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Candida-Candida and *Candida-Staphylococcus* species interactions in *in-vitro* dual-species biofilms

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***Abstract*---**Objective: In present study, interaction between different *Candida* species and also their interaction with *Staphylococcus* species were investigated in mono and dual-species biofilm model.

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Methods: Resistant and weak biofilms former *Candida*(*C. albicans*, *C. glabrata*, *C. krusi*) and *Staphylococcus* species (*S. aureus*, *S. epidermidis*, *S. saprophyticus*) alongwith ATCC isolates were used in mono and different dual-species combinations for estimation of biofilms by crystal violet (CV) biofilm biomass assay and XTT reduction assay. Aspartyl proteinase activity of *Candida* species was also measured in these developed biofilms. Results: CV assay showed increased in biofilm biomass after 48 h of incubation in developed mono and dual-species biofilms. XTT reduction assay showed overall decreasing trend in metabolic rate in biofilms after 48 h of incubation. All three *Candida* species with each other in dual-species *in-vitro* biofilms showed antagonistic behaviour. Biofilm biomass production was raised for *C. albicans* and *C. glabrata* dual-specie biofilm with all*Staphylococcus* species except with clinical isolate of *S. aureus* and in *C. glabrata-S. epidermidis* dual-specie biofilm. ATCC and clinical isolate of *Candida* showed markedly decrease aspartyle proteinase activity when co-cultured with*Staphylococcus*compared to when it was cultured alone. Conclusion: In consortium species change their behaviour, expression of different virulence factors and can behave synergistically or antagonistically in dual-species biofilm model for their survival.

Keywords---Biofilm biomass, *Candida*, *Staphylococcus*, XTT

Introduction

Microbial cells can exist as both free living planktonic forms or as biofilms(1), embedding themselves in a self-produced hydrated polysaccharide and protein matrix (2,3). Due to biofilm formation several benefits are conferred to the microorganisms, ranging from shared metabolic pathways to protection from host defence mechanisms, environmental factors and toxic substances such as antimicrobials(4,5). These biofilm structures are of great interest and importance in clinical scenarios as these biofilm play role in development of various infectious disorders and increased resistance to significantly higher concentration of antimicrobial agents (1)(6,7). Biofilm can form on both abiotic (medical devices) and biotic surfaces (mucosal surfaces). National Institute of Health, USA, estimated that approximately 80% of the human infections arise due to pathogenic biofilms (8).

In biofilm development commensal flora are mainly involved, including viral, bacterial and fungal species. In more than 70% of the biofilm related infections, Gram-positive especially *Staphylococcus* species are involved. Among fungal species commonly studied biofilm forming specie is *C. albicans*. Other biofilm forming *Candida* species include *C. parapsilosis*, *C. krusei. C. tropicalis* and *C. glabrata*. Fungal biofilm related infection commonly effects oral cavity, lungs, burn wounds, reproductive tract, gastrointestinal tract, intravascular catheters, skin and urinary catheters (9).

C. albicans is common human oral commensal specie, which has been a best studied fungal model for biofilm development. This fungus can become pathogen under unhygienic and immunocompromised conditions. It is commonly isolated from biofilms of root caries and infected gingival crevices and involve in its pathology. *C. albicans* has several cariogenic characteristics like high acid tolerance, ability to bind collagen and dentin collagen degradation by secreted aspartyle proteinase. Despite its known role in caries, its significance is often ignored because of its small number in oral cavity(10,11,12).

Mostly *C. albicans* biofilm based studies are *in vitro* as mono-specie biofilms developed on abiotic surfaces (13). Recent studies on biotic surfaces, such as oral and vaginal mucosal tissues show that these surfaces are excellent for biofilm development especially for multiple microbial biofilms (14). *C. albicans* is involved in several mixed-species infections like in periodontitis (15). Currently, dual-speciesbiofilmformation between *C. albicans* and *S. aureus* is commonly studied for their pathogenic potential (16). However, limited data is available in literature related to other oral commensal *Candida* species interaction with each other and with coagulase negative *Staphylococcus* species (CoNS) in dual-species biofilm. There is a need to explore these interactions in comprehensive way to assess the possible intra and inter-species effects of these oral microbial communities in biofilms development leading to oral infections. In present study, interaction between different *Candida* species and also their interaction with *Staphylococcus* species isolated from saliva weredissectedin dual species-biofilm model by crystal violet (CV) and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazoliumhydroxide (XTT)assay. In addition, estimation of aspartyl proteinase activity in single and dual-species biofilm were also evaluated as they are a major contributor in pathogenesis of oral fungal disorders.

