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Environmental pH influences *Candida albicans* biofilms regarding its structure, virulence and susceptibility to fluconazole

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ABSTRACT

Candida albicans colonizes sites with different environmental pH. However, it is unclear how these conditions can interfere on biofilms. This study aimed to evaluate the influence of environmental pH on behavior of *C. albicans* regarding its structure, virulence and susceptibility to fluconazole (FLZ). Minimal inhibitory concentration, minimal fungicidal concentration and time kill were used to evaluate the susceptibility to FLZ in planktonic cells under three pH values (4.0, 5.5, 7.0). These pH values were used for biofilms analysis. *C. albicans* ATCC 90028 was developed on poly(-methlymethacrylate) resin for 48 h. Then, 2.56 μg/mL of FLZ was added to experimental groups for 24 h, and biofilms were analyzed by cell quantification, bioactivity, secretion of proteinases and phospholipases and structure. All data were analyzed by two-way ANOVA, followed by Tukey's test ($\alpha = 0.05$). For planktonic cells, changes in environmental pH decreased the susceptibility to FLZ. *C. albicans* biofilms developed at pH 5.5 showed higher cell counts, bioactivity, bio-volume, average thickness and roughness coefficient ($p < 0.05$). In contrast, the presence of FLZ at pH 4.0 did not influence the structural parameters ($p > 0.05$), but increased secretion of proteinase and phospholipase ($p < 0.05$). Within the conditions studied, it was shown that environmental pH modulates the structure, virulence and susceptibility of *C. albicans* to FLZ.

1. Introduction

Candida albicans colonizes sites in humans with different environmental pH values, such as gastrointestinal tract (pH 2.0–7.7), vaginal mucosa (pH ~4.0) and blood (pH 7.4) [1]. In the oral cavity, the pH is approximately 7.0 [2], although temporary changes in environmental pH can be observed at specific sites, as under maxillary dentures [3,4]. It is well established that when *C. albicans* colonizes the upper denture surface, an acidic environment is recognized, which may contribute to the development of *Candida*-associated denture stomatitis (CADS) [3]. The treatment of CADS infections has been greatly facilitated with azole antifungal agents, in particular by the fluconazole (FLZ) [5].

In the pathogenesis of CADS, the colonization on the fitting surface of the denture by *C. albicans* and the following acid production can activate virulence attributes increasing the pathogenic potential of such fungal [4]. Acidic environments increase the secretion of proteinase and phospholipases [6–8], and such enzymes contribute to invasion of host tissues [9]. The pH is also a potent regulator of the transition of yeast into filamentous hyphae [10], and an acidic environment favors yeast growth, while an alkaline favors hyphae growth [11,12].

The environmental pH may lead to marked changes in fungal cell wall structures, which may influence the susceptibility of *C. albicans* biofilm to FLZ [13–15]. Therefore, different pH values may have an effect on cellular morphology that is involved in drug resistance [14,16]. Considering that is still unclear how different pH conditions can interfere on the parameters of *C. albicans* development, this study investigated the influence of environmental pH on the behavior of *C. albicans* regarding its structure, virulence and susceptibility to FLZ.

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2. Materials and methods

2.1. Experimental design

This *in vitro* study had a randomized and blinded design. The antifungal susceptibility to FLZ was performed for *C. albicans* planktonic cells and biofilms under three different environmental pH conditions (4.0, 5.5 and 7.0). The susceptibility of *C. albicans* planktonic cells to FLZ was evaluated by minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and time-kill tests. For biofilms analyses, *C. albicans* was allowed to colonize the poly (methylmethacrylate) (PMMA) resin disc surface for 48 h. Then, FLZ at 2.56 μg/mL was added to the culture medium for 24 h until 72 h of biofilm growth. Culture medium without FLZ was used as control. The cell quantification, metabolic activity, enzymatic activity and structure were analyzed after the 72 h of biofilm development. All of the experiments were performed in triplicate of three independent experiments on different days ($n = 9$). Data were analyzed by two-way ANOVA followed by Tukey's test at 5% significance level.

