Biofilm formation by the interaction of fungi (*Candida tropicalis*) with various bacteria

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Academic editor
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ABSTRACT

Biofilms are composed of more than one species and are often called polymicrobial biofilms, so research is needed on the formation of polymicrobial biofilms, especially between fungi and bacteria. This study aimed to analyze the total biomass and metabolic activity of biofilms formed from the interaction of fungi (*Candida tropicalis*) with various bacteria, including *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Bacterial and fungal cultures were suspended in tryptic soy broth (TSB) medium, and biofilm was cultivated in two 96-well microplates for 48 hours at 37°C. The crystal violet assay was used to detect the total biomass biofilm, and the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) assay was used to obtain the metabolic activity of biofilm with the optical density (OD) value using an ELISA reader. The results of this study obtained significant data from both parameters. The highest average value was found in the *C. tropicalis* treatment group (2,412 ± 0.825 on the biomass test results; 1,525 ± 0.473 on the metabolic activity test results), while the lowest was seen in the *S. aureus* treatment group (0.512 ± 0.224 in the biomass test results; 0.978 ± 0.349 in the metabolic activity test results). In conclusion, it was confirmed that biofilm biomass could be generated strongly in each treatment in the total biomass parameter, while metabolic activity data suggested that each treatment could carry out cell proliferation.

INTRODUCTION

Fungal infections with bacteria, often known as polymicrobial infections have increased. Polymicrobial infection is an interaction caused by the formation of polymicrobial biofilms, especially between fungi and bacteria [1]. Polymicrobial biofilm is an interaction process of multispecies microbes to form a biofilm community [2]. Polymicrobial biofilm infections have a higher risk of death (about 70%) than infections caused by a single species of microbe (23%) [3]. Microbial biofilms are an important research area for two reasons. Firstly, understanding the form and existence of bacteria in multicellular communities is an intriguing basic science. Secondly, the formation of biofilms can cause problems in several fields, including medical and industrial. Additionally, biofilms can cause antimicrobial treatment resistance, decreased host response, and biocides resistance [4]. Several factors affect the type of interaction between fungi and bacteria depending on the species, strain, and environmental parameters such as temperature, pH, and glucose levels [1, 5]. *Candida tropicalis* is an opportunistic pathogen with a high infection rate. This microbe is one of the most

common pathogens of the non-albicans candida (NAC) species and has the highest adhesion ability compared to other NAC species [6–8].

In polymicrobial communities, several mechanisms can occur, including antagonism mechanisms that drive competition for nutrients and cooperative mutualism synergism mechanisms that provide a mutual benefit under certain conditions [9, 10]. The communication mechanism between microorganisms is often associated with quorum sensing (QS), a signaling molecule that plays a role in coordinating the expression of virulence and microbial survival [5, 11]. Each cell is capable of responding to quorum-sensing molecules (QSMs) [12, 13]. QSMs also play an important role in biofilm development, morphogenesis, cell population restriction, infection emergence, and dissemination processes [11, 14].

Candida spp. is an example of the interaction between fungi and bacteria, since it interacts with the gram-positive bacteria *Staphylococcus aureus*, which can mutually increase the level of virulence and resistance in the host [1]. Fungal and bacterial interactions can occur in various infections, for example, *Candida spp*. with *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and methicillin-resistant *S. aureus* (MRSA). The interaction between *Candida albicans* and MRSA can coexist synergistically to form biofilms [3, 15].

Since polymicrobial biofilms cause severe infection, this study analyzes the effect of polymicrobial biofilms derived from interactions between the fungi *C. tropicalis* and the bacteria *S. aureus*, MRSA, *P. aeruginosa*, and *A. baumanii* on total biomass and metabolic activity of biofilm formation.

MATERIALS AND METHODS

Ethical approval

This research has received ethical approval with certificate reference number: 138/HRECC.FODM/III/2022 on March 31, 2022, by the Faculty of Dental Medicine Health Research Ethical Clearance Commission Universitas Airlangga.