Material and Methods

Isolates used for mono and dual-species biofilm assays

For biofilm assays confirmed multiple drug resistant, weak biofilms former *Candida* and*Staphylococcus*species isolated from saliva samples along-with ATCC isolates obtained from Molecular Medicine Microbiology Laboratory, Quaid-i-Azam University, Islamabad, were used in mono and different dual-speciescombinations (Table 1).*Candida* isolateswere cultured for 48 h in Sabouraud dextrose broth (SDB) (Liofilchem, Italy) supplemented with 10% glucose, while *Staphylococcus* isolates for 24 h in nutrient broth supplemented with 10% glucose and incubated at 37°C for CV and XTT reduction assays. After incubation, centrifugation was done for 10 min at 5,000 rpm. Supernatant was removed and washing of pellet was done twice in sterile PBS (pH 7.4-7.6). The cells were resuspended in 1.0 mL of RPMI 1640 media. Inoculum was prepared with final cell density of 1.0×10^7 cells/mL, compared with 0.5 McFarland and adjusted tooptical density (OD) of 0.38 at wavelength 520 nm spectrophotometrically (Multiskan Go, thermo scientific).

Table 1
Fungal and bacterial combinations used for mono and dual-species biofilms

Mono-specie biofilm	Dual-species biofilm
ATCC *C. albicans* (90092)	ATCC *C. albicans* (90092) + ATCC *S. aureus* (25923)
ATCC *S. aureus* (25923)	*C. albicans* BK239+ *C. glabrata* BK214
C. albicans BK239	*C. albicans* BK239+ *C. krusei* BK229
C. glabrata BK214	*C. glabrata* BK214+ *C. krusei* BK229
C. krusei BK229	*C. albicans* BK239+ *S. aureus* BK79
S. aureus BK79	*C. albicans* BK239+ *S. epidermidis* BK155
S. epidermidis BK155	*C. albicans* BK239+ *S. saprophyticus* BK134
	C. glabrata BK214+ *S. aureus* BK79
	C. glabrata BK214+ *S. epidermidis* BK155
S. saprophyticus BK134	*C. glabrata* BK214+ *S. saprophyticus* BK134
	C. krusei BK229+ *S. aureus* BK79
	C. krusei BK229+ *S. epidermidis* BK155
	C. krusei BK229+ *S. saprophyticus* BK134

Crystal violet (CV) biofilm biomass assay

CVbiofilm biomass assay was done by using 96-well microtiter plates, which contained 75 µL of cell suspension and 75 µL of RPMI 1640 for each strain. In dual-species assay, 75 µL of microorganism's suspension was used in the combinations. Three sterile RPMI 1640 containing wells without fungal and bacterial cultures were used as negative control. Plates were incubated for 24 h and 48 h at 37°C, separately. Media was removed carefully from wells after incubation period. Washing of wells were done three times with 200µL PBS (pH 7.2). For fixing adherent cells, 95% ethanol was added to each well and incubated (30 min) at room temperature. The adherent cells were stained with 1% w/v CV (200µL) and again incubated at room temperature (15 min). The wells of microtiter plate were washed to remove excess stain with deionized water. After washing, wells were air dried and to solubilize the CV stain, treated with 33% acetic acid followed by incubation for 15 min at room temperature. For each well, OD was determined at wavelength 492 nm (Multiskan Go, Thermoscientific). Isolates were inoculated in triplicates for biofilm formation and average of three reading was taken for result analysis by comparing ODs of isolates (17).

