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Candida albicans in Association with Pathogenic Bacteria to Form Mono and Polymicrobial Biofilms

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***Abstract*— Biofilm formation by the accumulation of various pathogens may be responsible for chronic and acute human infections. This research aimed to explain the role of *Candida albicans* in biofilm formation with pathogenic bacteria isolated from clinical samples. Ninety clinical samples were collected to isolate pathogenic bacteria and *Candida albicans*. The isolation and identification of these pathogens were performed using selective media and the VITEK 2 system. Flat-bottom microtiter plates were used to evaluate the ability of all isolates to form biofilms in case of monomicrobial and polymicrobial species. Three pathogenic bacteria, including *Staphylococcus aureus* (n=8), *Pseudomonas aeruginosa* (n=8), *and Escherichia coli* (n=4), as well as *C. albicans* (n=20), were isolated. All isolates were submitted to evaluate their ability to form monomicrobial and polymicrobial biofilms. In the case of monomicrobial species, *C. albicans* (100%) and bacterial species (80%) showed a high percentage of biofilm formation. However, *C. albicans* was prepared to make an adherence phase for each one of *S. aureus*, *P. aeruginosa*, and *E. coli*. In this experiment, all bacterial isolates improved their ability to form polymicrobial biofilms. in contrast, some bacterial isolates reduce their ability for forming polymicrobial biofilms if bacterial isolates are used as an adherence phase. In conclusion, the replacement of adherence phase of the polymicrobial biofilm causes either an increase or a decrease in the ability of organism to form a biofilm when compared to monomicrobial biofilms.**

Keywords— Biofilm, Monomicrobial biofilm, polymicrobial biofilm, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*

I. INTRODUCTION

Biofilms are defined as a community of microorganisms that can attach to both biotic or abiotic surfaces, and are encased in a self-produced extracellular polymeric matrix [1]. The national institute of health (NIH) estimated that 65% of all microbial infections are associated with biofilm formation, which is strongly linked to antimicrobial resistance, making treatment of such infections difficult [2].

Bacteria in biofilms can employ a variety of survival strategies to avoid the host defense systems. They may cause topical tissue damage and an acute infection by remaining latent and hidden from the immune system. Infections caused by biofilms occur when bacteria attach to medical devices such as urinary catheters, prosthetic joints, and heart valves [3]. On the other hand, *Candida albicans* is the most common cause of candidiasis in most clinical settings. *C. albicans* is a dimorphic microorganism that can take the form of yeast or hyphal cells and serves as the foundation of a complex multicellular biofilm. Candidiasis is caused by *C. albicans*, an opportunistic infection that can be acute, subacute, or chronic, and usually results in life-threatening mycoses [4]. The ability of these yeasts to form biofilms on medical devices has a significant impact on their ability to cause human disease [5].

Biofilms caused by a single microbial species or a mix of bacterial and fungal species have significantly increased, contributing to high levels of morbidity and mortality [6]. The presence of both eukaryotic and prokaryotic pathogens makes infections difficult to diagnose and treat, necessitating complex multi-drug treatment strategies [7]. The aim of this study was to evaluate *in-vitro* monomicrobial and polymicrobial biofilms formation among three pathogenic bacteria and *C. albicans* regarding the adherence phase, which is the most important step in biofilm formation.

II. MATERIALS AND METHODS

A. Ethical Approval

This research was performed based on the approval form of a Research Protocol by the ministry of Health and Environment (Form number 02/2021) in the republic of Iraq. This form was signed by the authors and the Thi-Qar health department.

B. Microorganism's isolates and identification

A total of 90 clinical samples were collected from patients suffering from respiratory tract infection, urinary tract infections, nail infections, and burn patients received at the department of Microbiology / teaching Al-Hussein hospital in Thi-Qar. All samples were cultivated on MacConkey agar (Oxoid, USA), Eosin Methylene Blue (EMB) agar (HiMedia, India), Mannitol salt agar (Oxoid, UK), and nutrient agar (HiMedia, India), for the isolation

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of *E. coli*, *S. aureus*, and *P. aeruginosa* [8]. VITEK 2 system (Biomerieux, France), as described by [9], was used to confirm the identification of bacterial isolates. C. albicans was identified based on colony colorimetric assay on CHROM*Candida* agar (HiMedia, India), germ tube test and growth at 45 Ċ for 48 hours [10].