Sample collection

The strains were obtained from the Microbiology Laboratory, Faculty of Medicine, Universitas Airlangga, Indonesia. Strains were stored at 4°C in saboraud dextrose agar (SDA) medium (Oxoid) for *Candida tropicalis* strain and tryptic soy agar (TSA, OXOID) medium for bacterial strains. Each strain is a clinical isolate from Dr. Soetomo General Hospital, Surabaya, Indonesia. This research was conducted at the Microbiology Laboratory, Faculty of Medicine, Universitas Airlangga, Indonesia from March to May 2022.

Preparation of microbial suspension

Prior to inoculation, all strains were re-cultured from the initial culture to their respective growth medium: SDA medium for *Candida tropicalis*, and TSA medium for bacterial strains. Then all that had been re-cultured were incubated aerobically at 37°C (Binder Model BD 400) for 24 h [16]. After incubation, the suspension of each strain was formed by inoculating each strain into 5 ml of tryptic soy broth (TSB) medium (OXOID, CM0129B), and incubated aerobically at 37°C for 24 h. Microbes from the suspension

were vortexed and compared for turbidity with a McFarland 0.5 solution (equivalent to 1×10^7 CFU/ml) [17].

Biofilm cultivation

The microbial suspension was used in the biofilm cultivation process on two different 96-well microplates (NEST, flat bottom TC sterile, Nest Biotechnology Co., Ltd.). The first microplate was used for the CV assay parameters and the second microplate was used for the MTT assay parameters. The biofilm cultivation step was carried out by inoculating each microbial suspension into 9 treatment wells: one well for the control group (containing only 150 μL of TSB medium), five wells given a single-species suspension of 150 μL each (*C. tropicalis*, *S. aureus*, MRSA, *P. aeruginosa*, and *A. baumanii*), and four wells were inoculated with 75 μL of mixed-species suspension to obtain a volume of 150 μL in one well. Each treatment was repeated 6 times, so there were 60 filled wells. After the process, the microplate was closed, covered with plastic wrap, and incubated at 37 degrees Celsius for 48 hours without shaking [18, 19].

Crystal violet assay

In this method, the total biomass was obtained by removing the non-adherent cells from each well and rinsing the adherent cells using phosphate-buffered saline (PBS, pH 7.2) three times. Subsequently, the microplate was air-dried for approximately 30 minutes. A total of 150 μL of CV solution (0.4% (w/v), Ceristain C.I 42555) was poured into each well, allowed to stand for 15 minutes, then the microplate was rinsed under running water and air-dried at room temperature. After drying the microplate, each treatment well was fixed by adding 150 μL of 95% ethanol (Onemed, Jayamas medical industry, Indonesia). Following this procedure, the microplate was ready for reading the absorbance value with an ELISA reader (Thermo multiscan Go 1510, Thermo Fisher Scientific, Inc.) at 570 nm [16, 19, 20].

MTT assay

This method is a colorimetric test that provides a value for metabolic activity due to the reduction of the MTT tetrazolium salt. The first step of the method was to remove non-adherent cells in each well and rinsed with PBS (pH 7.2) twice. Furthermore, 15 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Code M6494, invitrogen™) which had been dissolved with PBS was poured into each well including control wells. Then the microplates were incubated at room temperature 37°C for 4 hours in the dark. After incubation, 150 μL of dimethyl sulfoxide solution (DMSO, Vivatis code PC0906, Vivantis Technologies Sdn Bhd-Malaysia) was added to the wells to remove the remaining medium and dissolve the MTT formazan crystals. Furthermore, the color change from yellow to purplish in each well was observed on the microplate. After this process, the microplate was ready to read the absorbance value using an ELISA reader (BioTek 50TM, MERCK) at 540 nm [21–23].