XTT reduction assay

For this assay, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazoliumhydroxide (XTT) was used to assess metabolic activity of adhere biofilm cells. XTT (Sigma) reagent solution was prepared (1.0 mg/mL) in ultrapure water, filtered and stored at -70°C until use. Solution of phenazine methosulfate (PMS) stock was prepared freshly (0.32 mg/mL in deionized water) and stored in a dark. XTT assay was done by using 96-well microtiter plates. Biofilm were developed after 24 h and 48 h of incubation by following the procedure used for CV assay. Media was removed from each well after incubation and washed thrice with 150 µL of PBS (0.1 M, pH 7). Later, 90 µL of XTT solution and 10 µL of freshly prepared PMS were added. Plates were incubated for one h at

37°C in dark and absorbance was measured at 492 nm (Multiskan Go, Thermoscientific). All isolates were tested for XTT reduction assay in triplicates.

Aspartyl proteinase activity

Candida species and bacterial strains were firstly cultured in 5.0 mL brain heart infusion broth CM 1135(BHI) and incubation was done for 24 h at 37 °C. After incubation, washing with PBS was done twice by centrifugation for 5 min at 5000 rpm. The cells suspension density of 10^7 cells/mL was inoculated in 5.0 mL of BHI broth and incubated for 24 h and 48 h at 37°C. After incubation, 0.1 mL broth was transferred to other eppendroff and 0.4 mL of 0.1 M sodium citrate buffer (pH 3.2) containing 1% w/v bovine serum albumin was added and incubation was done for 15 min at 37°C. After incubation, 5% w/v trichloroacetic acid (0.5 mL) was added to stop the reaction and centrifugation was done for 10 min at 3000 rpm, and OD was measured at 280 nm against distilled water. All isolates were tested in triplicates.

Statistical analysis

Means, Standard deviation (SD) and mean standard error was calculated for descriptive data and categorical data by using IBM SPSS (version 21).

Results

Candida-Candida interactions in dual-species biofilm by CV and XTT assay

CV assay results showed increased in biofilm biomass after 48 h of incubation in mono and dual-species biofilm. For *C. albicans* BK239 and *C. glabrata* BK214 decrease in biofilm biomass (OD=0.172±0.010 after 24 h and OD=0.270±0.016 after 48 h) was recorded, when co-cultured.Alone *C. krusei* BK229 showed maximum biofilm biomass formation among *Candida* isolates, but its biofilm biomass was markedly decreased in dual-species biofilm with *C. albicans* BK239 and *C. glabrata* BK214 (Table 2).

XTT assay showed overall decreasing trend in metabolic rate in biofilms after 48 h of incubation compared to 24 h. Metabolic activity of *C. albicans* (BK239)-*C. glabrata* (BK214) dual-species biofilm increased after 24 h and 48 h of incubation compared to mono-specie assay. *C. krusei* (BK229) biofilms showed highest metabolic rate in mono-species biofilm, which increased further after 24 h of incubation in dual-species biofilms. However, after 48 h of incubation their biofilms showed slight reduction in metabolic rate in dual-species biofilm with both *C. albicans* 239BK (from OD=0.338±0.035 to OD=0.300±0.032) and *C. glabrata* (OD=0.338±0.035 to OD=0.272±0.004) (Table 2).

Table 2
Detection of biofilm formation (CV assay) and metabolic activity (XTT reduction assay) expressed by *Candida* mono and dual-species biofilm after 24 h and 48 h of incubation