C. Biofilm formation assay with Crystal violet

All bacterial and *Candida* isolates were activated by inoculating a colony into tubes containing 2 ml of Brain Heart Infusion Broth medium (HiMedia, India) and incubated for 24 hours at 37 Ċ. Then they were diluted by fresh BHIB broth medium, in a ratio of 1:20, and 200 µl of each isolate was placed into sterile 96- wells flat bottom microtiter plates (Triplicate for each isolate), and again incubated for 24 hours at 37 Ċ. Microtiter plate was emptied and rinsed three times with distilled water and left inverted for 2 minutes to dry. After that, 200 µl of 1% crystal violet (Oxoid, USA) solution was added to each well and was incubated the microtiter plate for 15 minutes at room temperature. The microtiter plate was again emptied and rinsed three times with distilled water and left inverted for 2 minutes to dry. Then, each well was filled with 200 µl of a mixture of acetone and ethanol (Oxoid, USA) (v:v) 20:80. Finally, an ELISA Microplate reader (Genex Lab. USA) with a wavelength of 450nm was used to read the optical density OD of cells that contributed to biofilm formation. A set of wells with sterile BHIB were used as negative control [11] [12].The optical density cut-off (ODc) was calculated through three standard deviations above the mean OD of the measured negative control. Based on these result, isolates were classified into four classes: non-biofilm-former (OD $\leqslant$ ODc); weak biofilm former (ODc < OD $\leqslant 2 \times$ ODc); moderate biofilm former ($2 \times$ODc < OD $\leqslant 4 \times$ODc); and strong biofilm former (OD > 4 ODc) [13][14].

D. Polymicrobial Biofilm Formation

The same method mentioned above was used to determine polymicrobial biofilm formation by adding one species of three bacterial genera with *C. albicans* in the same well of the Microtiter plate. This method was performed by allowing *C. albicans* isolates to form biofilms as an adherence phase for 24 hrs. at 37 Ċ, then the microtiter plate was emptied and filled with fresh BHIB. Each well of the microtiter plate was inoculated with one species of bacteria and incubated for 24 hrs. at 37 ºC . This step was repeated, but the bacterial species were grown as an adherence phase for *C. albicans*. Optical density was read as mentioned above as well as ODc was calculated to find out the four categories of biofilms.

E. Statistics Analysis

The significant differences among biofilm groups (p-value at level 0.05) were calculated using the Chi-square test. The t-test was also used to show the significant differences between *C. albicans* and bacterial species based on alteration of the organism state in biofilm formation. Statistical analysis was performed using IBM SPSS Statistics software v. 22.

III. Results

In the current study, 20 bacterial isolates were identified, including *S. aureus* (8 isolates), *P. aeruginosa* (8 isolates), and *E. coli* (4 isolates), as well as 20 *C. albicans* isolates. They were subjected to monomicrobial and polymicrobial biofilm formation. Monomicrobial biofilm formation was observed in all *C. albicans* and *E. coli* isolates. However, five out of eight isolates from each *S. aureus* and *P. aeruginosa* formed monomicrobial biofilms, distributed into weak and moderate biofilms (Table 1).

In general, *C. albicans* isolates are distinguished by their ability to form strong biofilms compared to bacterial species. Polymicrobial biofilms formation was performed via two procedures based on adherence phase, once adherence phase by bacteria species followed by mature phase with *C. albicans*, and once more of adherence phase by *C. albicans* followed by mature phase with bacteria species. When *C. albicans* was allowed to form the mature phase of biofilm on adherence phase of bacteria species, there was decrease in the ability of some isolates to form biofilms. For example, isolate 1 and 2 of *C. albicans* decrease its ability to form biofilm from moderate to weak with adherence phase by *S. aureus* isolates 1 and 2. The decrease biofilm formation was also recorded in three isolates of *C. albicans* with *P. aeruginosa*. On the other hand, three *C. albicans* isolates increased their ability to form biofilms from weak to moderate or strong biofilms with adherence phase by *S. aureus* isolates 3, 5 and 6 (Table 2). But all *E. coli* isolates led to decrease the ability of *C. albicans* to form biofilms (Table 2).