2.2. Fabrication of PMMA specimens

The specimens were fabricated using a water bath PMMA acrylic resin (QC-20 PMMA; Dentsply Ltd., Weybridge, England) according to manufacturers' directions and using a metal mould (10-mm diameter, 2-mm thick). Processed specimens were immersed in purified water for 48 h at 35 °C to release residual monomer [17]. Then, the specimen surfaces were ground in a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil) by using progressively smoother aluminum oxide papers (320, 400 and 600 grit). After, the specimens were ultrasonicated for 20 min to remove any contaminants. The specimens were disinfected with sodium hypochlorite 0.5% for 3 min and washed three times with distilled water.

2.3. Surface roughness measurements

The surface roughness of the specimens was measured by a profilometer (Surfcoder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan) accurate to 0.01 μm with a total measurement length of 3.2 mm and 0.5 mm/s. Three readings were made for each specimen, obtaining a mean value of 0.30 ± 0.04 μm [18]. It was standardized in order to avoid any roughness interference with the *C. albicans* adherence and/or biofilm accumulation.

2.4. Preparation of C. albicans suspension

C. albicans reference strain (ATCC 90028) was selected for this study. Before the experimental procedures, the identity of this strain was confirmed by CHROMagar® *Candida* test (Difco, Detroit, MI, USA) and carbohydrate assimilation test using Vitek-2 identification system (bioMérieux, Marcy l'Etoile, France).

C. albicans strain was aerobically cultured under agitation for 18–20 h at 35 °C into Yeast Nitrogen Base culture medium (YNB; Difco) supplemented with 50 mM glucose. Then, *C. albicans* cells were washed twice with phosphate-buffered saline (PBS; pH 7.2). The cells were resuspended in PBS (for susceptibility tests) or YNB supplemented with 100 mM glucose (for biofilm assays). A final suspension of $\sim 10^7$ cells/mL was optically adjusted at 520 nm (OD = 0.25) using a spectrophotometer (Spectronic 20; Bausch & Lomb, Rochester, NY, USA) [19].

2.5. Susceptibility tests to FLZ

The MIC of FLZ against *C. albicans* was determined using M27-A3 standards [20]. Briefly, RPMI 1640 culture medium buffered at pH 4.0, 5.5 or 7.0 and unbuffered (initial pH of 7.4) were used. The final concentrations of FLZ ranged from 0.125 to 64 μg/mL. The plates were incubated for 48 h at 35 °C. The MIC was determined visually being defined as the lowest FLZ concentration that inhibited 90% growth in comparison with the growth control.

The MFC was obtained by plating 100 μL from MIC wells with more than 90% of growth inhibition in comparison with the growth control onto Sabouraud Dextrose Agar (SDA; Difco) plates following 24 h of incubation. The MFC was defined as the lowest FLZ concentration that resulted in ≈99% killing.

The period necessary for FLZ to inhibit the growing of *C. albicans* cells was determined by time-kill assay [21]. For this, the experiment was performed at the MIC concentrations, which means that the concentration of FLZ was in accordance to each pH. RPMI was prepared at MIC concentration for each group and mixed with *C. albicans* standard suspensions at a final concentration of 10^3 cells/mL.

Time-kill was evaluated at 0, 2, 4, 6, 8, 12 and 24 h of exposure. In each period, an aliquot of 100 μL was serially diluted in PBS, plated on SDA and incubated aerobically for 24 h at 35 °C. Yeast cells were counted using a stereomicroscope (Coleman Comp. Imp., Santo André, São Paulo, Brazil), and the results were expressed in cells/mL. Suspensions without FLZ were considered as controls.

2.6. Biofilm development

To mimic the oral cavity, the specimens were coated with human salivary pellicle prior to biofilm development. For this, stimulated human saliva was collected from two healthy volunteers, centrifuged (10,000 × *g* 5 min at 4 °C), and the supernatant was filtered-sterilized and immediately used. The volunteers provided written formal consent according to a protocol approved by the Ethics Committee in Research of Piracicaba Dental School (#113/2012).