Candida mono and dual-species combination		CV assay ODs after 24 h	CV assay ODs after 48 h	XTT assay ODs after 24 h	XTT assay ODs after 48 h
C. albicans BK239	Mean±SD	0.239±0.037	0.320±0.023	0.142±0.006	0.119±0.018
	Std. error	0.021	0.0133	0.003	0.010
C. glabrata BK214	Mean±SD	0.165±0.016	0.390±0.092	0.105±0.002	0.112±0.011
	Std. error	0.009	0.053	0.0011	0.006
*C. krusei*BK229	Mean±SD	0.534±0.026	0.971±0.188	0.403±0.054	0.338±0.035
	Std. error	0.015	0.108	0.031	0.020
C. albicans BK239+*C. glabrata* BK214	Mean±SD	0.172±0.010	0.270±0.016	0.306±0.041	0.300±0.032
	Std. error	0.006	0.009	0.024	0.018
C. albicans BK239+*C. krusei* BK229	Mean±SD	0.333±0.05	0.847±0.097	0.511±0.041	0.303±0.050
	Std. error	0.030	0.056	0.023	0.028
C. glabrata BK214+*C. krusei* BK229	Mean±SD	0.187±0.02	0.688±0.320	0.423±0.017	0.272±0.004
	Std. error	0.013	0.185	0.009	0.002
Blank	Mean±SD	0.083±0.005	0.168±0.001	0.063±0.003	0.059±0.002
	Std. error	0.003	0.0005	0.001	0.001

Aspartyle proteinase activity in *Candida-Candida* dual-species biofilm

Aspartyl proteinase activity was not much affected after 48 h of incubation both in mono and dual-species biofilm. Highest aspartyl proteinase activity was expressed by *C. albicans* BK239 (0.147±0.010) after 24 h of incubation. *C. albicans* BK239-*C. glabrata* BK214 and *C. albicans* BK239-*C. glabrata* BK229 dual-species biofilm showed decrease in activity after 24 h and 48 h of incubation. Decrease activity was also seen in *C. glabrata* BK214-*C. krusei* BK229 dual-species biofilm after 24 h and 48 h of incubation (Table 3).

Table 3
Estimation of aspartyl proteinase activity displayed by *Candida* in mono and dual-species biofilm combinations after 24 h and 48 h of incubation

Candida mono and dual-species combinations		ODs of aspartyl proteinase activity after 24 h	ODs of aspartyl proteinase activity after 48 h
C. albicans BK239	Mean±SD	0.147±0.010	0.125±0.008
	Std. error	0.006	0.005
C. glabrata BK214	Mean±SD	0.111±0.005	0.131±0.01
	Std. error	0.003	0.005
C. krusei BK229	Mean±SD	0.097±0.005	0.113±0.008
	Std. error	0.003	0.005
C. albicans BK239+	Mean±SD	0.116±0.01	0.116±0.008

C. glabrata BK214	Std. error	0.006	0.004
C. albicans BK239+	Mean±SD	0.109±0.006	0.119±0.007
C. Krusei BK229	Std. error	0.003	0.004
C. glabrata BK214+	Mean±SD	0.095±0.002	0.098±0.006
C. krusei BK229	Std. error	0.001	0.003

***Candida-Staphylococcus* interactions in dual-species biofilm by CV assay**

Biofilm biomass production was enhanced after 48 h of incubation for both mono and dual-species biofilm. CV assay results showed that compared to mono-specie biofilm,absorbance was greatly increased when ATCC *C. albicans* (90029) was co-cultured with ATCC *S. aureus* (25923) (OD=0.425±0.042 after 24 h and OD=1.100±0.208 after 48 h). *C. albicans* (BK239) showed increased in biofilm biomass in dual-species biofilm with *Staphylococcus* species except for *S. aureus* BK79, in which it showed reduction in biofilm biomass after 24 h and 48 h of incubation (OD=0.239±0.037 after 24 h and OD=0.320±0.023 after 48 h to OD=0.190±0.027 after 24 h and 0.283±0.136 after 48 h) compared to mono-specie biofilm (Table 4a). Among other *Candida* species, *C. glabrata* (BK214) showed increased in biofilm biomass in dual-species biofilms with *Staphylococcus* species except for *S. aureus* BK79, in which it showed reduction in biofilm biomass after 48 h of incubation (OD=0.390±0.092 to OD=0.283±0.136) compared to mono-specie biofilm. *C. krusei* BK229 showed reduction in biofilm biomass in dual-species biofilm with all *Staphylococcus* species (Table 4b).