Concerning Polymicrobial biofilms formation by bacterial species on adherence phase by *C. albicans*, it was observed that bacteria species increased their ability to form biofilms. Firstly, three isolates from each *S. aureus* and *P. aeruginosa* did not form biofilm in the case of monomicrobial biofilm formation. However, these isolates of *S. aureus* and *P. aeruginosa* were moved their biofilm categories from non-biofilms to moderate and strong biofilms in case of adherence biofilm phase formed by *C. albicans*. Other isolates of bacteria also changed their status from weak or moderate to strong biofilms with an adherence biofilm phase formed by *C. albicans* (Table 3).

In general, 15 % of *C. albicans* isolates (n=20) have improved their ability to form biofilm with an adherence phase by bacterial species. In contrast, 85 % of bacterial species (n=20) have improved their ability to form biofilm with the adherence phase by *C. albicans*. The most important result was observed that 40 % of *C. albicans* isolates were decreased their ability in biofilm formation with adherence phase by bacteria species, while the percentage of bacteria species isolates that form biofilms became 100 % with adherence phase by *C. albicans* (Table 4).

TABLE I. Distribution of isolates based on their ability to form Monomicrobial biofilms (Chi-square p-value at level 0.05 = 0.03)

Organism	Non-biofilm	Weak biofilm	Moderate biofilm	Strong biofilm	Total
S. aureus	3	2	2	1	8
P. aeruginosa	3	4	1	0	8
E. coli	0	2	2	0	4
C. albicans	0	5	6	9	20

TABLE 2. POLYMICROBIAL BIOFILM FORMATION BY *C. ALBICANS* DURING THE ADHERENCE PHASE BY BACTERIAL SPECIES AND MONOMICROBIAL BIOFILM FORMATION BY *C. ALBICANS*.

Mature phase by *C. albicans*	Adherence phase by Bacterial species	Non-biofilm	Weak biofilm	Moderate biofilm	Strong biofilm
1	*S. aureus* 1	0	2	1	0
2	*S. aureus* 2	0	2	1	0
3	*S. aureus* 3	0	1	2	0
4	*S. aureus* 4	0	0	2	1
5	*S. aureus* 5	0	1	0	2
6	*S. aureus* 6	0	1	2	0
7	*S. aureus* 7	0	0	1,2	0
8	*S. aureus* 8	0	2	1	0
9	*P. aeruginosa* 1	0	0	0	1,2
10	*P. aeruginosa* 2	0	0	2	1
11	*P. aeruginosa* 3	0	1	2	0
12	*P. aeruginosa* 4	0	0	2	1
13	*P. aeruginosa* 5	0	0	1,2	0
14	*P. aeruginosa* 6	0	0	1,2	0
15	*P. aeruginosa* 7	0	1	0	2
16	*P. aeruginosa* 8	0	0	2	1
17	*E. coli* 1	0	0	2	1
18	*E. coli* 2	0	0	2	1
19	*E. coli* 3	0	0	2	1
20	*E. coli* 4	0	0	2	1

0: No biofilm formed.
1: Formation of a Monomicrobial biofilm by *C. albicans.*
2: Formation of polymicrobial biofilm via an adherence phase by bacterial species and a mature phase by *C. albicans.*
-The color gradient shows the stages of biofilm formation.

TABLE 3. POLYMICROBIAL BIOFILM FORMATION BY BACTERIAL SPECIES DURING THE ADHERENCE PHASE BY *C. ALBICANS* AND MONOMICROBIAL BIOFILM FORMATION BY BACTERIAL SPECIES.