Data analysis for crystal violet assay

The data obtained from the crystal violet assay was analyzed using the microtiter plate assay method by calculating the cut-off OD (ODc) value. In this method the biomass

value was obtained and grouped based on its ability to produce biofilms with the following formula format [19,24]:

$$OD_C = \overline{X}OD_{Control} + 3SD_{Control,} \qquad (1)$$

$$OD_{isolate} = \overline{X}OD_{treatment} - OD_{C.} \qquad (2)$$

Based on the results obtained from the formula, the interpretation of the OD isolate values was categorized into 4 groups as follows:

$OD_{isolate} \leq OD_C$ (0) no biofilm forming,

$OD_C < OD_{Isolate} \leq 2 \times OD_C$ (+ or 1) weak biofilm forming,

$2 \times OD_C < OD_{Isolate} \leq 4 \times OD_C$ (++ or 2) moderate biofilm forming,

$4 \times OD_C < OD_{Isolate}$ (+++ or 3) high biofilm forming.

Data analysis for MTT assay

The data obtained from this method is the average value of OD in each well which was used to determine the level of metabolic activity which is characterized by the presence of cell proliferation, with the following formula [22,23],

$$OD_{result} = \overline{X}OD_{isolate} - \overline{X}OD_{control} \qquad (1)$$

Based on the results obtained from the formula, the interpretation of the OD results can be categorized into the following 3 groups:

$OD_{result} < 0.75$ Low cell proliferation,

$0.75 \leq OD_{result} \geq 1.25$ Normal cell proliferation,

$OD_{result} > 1.25$ Increased cell proliferation.

Statistical analysis

In this study, each microplate underwent 10 treatments with 6 repetitions. Each result obtained is presented as mean ± standard deviation. The results obtained from the CV assay and MTT assay in the form of OD values were analyzed for differences using one-way ANOVA with Tukey's post hoc follow-up test with SPSS version 16.0 software application with 95% confidence degree (P-value <0.05 is considered to be significantly different).

RESULTS

Effect of biofilm formation on total biomass as measured by CV assay

In this study, cultivation was carried out on single-species and fungal-bacterial biofilms (*Candida tropicalis*, *Staphylococcus aureus*, MRSA, *Pseudomonas aeruginosa*, and *Acinetobacter baumanii*). The biofilms were grown in 96-well microplates and incubated for 48 h. Crystal violet staining was performed, and the absorbance value was measured three times with an ELISA reader at 570 nm. The results showed that biofilm

biomass was formed in both treatments (single-species and mixed-species), except in control wells (TSB medium only), with the difference between the mean OD isolate and the mean ODc being 0.072 (data not shown). This indicates that each OD isolate value is four times greater than the ODc value (0.072). Based on the previously described formula, each treatment is included in the high biofilm-forming (HBF) category.

Each treatment data set was presented as mean ± SD from the highest to the lowest value, respectively. Single-species treatment of *Candida tropicalis* had the highest total biomass value (2,412 ± 0.825) and *S. aureus* had the lowest value (0.512 ± 0.224) treatment (Table 1).

Table 1. Biofilm formation for each treatment was measured by CV staining and MTT method.

Species	CV values*	CV category	MTT values*	MTT category
C. tropicalis	2.412 ± 0.825[b]		1.525 ± 0.473[b]	ICP
C. tropicalis x MRSA	2.173 ± 0.515[c]		1.508 ± 0.380[c]	ICP
C. tropicalis x *S. aureus*	2.008 ± 0.212[c]		1.481 ± 0.364[c]	ICP
C. tropicalis x *A. baumannii*	1.913 ± 0.366[c]		1.423 ± 0.304[c]	ICP
P. aeruginosa	1.854 ± 0.488[b]	HBF	1.239 ± 0.500[b]	NCP
C. tropicalis x *P. aeruginosa*	1.163 ± 0.243[c]		1.144 ± 0.095[c]	NCP
A. baumannii	0.975 ± 0.471[b]		1.114 ± 0.313[b]	NCP
MRSA	0.653 ± 0.332[b]		0.995 ± 0.165[b]	NCP
S. aureus	0.512 ± 0.224[b]		0.978 ± 0.349[b]	NCP
Control group	0.048 ± 0.008[a]	-	0.079 ± 0.014[a]	-

Tukey post hoc test showed= [a]Control group. [b]Single-species treatment. [c]Mixed-species treatment. CV values = displays the absorbance value with Crystal violet assay with *P-value < 0.05 from the data considered statistically significant. MTT values = displays the absorbance value with the MTT assay with *P-value < 0.05 from the data considered statistically significant. HBF = high biofilm-forming. ICP = increased cell proliferation. NCP = normal cell proliferation. Data expressed as mean ± SD.