***Candida-Staphylococcus* interactions in dual-species biofilm by XTT assay**

Metabolic activity was reduced overall after 48 h of incubation compared to 24 h, except for *Staphylococcus* mono-species biofilms and *C. glabrata* BK214-*S. aureus* BK79 dual-species biofilm. Metabolic rate of ATCC *C. albicans* (90029) was enhanced after 24 h and 48 h of incubation in dual-species biofilm with ATCC *S. aureus* 25922 (OD=0.511±0.007 after 24 h andOD=0.430±0.059 after 48 h). Metabolic activity of *C. albicans* (CA239) with all *Staphylococcus* species was enhanced after 24 h and 48 h of incubation compared to mono-specie, however, *S. saprophyticus* showed decrease in metabolic rate when co-cultured with *C. albicans* BK239 (Table 4a). Metabolic activity of *C. glabrata* (BK214) was not much affected in dual-species biofilm except with *S. saprophyticus*, with which it showed enhanced activity. *C. krusei* BK229 showed increase in metabolic rate in dual-species biofilm with *Staphylococcus* species except for *S. saprophyticus* BK134, with which it showed reduction after 24 h incubation. *S. saprophyticus* showed decreased in metabolic activity in dual-species biofilm with all *Candida* species (Table 4b).

Table 4a

Detection of biofilm formation (CV assay) and metabolic activity (XTT reduction assay) expressed by *C. albicans-Staphylococcus* mono and dual-species biofilm combinations after 24 h and 48 h of incubation

C. albicans-Staphylococcus species combinations		CV assay ODs after 24h	CV assay ODs after 48h	XTT assay ODs after 24h	XTT assay ODs after 48h
ATCC C. *albicans* (90029)	Mean±SD	0.282±0.261	0.877±0.128	0.368±0.011	0.245±0.003
	Std. error	0.015	0.074	0.006	0.002
ATCC *S. aureus* (25922)	Mean±SD	0.137±0.022	0.257±0.052	0.298±0.068	0.090±0.003
	Std. error	0.01	0.03	0.039	0.001
ATCC *C. albicans* (90029) +ATCC *S. aureus* (25922)	Mean±SD	0.425±0.042	1.100±0.208	0.511±0.007	0.430±0.059
	Std. error	0.02	0.12	0.004	0.034
C. albicans BK239	Mean±SD	0.239±0.037	0.320±0.023	0.142±0.006	0.119±0.018
	Std. error	0.0217	0.0133	0.003	0.010
S. aureus BK79	Mean±SD	0.159±0.016	0.365±0.036	0.110±0.013	0.112±0.003
	Std. error	0.009	0.02	0.007	0.001
S. epidermidis BK155	Mean±SD	0.116±0.016	0.329±0.063	0.159±0.082	0.070±0.014
	Std. error	0.009	0.03	0.047	0.008
S. saprophyticus BK134	Mean±SD	0.151±0.014	0.189±0.017	0.852±0.030	1.101±0.061
	Std. error	0.008	0.01	0.01	0.035
C.albicans BK239+*S. aureus* BK79	Mean±SD	0.190±0.027	0.283±0.136	0.754±0.066	0.762±0.029
	Std. error	0.015	0.078	0.038	0.01
C. albicans BK239+ *S. epidermidis* BK155	Mean±SD	0.299±0.017	0.506±0.110	0.293±0.019	0.178±0.001
	Std. error	0.009	0.064	0.01	0.0005
C. albicans BK239+ *S. saprophytics*BK134	Mean±SD	0.243±0.014	0.369±0.041	0.315±0.006	0.203±0.098
	Std. error	0.008	0.02	0.003	0.05
Blank	Mean±SD	0.083±0.005	0.168±0.001	0.063±0.003	0.059±0.002
	Std. error	0.003	0.0005	0.001	0.001

Table 4b

Detection of biofilm formation (CV assay) and metabolic activity (XTT reduction assay) expressed by non-albicans *Candida-Staphylococcus* mono and dual-species biofilm combinations after 24 h and 48 h of incubation