Under aseptic conditions, the specimens were horizontally placed into a 24-well culture plate containing saliva and incubated under agitation for 1 h at 35 °C to form the salivary pellicle [19]. After, saliva-coated specimens were transferred to plates containing *C. albicans* cell suspension (10^7 cells/mL) prepared in YNB supplemented with 100 mM glucose, and incubated aerobically under agitation at 35 °C for 1.5 h (adhesion phase). The specimens were washed twice with PBS and transferred to plates containing fresh YNB medium buffered with 0.1 M sodium phosphate at pH 4.0, 5.5 or 7.0. These sets were incubated under agitation for 48 h at 35 °C. At the end of each 24 h period, the specimens were washed with PBS, and fresh medium was added. After 48 h of biofilm development, FLZ at 2.56 μg/mL (concentration bioavailable in saliva) [22] was added to culture medium on the experimental groups. The specimens were incubated under agitation for 24 h at 35 °C. After 72 h, the biofilms were analyzed for its cell counts, metabolic activity, secretion of proteinases and phospholipases, bio-volume, average thickness and roughness coefficient.

2.7. Biofilm analyses

For cell counts, biofilm-containing specimens were immersed in PBS and sonicated (7 W, for 30 s) to disrupt the biofilm structure. The suspensions were serially diluted in PBS, and samples were plated in triplicate onto SDA. The plates were incubated aerobically for 24 h at 35 °C. Yeast cells were counted using a stereomicroscope, and the results were expressed in cells/mL.

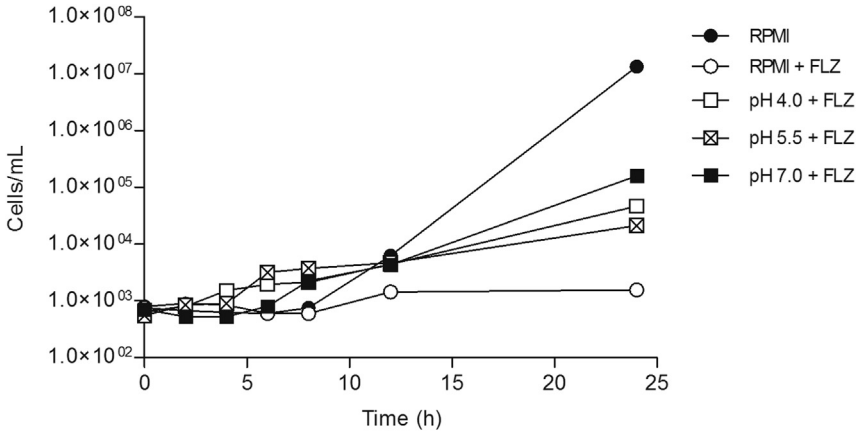

Fig. 1. Resistance curve of *C. albicans* to fluconazole in RPMI medium unbuffered or buffered in different pH.

The metabolic activity was performed with an adapted XTT assay [23]. Briefly, biofilm-containing specimens were placed into a 24-well culture plate with XTT solution (PBS supplemented with 200 mM glucose, 1 mg/mL XTT, and 0.4 mM menadione). The plates were protected from light and incubated under agitation for 3 h at 35 °C. Colorimetric changes in the supernatant were measured by using a spectrophotometer at 492 nm.

For enzymatic activity measurements, biofilm-containing specimens were sonicated, and the suspensions were centrifuged (10,000 × *g* 5 min). The measurements were performed using the supernatant. The proteinase activity was determined as described previously [24]. Briefly, the supernatant was mixed with 1% azocasein at 1:9 (v/v) for 1 h at 35 °C. After, the reaction was stopped by adding 500 μL of 10% trichloroacetic acid, followed by centrifugation (10,000 × *g* 5 min). After, 500 μL of the supernatant was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The proteinase activity was evaluated spectrophotometrically at 440 nm. The specific proteinase activity was defined as the amount of enzyme that elicited and an increase of 0.001 units of absorbance per minute of digestion by XTT reduction [25].

The phospholipase activity was determined as previously described [26]. Briefly, the biofilm supernatants were mixed with an equal volume of phosphatidylcholine substrate and incubated for 1 h at 35 °C. The phospholipase activity was evaluated spectrophotometrically at 630 nm. The specific phospholipase activity was established as the absorbance shift per minute of reaction by XTT reduction [25].