Based on the results described in Table 1, the graph (Figure 1) explains the differences in the OD-treatment values from the highest in *C. tropicalis* to the lowest in *S. aureus*. The X-axis represents the type of treatment species, while the Y-axis shows the OD-treatment value at a wavelength of 570 nm. The error bar indicates the standard deviation value. The one-way ANOVA statistical test showed a significant (P-value <0.05) difference between the single-species and mixed-species biofilm treatments.
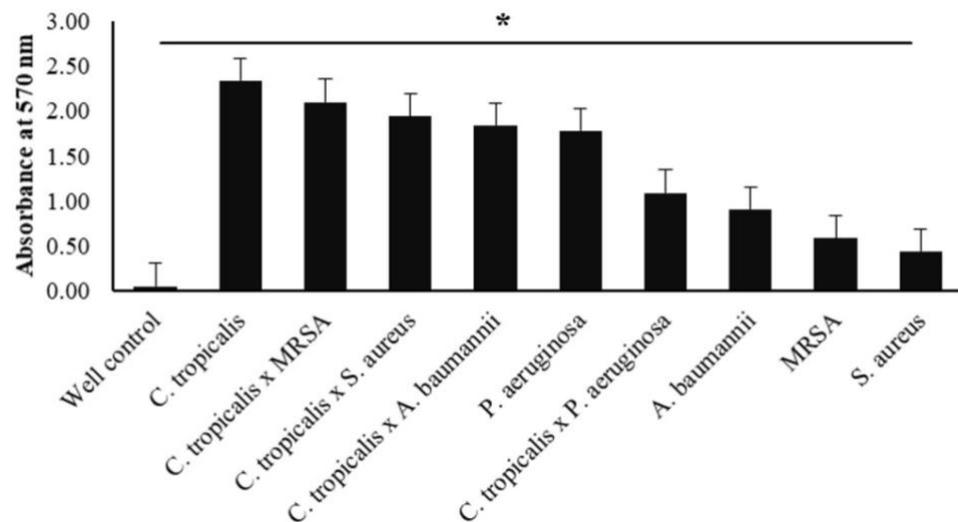

Figure 1. Total biomass by CV staining for single-species and mixed-species biofilms where OD isolate > 4xODc (0.072) = high biofilm forming. Data were obtained based on *P-value < 0.05 (compared with control) which was considered statistically significant.

Effect of biofilm formation on metabolic activity by MTT assay

In this method, single-species and mixed-species biofilm cultivation were carried out on *Candida tropicalis*, *Staphylococcus aureus*, MRSA, *Pseudomonas aeruginosa*, and *Acinetobacter baumanii* strains. The biofilm was grown in a 96-well microplate for 48 hours before the tetrazolium salt method test (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide). The presence of metabolic activity in the biofilm is indicated by the reduction or discoloration of the tetrazolium salt from yellow to blue formazan. These results were obtained by measuring the OD value three times with an ELISA reader at 540 nm, then calculating the difference between the average value of OD in each treatment with the average value of OD-control, which shows 0.079 (table 1).

The results are presented as mean ± SD for each treatment, following the previously described formula regarding grouping the results of cell proliferation activity. Thus, this study found the category of increased cell proliferation (ICP) in the following treatments, *C. tropicalis* (1,525 ± 0.473), *C. tropicalis* x MRSA (1,508 ± 0.380), *C. tropicalis* x *S. aureus* (1,481 ± 0.364), and *C. tropicalis* x *A. baumannii* (1,423 ± 0.304). In addition, this study also found the results of cell proliferation which were included in the normal cell proliferation (NCP) category in the treatment of *P. aeruginosa* (1,239 ± 0.500), *C. tropicalis* x *P. aeruginosa* (1.144 ± 0.095), *A. baumannii* (1.114 ± 0.313), MRSA (0.995 ± 0.165), and *S. aureus* (0.995 ± 0.165) (Table 1).