Non. *albicans-Staphylococcus* species combinations		CV assay ODs after 24 h	CV assay ODs after 48 h	XTT assay ODs after 24 h	XTT assay ODs after 48 h
C. glabrata BK214	Mean±SD	0.165±0.016	0.390±0.092	0.105±0.002	0.112±0.011
	Std. error	0.009	0.053	0.0011	0.006
*C. krusei*BK229	Mean±SD	0.534±0.026	0.971±0.188	0.403±0.054	0.338±0.035
	Std. error	0.015	0.108	0.031	0.020
C. glabrata BK214+ *S. aureus* BK79	Mean±SD	0.190±0.027	0.283±0.136	0.107±0.009	0.125±0.005
	Std. error	0.015	0.078	0.005	0.003

C. glabrata BK214+ *S. epidermidis* BK155	Mean±SD	0.299±0.017	0.506±0.110	0.116±0.016	0.115±0.017
	Std. error	0.009	0.064	0.009	0.009
C. glabrata BK214+ *S. saprophytics*BK134	Mean±SD	0.243±0.014	0.369±0.041	0.292±0.04	0.238±0.103
	Std. error	0.008	0.02	0.02	0.059
C. krusei BK229+ *S. aureus* BK79	Mean±SD	0.190±0.027	0.283±0.136	0.459±0.077	0.357±0.023
	Std. error	0.015	0.078	0.04	0.013
C. krusei BK229+ *S. epidermidis* BK155	Mean±SD	0.299±0.017	0.506±0.110	0.443±0.001	0.366±0.045
	Std. error	0.009	0.064	0.0008	0.02
*C. krusei*BK229+ *S. saprophytics*BK134	Mean±SD	0.243±0.014	0.369±0.041	0.370±0.004	0.353±0.045
	Std. error	0.008	0.02	0.002	0.02
Blank	Mean±SD	0.083±0.005	0.168±0.001	0.063±0.003	0.059±0.002
	Std. error	0.003	0.0005	0.001	0.001

Aspartyle proteinase activity by *Candida* in *Candida-Staphylococcus* dual-species biofilm

In dual-species biofilm, both ATCC and clinical isolate of *Candida* showed markedly decrease activity with all *Staphylococcus* isolates compared to when it was cultured alone (Table 5).

Table 5
Estimation of aspartyl proteinase activity displayed by *Candida* and *Staphylococcus* species in mono and dual-species biofilm combinations after 24 h and 48 h of incubation

Candida-Staphylococcus species combinations for mono and dual-species biofilms		ODs of aspartyl proteinase activity after 24 h	ODs of aspartyl proteinase activity after 48 h
C. albicans-Staphylococcus species combinations			
ATCC C. *albicans* (90029)	Mean±SD	0.247±0.012	0.120±0.003
	Std. error	0.007	0.001
ATCC *S. aureus* (25922)	Mean±SD		
	Std. error	-	-
ATCC *C. albicans* (90029) +ATCC *S. aureus* (25922)	Mean±SD	0.062±0.023	0.072±0.017
	Std. error	0.013	0.009
C. albicans BK239	Mean±SD	0.147±0.010	0.125±0.008
	Std. error	0.006	0.005
C. albicans BK239+ *S. aureus* BK79	Mean±SD	0.070±0.019	0.093±0.003
	Std. error	0.01	0.002
C. albicans BK239+ *S. epidermidis* BK155	Mean±SD	0.057±0.018	0.084±0.004
	Std. error	0.01	0.002
C. albicans BK239+*S.*	Mean±SD	0.063±0.011	0.091±0.018

saprophyticus BK134	Std. error	0.006	0.01
Non. Albicans *Candida- Staphylococcus* species combinations			
C. glabrata BK214	Mean±SD	0.1111±0.005	0.131±0.01
	Std. error	0.003	0.005
*C. krusei*BK229	Mean±SD	0.097±0.005	0.113±0.008
	Std. error	0.003	0.005
C. glabrata BK214+*S. aureus* BK79	Mean±SD	0.019±0.008	0.129±0.037
	Std. error	0.004	0.02
C. glabrata BK214+*S. epidermidis* BK155	Mean±SD	0.054±0.007	0.090±0.008
	Std. error	0.004	0.004
C. glabrata BK214+*S. saprophyticus* BK134	Mean±SD	0.072±0.022	0.080±0.005
	Std. error	0.01	0.002
C. krusei BK229+*S. aureus* BK79	Mean±SD	0.053±0.014	0.068±0.006
	Std. error	0.008	0.003
C. krusei BK229+*S. epidermidis* BK155	Mean±SD	0.069±0.013	0.096±0.010
	Std. error	0.007	0.005
*C. krusei*BK229+*S. saprophytics*BK134	Mean±SD	0.072±0.028	0.109±0.021
	Std. error	0.016	0.012

