progressive reduction in protein levels over time suggests that the combinations of treatments with aPDT are necessary to achieve a distinguished impact on microbial cells and their ECM composition. This outcome aligns with findings from biofilm eradication studies, emphasizing that single treatments may fail to induce significant matrix degradation, requiring sequential or combination therapies for optimal efficacy [50].

The concept of WSP for the ECM consists of loosely bound exopolysaccharides, while ASP represents structural polysaccharides tightly associated with biofilm stability [44]. After multiple applications, this study showed a reduction in both exopolysaccharides, being the most significant for WSP in So+aPDT and Dn+So+aPDT groups. Considering WSP as a specific component contributing to ECM structure and function, its degradation increases ECM porosity and allows antimicrobial agents to penetrate more deeply into the biofilm layers [51]. Then, the result of present study suggests that the enzyme facilitated the penetration by initially targeting eDNA entangled to WSP, which, as it detangled, subsequently promoted the breakdown of ECM and enabled the accumulation of DNase within the biofilm structure over successive

applications, thereby enhancing PDT's penetration. In contrast to previous investigations in which a single DNase application before aPDT did not alter ASP levels, our findings demonstrate that successive treatments significantly enhanced ASP degradation, particularly when DNase was combined with mechanical sonication [30]. These results indicate that, despite its structural robustness, ASP can be disrupted by repeated applications, with the most pronounced reduction observed when mechanical sonication was combined with DNase treatment.

It has been suggested that in the biofilm ECM, eDNA can become entrapped and bind to polysaccharides of the MGCx complex [52]. Our findings support this once DNase combined with aPDT simultaneously reduced WSP and eDNA concentrations in the ECM. A previous study observed that aPDT effectively reduced the amount of WSP in the ECM and that the combination with DNase enhanced the reduction of WSP [42]. DNase hydrolyzes eDNA chains and interferes with the organization of WSP and eDNA, reducing the amount of these polysaccharides to which eDNA molecules are attached [31]. In this way, the results of this study are consistent with a previous investigation in which a 5-minute DNase pretreatment followed by aPDT also significantly reduced eDNA content in *C. albicans* biofilms [30]. This reduction in eDNA content can be considered an essential factor in ECM weakening, as eDNA is linked to ECM integrity and biofilm resistance to conventional antifungal agents [29].

In our study, biofilms subjected to DNase treatment (Dn+P+L+ group), sonication (SO+P+L+ group), or a combination of both (Dn+So+ P+L+ group) before aPDT exhibited greater structural biofilm disruption compared to the group treated with aPDT (P+L+). The presented SEM images demonstrated the appearance of deformities in the cell walls, indicating that the disruption and weakening strategies were effective. Additionally, a reduction in the number of cells and cell clusters was also observed, demonstrating an improvement in the effectiveness of the aPDT treatment. Structural damage with deformities in cell walls and reduced ECM components were identified, and it was proved that aPDT could increase the treatment susceptibility [53]. The present study demonstrated that applying sonication prior to aPDT enhanced ECM disruption, leading to greater reductions in cell clusters and structural deformities compared to the groups without mechanical disruption. Moreover, SEM observations corroborate the colony count results, indicating that mechanical and enzymatic destabilization of the ECM can significantly enhance aPDT efficacy against *C. albicans* biofilms.

5. Conclusion and future directions

Combining application of enzymatic (DNase) and mechanical disruption (sonication) significantly enhances the efficacy of aPDT against *C. albicans* biofilms. Repeated treatments promoted a progressive reduction in fungal cell viability and biofilm structural integrity, likely resulting from increased ECM permeability and enhanced photosensitizer penetration. Notably, the synergistic interaction between DNase and sonication was particularly effective in promoting ECM breakdown and increasing microbial susceptibility to oxidative damage induced by aPDT. These results underscore the potential of sequential and combined therapeutic approaches in overcoming the limitations of conventional treatments, offering a promising alternative for managing persistent fungal biofilms. Clinically, this protocol could be translated to decontamination using a brief DNase rinse and low-frequency ultrasound, followed by short aPDT cycles for treating denture stomatitis or oral candidiasis. Future studies should optimize dosing/delivery formats (rinse/gel, ultrasonic mouthpiece or tray) and evaluate safety and efficacy in randomized clinical trials.

Ethical approval

Not applicable.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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