As described in table 1, the graph (Figure 2) shows the highest OD value (treatment with ICP category for *C. tropicalis*) to the lowest OD value (treatment with NCP category for *S. aureus*). The X-axis indicates the treatment of species, while the Y-axis shows the OD value at a wavelength of 540 nm. The error bar indicates the standard deviation value (Figure 2). The one-way ANOVA statistical test showed a significant (P-value <0.05) difference between the single-species and mixed-species.

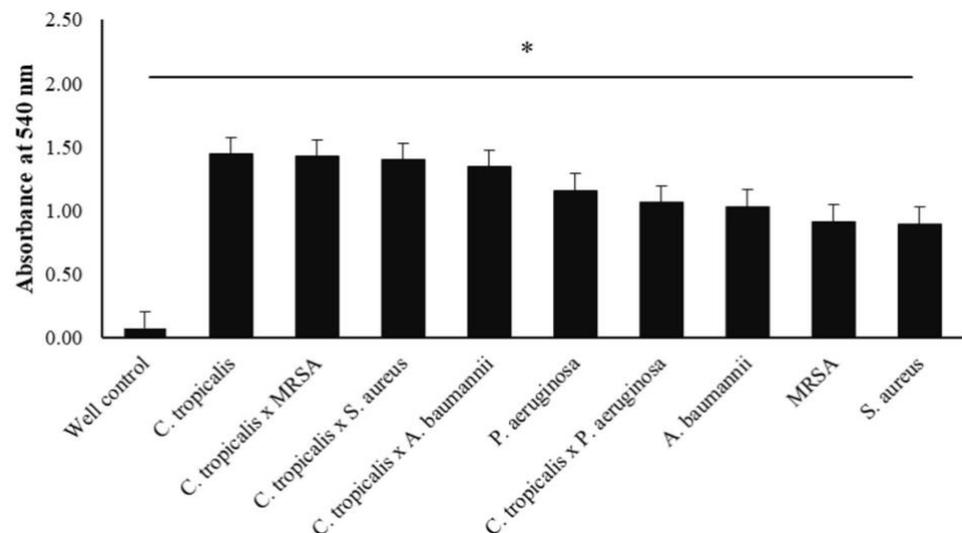**Figure 2.** Metabolic activity by MTT assay for single-species and mixed-species biofilms where OD < 0.079=low cell proliferation, 0.079 < OD < 1.25= Normal cell proliferation, OD > 1.25= Increased cell proliferation. Data was obtained based on *P-value < 0.05 (compared with control) which was stated to be statistically significant.

DISCUSSION

In this study, the formation of *in-vitro* biofilms showed significant results. These findings indicated that each strain was able to form biofilms. The crystal violet dye binds to negatively charged surface molecules from living and dead cells, and exopolysaccharides can be used to calculate total biomass [25, 26]. MTT assay method reduces yellow-colored tetrazolium salt to blue-colored formazan due to the activity of mitochondria or enzymes in plasma cells such as oxidoreductase, dehydrogenase, and oxidase. So in this method cell, proliferation and viability were calculated using this method [27, 28].

This study demonstrates that the single-species *C. tropicalis* treatment yielded the highest absorbance value for both parameters. (Table 1). This is because the *C. tropicalis* strain is a yeast capable of producing hyphae for attachment [29]. According to another study, *C. tropicalis* exhibited higher adhesion than other *Candida* species [30]. Hyphae in *Candida* play a role in the preparation of exopolymer substances and blastopores, this is what causes *C. tropicalis* biofilms to form strongly [31]. The *C. tropicalis* strain formed strong biofilm [32].

Biofilms have complex structures that can adhere to both biotic and abiotic surfaces [29]. In vitro biofilm formation has three important steps namely, attachment, colonization, and cell proliferation [33]. Another study showed that there are five important steps in the formation of fungal and bacterial biofilms, namely, adsorption, adhesion, and formation of microcolonies along with the matrix. The maturation process is characterized by metabolic activity and the emergence of quorum sensing (QS) molecules, and the dispersion process [1]. Biofilm formation is based on the concept of microbial community formation, which is mediated by a cascade of molecular mechanisms and gene expression. The quorum sensing (QS) molecular signal contained in each microbe plays an important role in communication between microbes to form a population. [12,34].