Mature phase by Bacteria species	Adherence phase by *C. albicans*	Non-biofilm	Weak biofilm	Moderate biofilm	Strong biofilm
S. aureus 1	1	0	0	0	2
S. aureus 2	2	0	0	1,2	0
S. aureus 3	3	0	0	1	2
S. aureus 4	4	0	1	0	2
S. aureus 5	5	0	0	0	2
S. aureus 6	6	0	1	0	2
S. aureus 7	7	0	0	0	2
S. aureus 8	8	0	0	0	1,2
P. aeruginosa 1	9	0	0	2	0
P. aeruginosa 2	10	0	0	0	2
P. aeruginosa 3	11	0	0	0	2
P. aeruginosa 4	12	0	0	1	2
P. aeruginosa 5	13	0	1	0	2
P. aeruginosa 6	14	0	1	0	2
P. aeruginosa 7	15	0	1	0	2
P. aeruginosa 8	16	0	1	0	2
E. coli 1	17	0	1	0	2
E. coli 2	18	0	1	2	0
E. coli 3	19	0	0	1	2
E. coli 4	20	0	0	1,2	0

0: No biofilm formed.
1: Formation of a Monomicrobial biofilm by Bacterial species.
2: Formation of polymicrobial biofilm via an adherence phase by C. albicans and a mature phase by bacterial species.
-The color gradient shows the stages of biofilm formation.

TABLE 4. CASES THAT ILLUSTRATE THE CHANGE IN THE ABILITY OF ORGANISMS TO FORM BIOFILMS.

Alteration of Organism's state in polymicrobial biofilms formation	*C. albicans* (n=20) and adherence phase by bacteria species		Bacteria species (n=20) and adherence phase by *C. albicans*		*t*.test (p <0.05)
	No of isolates	%	No of isolates	%	
Weak to Moderate biofilm	1	15	1	85	0.05
Moderate to Strong biofilm	0		3		
Weak to Strong biofilm	2		7		
non-biofilm to strong biofilm	0		5		
non-biofilm to Moderate biofilm	0		1		
Stable (same category)	4	20	3	15	
Strong to Moderate biofilm	8	40	0	0	0.14
Moderate to weak biofilm	3		0		
Strong to weak biofilm	2		0		
Monomicrobial biofilm formation	20	100	16	80	
Polymicrobial biofilm formation	20	100	20	100	

IV. DISCUSSION

The current study found that bacteria and *C. albicans* can form robust biofilms and modify their ability in biofilm formation. These findings confirm that human pathogens isolated from important body regions have a high possibility of occurring of in biofilm infections by a single pathogen (Yeast or Bacteria) or by multiple pathogens.

Candida albicans activity in biofilm formation as a mono or polymicrobial biofilm with bacterial species was demonstrated. The prevalence of this functional ability may indicate that these types of isolates are consistent and persistent even when found in different communities. The majority of *C. albicans* infections, however, are related to its ability to form biofilms, which involve the adherence of yeast cells to substrates, followed by proliferation and filament formation, resulting in the formation of a network of cells covered in an extracellular matrix [15][16]. This study produced results that *C. albicans* was extremely capable of adhering to surfaces and catching bacteria species present in the same environment to build strong biofilms. One study indicated that biofilm formation in bacterial isolates was previously identified as a significant factor in persistent infections [17].

In terms of bacterial biofilm formation, *S. aureus*, *P. aeruginosa*, and *E. coli* formed biofilms in the case of monomicrobial biofilms. *P. aeruginosa* has a variety of mechanisms for surviving in a biofilm, which presents a significant challenge [18]. Many previous studies confirmed that *S. aureus* isolates formed strong biofilms [19], [20]. Several studies found that many *E. coli* isolates collected from clinical samples with relapsed infections produced biofilm. Bacterial biofilms are frequently associated with the long-term persistence of bacterial species in various environments [21].

Candida albicans that formed an adherence phase for bacteria species demonstrated a high affinity with bacteria

for biofilm formation. It was discovered that using *C. albicans* as an adherence phase for *S. aureus* and *P. aeruginosa* resulted in conversion of bacterial state from non-biofilm in case of monomicrobial to biofilm formation in case of polymicrobial biofilms, as well as improved biofilm formation for other isolates.