The biofilm structure was evaluated by confocal laser-scanning microscopy (CLSM; Leica Microsystems CMS, Mannheim, Germany). The biofilm-containing specimens were stained by SYTO-9 and propidium iodide with the Live/Dead *BacLight* viability kit (Invitrogen Molecular Probes, Eugene, OR, USA). The biofilms were incubated in the dark for 20 min at 35 °C [27]. A series of images were obtained at 1-μm intervals in the *z*-axis. At least five representative random optical fields were examined for each specimen. The resulting series of images was evaluated by the COMSTAT

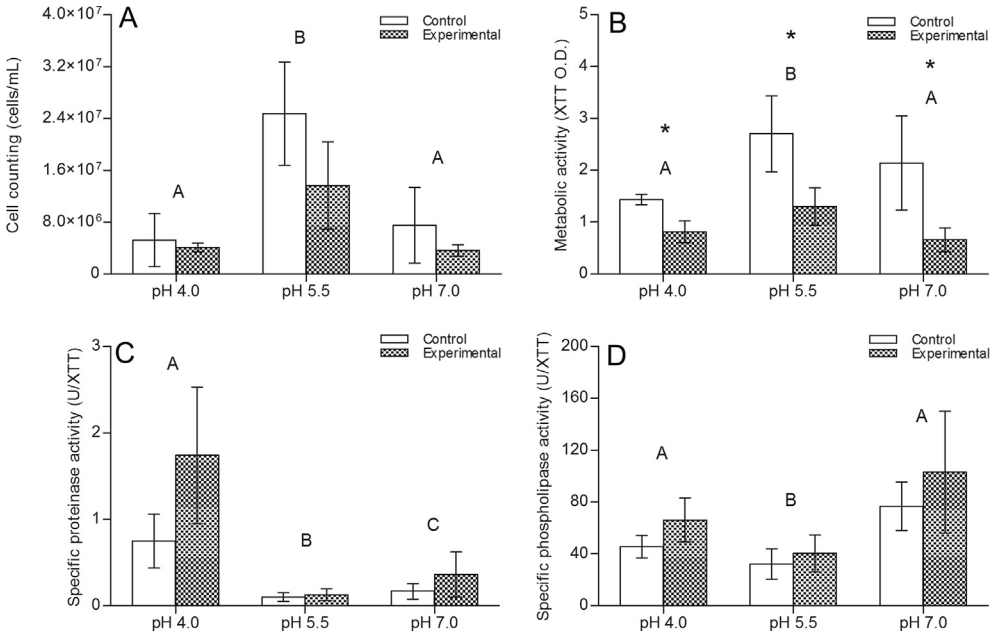

Fig. 2. Cell counting (cells/mL) (a), metabolic activity (OD 490 nm) (b), specific proteinase activity (U/XTT) (c), and specific phospholipase activity (U/XTT) (d) for *C. albicans* biofilms developed for 72 h on PMMA surface. Distinct upper case letters indicate statistically differences among the environmental pH ($p < 0.05$). Symbol (*) indicates statistically differences between biofilms exposed or not to fluconazole ($p < 0.05$).

software program to quantify structural parameters, such as the bio-volume ($\mu m^3/\mu m^2$), average thickness (μm) and roughness coefficient (μm) [28].

2.8. Statistical analysis

The statistical analysis was carried out using the SAS/LAB software package (SAS Software, version 9.0; Cary, NC, USA) with the significance level of 5%. The normality of error distribution and the degree of non-constant variance were checked for each response variable. Data were transformed as suggested by the software, as follows: cell counts, metabolic activity, proteinase and phospholipase (transformed by log10), bio-volume and average thickness (transformed by square root) and roughness coefficient (transformed by exponentiation, y^2).

All data were analyzed by two-way ANOVA, considering the environmental pH and presence or absence of FLZ as study factors, and the cell counts, metabolic activity, proteinase activity, phospholipase activity, bio-volume, average thickness and roughness coefficient as response variables. Tukey's HSD test was used for post-ANOVA comparisons.

3. Results

The MIC values of planktonic cells were 0.5 μg/mL at pH 4.0, 5.5 and unbuffered RPMI medium. It was noted a higher susceptibility to FLZ at pH 7.0 (0.125 μg/mL). All groups showed similar MFC values, being higher than 64 μg/mL. Time-kill test demonstrated a similar behavior between all groups until 4 h, followed by an exponential cell growth, mainly in RPMI group without FLZ. After 24 h it was observed that changes in environmental pH decreased the biofilm susceptibility to FLZ (Fig. 1).