One of the factors that influence the biofilm is cell attachment to the substrate. In *C. tropicalis*, the adhesion molecule and the regulation of the BCR1 and RBT5 genes play an important role in attachment [29, 35]. *S. aureus* strains can form biofilms on the host or other surfaces. This attachment is mediated by recognizing adhesive matrix molecules (MSCRAMMs), regulated by the ica locus gene and hemB mutation [36]. Furthermore, this strain can produce extracellular matrix factor and polysaccharide intercellular adhesin (PIA), which is regulated by the *Ica* gene and is involved in cell multiplication [37].

In this study, the absorbance values of the CV assay and MTT assay of mixed-species *C. tropicalis* with MRSA were higher than that of *C. tropicalis* with *S. aureus*. This was because the MRSA strain was more virulent in terms of mortality and morbidity than the S. aureus strain with MSSA [38]. MRSA is encoded by the mecA gene, which binds to the PBP 2A protein and confers resistance to methicillin and beta-lactam drugs [39]. This study supports previous findings that mixed-species biofilms had lower absorbance values than fungal biofilms because bacteria can prevent *Candida spp.* development [40].

Findings of this revealed that the mixed-species treatment of *C. tropicalis* with gram-positive bacteria MRSA and *S. aureus* had a higher absorbance value than the interaction of *C. tropicalis* with gram-negative bacteria *P. aeruginosa* and *A. baumannii*. The findings of this investigation differ from prior studies on *Candida albicans* with gram-negative (*E. coli* and *P. aeruginosa*) and gram-positive (*S. aureus*) bacteria. It showed that gram-negative bacteria were able to reduce *C. albicans* biofilm compared to

gram-positive bacteria [41]. *P. aeruginosa* has the virulence factor flagellin which plays a role in motility and has type III secretion [42]. *A. baumannii* has the polysaccharide capsule gene K1 [43]. Antagonism arises in mixed-species *P. aeruginosa* because this strain can kill hyphae and biofilms of *C. tropicalis*, which are triggered by phenazine compounds that damage cell wall integrity [44, 45]. However, this study is consistent with prior in vitro investigations on dual biofilms that show *P. aeruginosa* can limit the production of non-albicans Candida (NAC) biofilms such as *C. tropicalis*. This is because both microbes can release quorum sensing molecules and the presence of N- acyl homoserine lactone (AHL) in gram-negative bacteria. While in vitro conditions can decrease AHL in *P. aeruginosa*, inhibiting *Candida spp*. biofilms [46, 47].

In this experiment, single-species data on biomass or metabolic activity of bacteria exhibited a lower absorbance value than mixed species data [3]. In *A. baumanni* and MRSA, the lowest absorbance value was found in *S. aureus*. This is because *S. aureus* colonizes more slowly and forms biofilms on abiotic surfaces or under monoculture conditions. The colonization of this strain increases when in mixed biofilm conditions because the microcolonies of this strain can attach to *Candida spp*. hyphae. This indicated that *C. tropicalis* was able to facilitate the growth of *S. aureus* [48]. Based on the preceding description, this study found that there is a difference in total biomass value and metabolic activity between biofilm development from single-species and mixed-species treatments.

CONCLUSIONS

This study obtained the results that biofilms can be formed from single-species or mixed-species treatments. Each treatment showed significantly different results on the parameters of total biomass and metabolic activity. The limitation of this study is the inability to distinguish the constituent components of the biofilm that has been formed. So, for further research, it is recommended to add parameters for observing the biofilm layer with scanning electron microscopy to obtain a layer structure on the biofilm.

ACKNOWLEDGEMENT

AUTHOR CONTRIBUTIONS

DWA did the research proceed and conceived the manuscript, ADWW and RJS planned the idea for this research, S provided direction for data analysis, and WT and MRW helped to revise this manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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