Discussion

Most of the studies related to *Candida* biofilm development are *in vitro* based on mono-microbial biofilm models on abiotic surfaces. As ability of *C. albicans* to grow on various biotic surfaces as well as now on the indwelling catheters (abiotic surface) is becoming a significant issue, which can lead to fatal systemic infections, therefore, their role in oral surfaces is also needed to be deciphered. Species heterogeneity within polymicrobial biofilm makes it difficult to understand individual species contribution in disease pathogenesis (13). Besides *C. albicans*, other non-albicans species like *C. glabrata*, *C. Krusei* and *C. parapsilosis* are also frequently cultured from mouth and are involved in biofilm development on soft and hard tissues(18). In present study, potential of multidrug resistant weak biofilm former *Candida* speciesand *Staphylococcus* isolated from saliva samples of postpartum females to grow and survive in dual-species biofilm was analysed by *in vitro* biofilm model using CV biofilm biomass and XTT reduction assay. Dual-species biofilm between *Candida* species and oral bacteriawere developed on polystyrene surface and was investigated along with ATCC strains.

Mostly polymicrobial biofilm studies focus on relationship between bacterial and Candidal species, however, data is rare related to co-existence of more than two *Candida* species within a biofilm. In present work, dual-species biofilm formation between different *Candida* species was studied. Analysis showed that both *C. albicans* and *C. glabrata* exhibit reduction in biofilm biomass, however, their metabolic activity was raised in dual-species biofilm. Among *Candida* species maximum biofilm biomass was produced by *C. krusei*, which was greatly reduced in dual-species biofilm assay with both *Candida* species. Also, their metabolic activity was decreased in dual-species biofilm after 48h of incubation. *Candida* species might have antagonistic relation in developed polymicrobial biofilms.

Like present work, reduction in *C. albicans* biofilm forming ability was also reported by (19), when *C. glabrata* (77% reduction) and *C. krusei*(89% reduction) were co-cultured. Santos*et al.*, (2016), also showed similar trend of reduction in biofilm forming ability and metabolic activity in dual-species assays (*C. albicans* with *C. glabrata* and *C. krusei)*(20).Another study reported that *Candida* isolates from dental prostheses show reduction in biofilm forming ability and express antagonistic relationship in dual-species biofilm and their metabolic activity also significantly differ in mono and dual-species biofilm. In their study on*Candida-Candida* dual-species biofilm, hyphal production was reduced which is required for more invasive form of yeast (21). Possible reason for low biofilm biomass and metabolic activity expressed by *Candida* isolates in the present study could be due to microbial competitive behaviour for limited supply of nutrients in microtiter plates and for colonization sites available for *Candida* to grow in mixed-species biofilms. Furthermore, decreased survival due to oxygen stress in multi-layered biofilms might be contributing factor for antagonistic behaviour. Expression of high *C. krusei* biofilm biomass compared to other *Candida* species could be due to high initial colonization rate, high cell surface hydrophobicity and adherence to acrylic surfaces compared to other *Candida* species.

Contrary to present study, results were reported by a study based on *in vivo* mice model injected sublingually with *C. albicans* or *C. glabrata* alone and in combinations to check their ability to grow and develop into oropharyngeal candidiasis. These experiments showed that *C. glabrata* alone was unable to develop aggressive tongue infection; however, with pre-established *C. albicans* infection was greatly enhanced. There was also reduction in *C. albicans* along with decreased *C. glabrata* colonization (22). In present work, biofilms were developed *in vitro* on polystyrene surfaces. Effect of substratum on microbial biofilms development is already reported, like tongue as substratum provides nutritionally rich and balanced environment for greater support, growth and hyphal production of *Candida* species compared to *in vitro* polystyrene surfaces.