Biofilms developed at environmental pH 5.5 resulted in higher cell counts and bioactivity compared to the other conditions ($p < 0.05$). The presence of FLZ interfered in the bioactivity, decreasing the XTT values at all pH studied ($p < 0.001$, Fig. 2b).

The highest secretion of proteinase was observed in biofilms developed at pH 4.0 ($p < 0.001$), while the lowest was found at pH 5.5 followed by pH 7.0 ($p < 0.05$). The lowest secretion of phospholipase was observed at pH 5.5 ($p > 0.05$). However, FLZ did not influence the proteinase and phospholipase activity ($p > 0.05$, Fig. 2c, d).

Regarding the structural parameters, it was observed that environmental pH 5.5 resulted in higher values of bio-volume and average thickness compared to the other conditions ($p < 0.05$, Fig. 3a, b). In contrast, the presence of FLZ reduced up to 10 times the average thickness on biofilms developed at pH 7.0 ($p < 0.05$). A higher roughness coefficient was also observed in biofilms exposed to FLZ ($p < 0.001$), although FLZ has not influenced the biofilms developed at pH 4.0 ($p > 0.05$, Fig. 3c).

4. Discussion

In this study, we investigated the influence of environmental pH on *C. albicans* biofilms regarding its structure, virulence and susceptibility to FLZ.

Initially, we evaluated susceptibility of *C. albicans* planktonic cells to FLZ at pH 4.0, 5.5 and 7.0, although in accordance with the Clinical and Laboratory Standards Institute, the MIC test should be performed at pH 7.0 [20]. However, it is known that the oral mucosa underlying dentures shows an acidic environment with a pH ranging from up to 4.0 to 6.2 [3]. Thus, the MIC test performed only at pH 7.0 may not reflect the real susceptibility of *C. albicans to* fluconazol [29]. For this, we used buffered and unbuffered RPMI 1640 media in order to mimic the oral environment. Our hypothesis was confirmed by MIC results; a higher FLZ concentration is necessary in an acidic environment (pH 4.0 and 5.5) than in a neutral condition (pH 7.0), which is consistent with other reports about pH 4.0 and 7.0 [16,30]. This result can either be related with the chemical nature of FLZ, which is a weak base with a pK_a of 1.76, being rapidly protonated at pH values close to 4.0 [31] or the fact that pH provoke marked changes in cell wall, which may influence