However, *C. albicans* coexists with many bacterial species in different niches in the host, most notably *S. aureus*, which is a significant human bacterial pathogen that causes a wide range of diseases [22][23]. The results of this study agreed with previous study demonstrated that *S. aureus* alone is a poor biofilm formant, but when combined with *C. albicans*, it forms a substantial biofilm in which the fungus creates a scaffolding for the bacteria [24]. Another study suggested that the incidence of both *C. albicans* and *S. aureus* infections raised due to the increasing use of implanted medical devices, as the majority of these infections are caused by biofilms formed on medical implants [25].

Previous studies of mixed *C. albicans* and *S. aureus* biofilms in vitro and in vivo revealed that *S. aureus* has a high affinity for the *C. albicans* hyphal form, resulting in a dense and architecturally complex biofilm formed by these species' co-adherence and interaction [15]. In vitro research on the therapeutic implications of *C. albicans* and *S. aureus* interactions in biofilm found that *C. albicans* secreted matrix polysaccharides, primarily -1,3-glucan, that confer *S. aureus* with enhanced antimicrobial tolerance by impeding drug penetration through the biofilm [24]. However, the presence of co-infecting microorganisms in a biofilm can alter the environment, and polymicrobial interactions can result in augmented pathogenesis [26].

Although the interaction between *P. aeruginosa* and *C. albicans* was primarily antagonistic and complicated, synergistic effects can occur primarily through physical interactions and secreted factors by the quorum-sensing system [27]. Their genetic and phenotypic characteristics, combined with their proclivity to aggregate as recalcitrant polymicrobial biofilms, impose a significant burden on the infections in which they are present, prompting increased research interest in this area. For example, *P. aeruginosa* and *C. albicans* have been shown to colonize the lungs of cystic fibrosis patients and form biofilms on endotracheal surfaces [28]. *Candida albicans* colonizes the airway only in critically ill patients (elderly, immunocompromised, and/or hospitalized) receiving invasive mechanical ventilation. Furthermore, despite their antagonistic relationship, those with *C. albicans* tracheobronchial colonization are at an increased risk of severe ventilator associated pneumonia (VAP) infection caused by *P. aeruginosa* [29].

In the presence of *P. aeruginosa*, regurgitation of germ tube formation is common. The most common physical interaction between *P. aeruginosa* and *C. albicans* is extensive bacterial association with fungal hyphae [28]. Cell wall-associated compounds in bacteria, such as pili and lectin-carbohydrate interactions and mannan, cause the physical correlation between *P. aeruginosa* and *C. albicans* [30]. The current findings are consistent with previous research on *P. aeruginosa* biofilm formation, which has been reported with a high percentage of weak, moderate, and strong biofilms when compared to other bacteria species [31].

In case of *E. coli* with *C. albicans* as an adherence phase, biofilm formation increased for most of the isolates, indicating that *C. albicans* had effect on *E. coli* when forming polymicrobial biofilm. However, the biofilm formation rate decreased in *C. albicans* when E. coli was used as an adherence phase. One study discovered that *E. coli* co-culture inhibited the growth of *C. albicans* by six hours when compared to *C. albicans* grown alone. Surprisingly, after six hours of culture, the number of viable *Candida* cells began to decline [32]. *C. albicans* isolates that were positive for biofilm formation using the Congo red method had a positive effect on E. coli growth [33].

In general, bacterial association with fungal hyphae is thought to result from nutrient competition in the first hour of co-isolation, followed by the bacterial-fungal interaction, parasitism [34]. Lipopolysaccharide (LPS), another component of the bacterial cell wall, plays a role in polymicrobial crosslinking by interfering with fungal hyphae, metabolism, and growth [35].

V. Conclusion

In conclusion, in this study, a novel approach to investigate the formation of polymicrobial biofilms that are dependent on the adherence phase of bacterial or fungal species was followed. The replacement of the adherence phase of the polymicrobial biofilm results in either an increase or a decrease in the organism's ability to form a biofilm compared to monomicrobial biofilms. However, the authors suggest applying this approach using biotic surfaces. The ability of these pathogenic agents to resist antimicrobial agents must be evaluated.

Conflict of interest

The authors declare that they have no conflict of interest.

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