In next part, *Candidal-Staphylococcal* dual-species biofilm were analysed. Both ATCC isolates of *Candida* and *Staphylococcus* showed increased biofilm biomass production and metabolic activity in dual-species biofilm. Isolate of *C. albicans* and *C. glabrata* expressed increased biofilm biomass production and metabolic activity in dual-species biofilm with *Staphylococcus* species (except with *S. aureus*). Although *C. krusei* showed decrease in biofilm biomass, however, its metabolic activity was raised with *Staphylococcus* species except with *S. saprophyticus*.

Mostly literature, which is available is based on involvement of *C. albicans*-*S. aureus* dual-species biofilm in different pathologies such as periodontitis, keratitis, denture stomatitis, cystic fibrosis, UTIs, ventilator associated pneumonia and burn wound infections. A study by Zago *et al.*, (2015), reported synergistic growth effect in dual-species biofilm between *C. albicans*-MRSA and MSSA strains. A high biofilm biomass and metabolic activity was found in *C. albicans*-*S. aureus* dual-species biofilm (23). Another study demonstrated similar agonistic relationship in an *in vitro* biofilm assay of *C. albicans*-*S. aureus* in murine oral cavity. It was shown that *S. aureus* adhered more to *Candida* hyphal due to adhesion molecules called Als3p. Moreover, due to this adhesion transport of *S. aureus* across mucosal barrier and disseminate to other body site to cause systematic infections is enhanced (24). ATCC strains employed in present work exhibited the similar agonistic relationship in dual-species biofilm, however, local isolates used produced antagonistic relationship. The reason might be the differences in biofilm forming conditions (*in vitro* vs *in vivo*), short time duration required for *Candida* to showed phenotypic switching in a nutrient deficient culture media and to produces excessive EPS. Also strains used in both studies were from different sources as isolates from saliva samples were used in current work in comparison to ATCC cultures.

A study by Holt *et al.*, (2017), demonstrated a role of dual-species biofilmof *C. albicans*-*S. epidermidis* in nematode model. After mono and dual-species inoculation, survival of nematode after infection was assessed. In comparison to single-specie inoculation, dual-species inoculation significantly enhanced the virulence and reduced nematode survival rate (25). Hyphal production by *C. albicans* and EPS overproduction by *S. epidermidis* together enhanced the virulence in dual-species biofilm (13). An enhanced biofilm forming activity of dual-species*C. albicans*-*S. epidermidis* was also reported by El-Azizi *et al.*, in 2004. *C. albicans* from present study also showed enhanced biofilm biomass, protein content and metabolic activity in dual-species biofilm with *S. epidermidis*, indicating synergistic relationship between these two microorganisms in dual-species biofilm(26).

As biofilm formation play important role in development of dental caries and gingivitis, this was an attempt to develop a model to evaluate role of different microorganisms in the biofilm's development of these attached communities. Limited data is available in literature related to association of oral *Candida* species with commensal or pathogenic oral bacteria and ultimately their role in biofilm related oral disorders. In present study, overall, decrease in biofilm biomass was seen in *Candida*-*Candida*dual-speciesbiofilm. However, with *Staphylococcus* species increase in biofilm biomass was seen in all combinations except for clinical isolates of*C. albicans*-*S. aureus*, *C. glabrata*-*S. aureus*and *C. glabrata*-*S. epidermidis*dual-species biofilm. These findings suggest that in dual-species consortia microorganisms can behave synergistically or antagonistically for their survival and disease pathogenesis. The present study was carried outto explore the possible interaction of the *Candida* and oral bacteria in mixed-species biofilm formation as a virulence factor for pathogenesis of dental disorder by using *in vitro* model. Oral cavity provides the ecological niche which is totally different from *invitro* models. To understand the interaction between microbes in the multi-species consortia, there is a need to develop appropriate *in vivo* animal models which should be coupled with advance metagenomic, metaproteomic and metametabolomic techniques.

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Conflict of Interest

None to declare

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