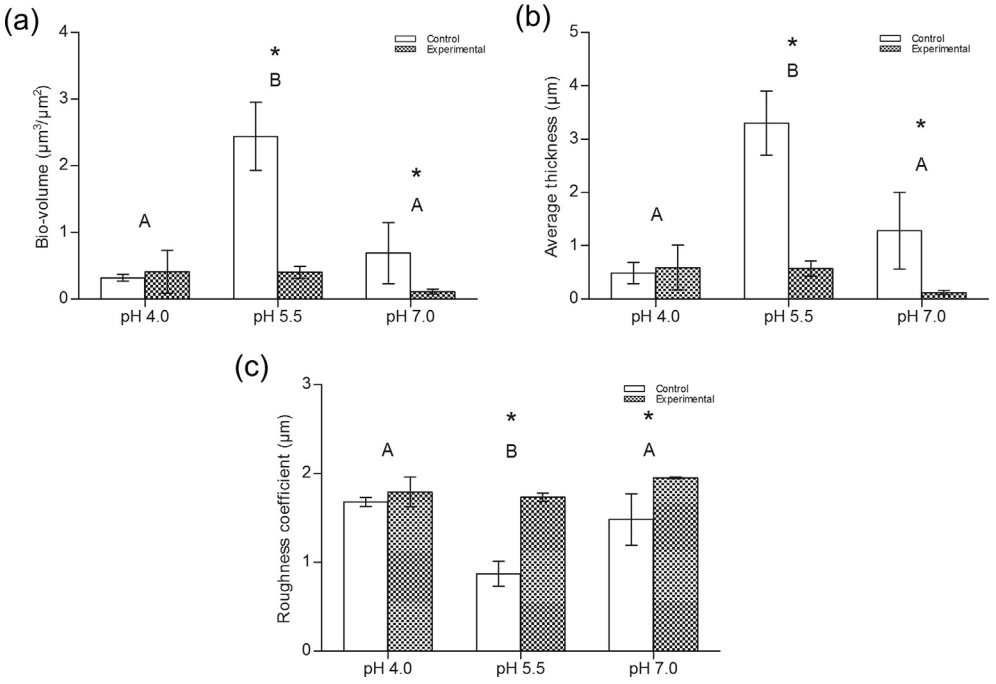

Fig. 3. Bio-volume ($\mu m^3/\mu m^2$) (a), average thickness (μm) (b), and roughness coefficient (μm) (c) for structural parameters of *C. albicans* biofilms developed for 72 h on PMMA surface (Means ± SD; $n = 6$). Distinct upper case letters indicate statistically differences among the environmental pH ($p < 0.05$). Symbol (*) indicates statistically differences between biofilms exposed or not to fluconazole ($p < 0.05$).

the susceptibility of *C. albicans* cells to FLZ [13–15]. Therefore, the association of these factors could explain the different concentrations required for cells inhibition.

The resistance curve obtained with the time-kill test showed that at early stages of exposure (i.e. until 4 h) the cells had a similar behavior under all experimental conditions, probably the time that *C. albicans* planktonic cells led to adapt to the environment. After this period, it was observed that the pH markedly influenced the susceptibility to FLZ. Probably, at 24 h, the FLZ was completely protonated, showing considerable cell grown at this time. In this context, may be assumed that the higher viable cells at pH 7.0 in relation to lower pH may reflect the lowest FLZ concentration used. It is important to consider that planktonic cells are more susceptible to antifungal agents than biofilms [16] and, therefore, lower drug concentrations were used. Thus, to biofilm susceptibility, a higher concentration of FLZ was used (2.56 μg/mL), considering the concentration of this drug bioavailable in saliva [22].

The biofilm cell counts and metabolic activity showed that the environmental pH 5.5 resulted in higher cell quantification and activity compared to pH 4.0 and 7.0. Actually, the environmental pH serves as a potential inducer of differentiation and development [11] and, according to our results, we believe that the pH 5.5 may be more favorable for biofilm formation. When the susceptibility to FLZ was investigated, it was found a significant reduction on metabolic activity after drug exposure at all pH conditions. These results can be attributed to the fungistatic nature of FLZ since it is capable to interfere in the ergosterol biosynthesis by acting on fungal cytochrome P450 which is responsible for mitochondrial activities [29,32].

Secretion of enzymes, particularly proteinase and phospholipase, is considered the main virulence factor of *C. albicans* as these enzymes increase the ability of the fungi to colonize and penetrate tissues [9,33]. The proteinase is activate intraorally in low pH niches, such as under maxillary dentures [3], and this phenomena was also observed in our results by the increased proteinase activity at pH 4.0. The phospholipase had increased secretion also at pH 7.0, being possible that *C. albicans* has hydrolytic phospholipase genes expressed under a variety of environmental conditions that provide sufficient flexibility to survive and promote infection at host sites with higher pH values [34,35]. No differences were observed in proteinase and phospholipase secretion at all pH conditions in the presence of FLZ. Considering that the same concentration of FLZ was employed (2.56 μg/mL, the concentration bioavailable in saliva) [22], we can hypothesize that the stress situations found by the different environmental pH can overrule the antifungal activity [30,36].

Regarding the biofilm structure, the presence of bulky and thick biofilms developed at environmental pH 5.5 could be related to the highest cell counts. Furthermore, considering that pH 5.5 is more favorable for biofilm formation, we predicted that this pH could affect the expression of morphology-specific genes necessary for robust biofilm development [4]. When the susceptibility to FLZ was investigated, it was found a significant reduction on bio-volume and thickness, and increase in roughness coefficient at pH 5.5 and 7.0 after drug exposure. These results can indicate a more dispersed and irregular cells conformation, which could facilitate the drug penetration [37,38].

Within the limitations of this study, it was concluded that the environmental pH modulates the structure, virulence and susceptibility of *C. albicans* biofilms to FLZ.

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