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Metabolomics-driven approaches on interactions between *Enterococcus faecalis* and *Candida albicans* biofilms

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ABSTRACT
INTRODUCTION: We aimed to search the impact of *Enterococcus faecalis* on the cell growth and hyphal formation of *Candida albicans* and to understand the exact mechanism of Candidal inhibition by the existence of E. faecalis by metabolomic analysis.
METHODS: Single and dual biofilms of *E. faecalis* and *C. albicans* were formed in a microtiter plate and the metabolite profile of both biofilms was determined by GS-MS. The hyphal cell growth of *C. albicans* after treatment with both the supernatant and biofilm cells of *E. faecalis* was examined microscopically.
The expression levels of Efg1 and the images of *C. albicans* cell wall in single and dual biofilms were determined by RT-qPCR and TEM, respectively. The violacein levels produced by *Chromobacterium violaceum* were measured to determine the quorum sensing (QS) inhibitory activity of single and dual biofilms.
RESULTS: The biofilm cell growth, Efg1 expression and hyphal development of *C. albicans* were inhibited by *E. faecalis*. Compared to single biofilms, carbohydrate, amino acid, and polyamine changes were observed in the dual biofilm for both microorganisms. Putrescine and pipecolic acid were detected at high levels in dual biofilm. The thicker β-glucan chitin and denser and narrower fibrillar mannan layer of *C. albicans* cell wall were found to be in dual biofilm. QS inhibitory activity was found to be higher in dual biofilm suspensions of *E. faecalis* and *C. albicans* compared to their single biofilms.
DISCUSSION AND CONCLUSION: *E. faecalis* inhibited the hyphal development and biofilm formation of C. albicans. Biofilm suspensions of *C. albicans* and *E. faecalis* showed an anti-QS activity which increased even further in the environment where the two species coexisted. Investigation of putrescine and pipecolic acid can be an important step to understand the inhibition of *C. albicans* by bacteria.

Keywords: Dual-biofilm, *C. albicans*, *E. faecalis*, fungal inhibition, metabolomic.

1. Introduction

Biofilms formed in nonsterile mucosal sites are polymicrobial and interspecies interactions in biofilms show variations. They can interact either in a synergistic or antagonistic manner [1-4]. *Candida albicans* and *Enterococcus faecalis* are frequently found together in biofilm related infections [5-7.] They have common features such as strong biofilm forming capability that complicates the treatment of chronic infections especially infections associated with foreign bodies [8,9].

Microbial metabolomics has attracted great attention in microbiology in recent years [10,11]. In recent years to better understand the biofilm structure of microorganisms, metabolic differences between planktonic and biofilm forms of the same microorganism have been investigated but the results of the polymicrobial biofilm environment containing multiple species have not been reported in the literature yet [6].

Studies concerning the details of the relationship between *C. albicans* and *E. faecalis* are limited [4]. Our goal is to investigate the interactions at the metabolic level in the dual-species biofilm model formed by *E. faecalis* and *C. albicans*. The metabolic profile that both cells exhibit alone and in a common biofilm environment were compared by GC-MS based metabolic analysis. Besides metabolomics analysis, the effects of each other were also investigated by several analysis including microscopy, quorum sensing and mRNA expression.

2. Materials and Methods

2.1. Microbial strains

Enterococcus faecalis ATCC 47077/OG1RF and *Candida albicans* ATCC MYA-2876 were cultured in brain heart infusion broth (BHI) (Oxoid, Basingstoke, UK) overnight at 37°C. *Chromobacterium violaceum* ATCC 12472 was grown in Luria Bertani (LB) broth (Merck, Darmstadt, Germany).

2.2. Evaluation the impact of *E. facealis* on *C. albicans* hyphal morphogenesis

C. albicans were cultured in Yeast extract-peptone-dextrose broth (YPD) (Merck, Darmstadt, Germany) at 30°C for 24 hours. The inoculum suspension of the cell pellet was prepared in RPMI medium as 10^5 cfu/ml. After the addition of 1 ml of the inoculum to the wells of cell culture slides which were coated with 20% fetal bovine serum (FBS), they were incubated for 90 minutes at 30^0C. After the incubation period, the wells were rinsed with phosphate-buffered saline (PBS), then RPMI medium containing 20% FBS and *E. faecalis* supernatant at a ratio of 1:1 (v/v) were transferred into wells.

To evaluate the direct effect of *E. faecalis* cells on hyphal cells, 50 µl of *E. faecalis* suspension were transferred to *C.albicans* which had previously adhered to slides via incubation for 90 minutes. Finally, 950 µl of Spider medium containing 20% FBS was transferred onto slides and incubated at 37^0C for 24 hours (4). To assess the impact of *E. faecalis* supernatant on the development of *C. albicans* hyphal cells, the supernatant of *E. faecalis* was used instead of its cell suspension in the same method above. Slides containing biofilms were rinsed with PBS and microscopic images were acquired using an inverted microscope (Thermo Scientific).

2.3. Development of single and dual biofilm models

Inoculum suspensions with final concentrations of ~ 10^6 cfu/ml for *E. faecalis* and 10^5 cfu/ml for *C. albicans* were made in BHI. The mature biofilms were formed as described previously [12]. Our experimental conditions include the biofilm formation of *E. facealis* and *C. albicans* alone and culturing both microorganisms together.

For the quantification of the biofilm cells, plates containing biofilms were sonicated after 5

minutes of vortexing, thereby allowing biofilm cells to break out of the wells [20]. Tryptic soy agar (TSA), (Merck, Darmstadt, Germany) and Sabouraud dextrose agar (SDA), (Merck, Darmstadt, Germany) were used for enumeration of single-species *E. faecalis* and *C. albicans* biofilm cells respectively. For the enumeration of *E. faecalis* and *C. albicans* cells in the dual-species biofilms, TSA media with amphotericin B (0.025 mg/mL) and SDA media with vancomycin (0.100 mg/mL) were used respectively.

2.4. Quantitative real-time PCR

C. albicans biofilms (single and dual) were harvested as described above. The mRNA expression changes of *Efg1* in C. albicans biofilms were evaluated using qPCR method adopted from previously reported study [12]. The sequence of each primer was compared in *C. albicans* database using BLAST to assess its specifity[13-14]

2.5. Quantification of violacein in single and dual-species biofilms

The production of purple-colored violacein which is regulated by the quorum-sensing system in *C. violaceum* is an easily observable and measurable marker and is widely used in QS research [15]. In the presented study, after obtaining *E. faecalis* and *C. albicans* cells and supernatans in single and dual-species biofilms as described above, quorum sensing activities were evaluated by slightly modified violacein measurement analysis referenced by Ganesh et al [15]. The amounts of violacein produced by *C. violaceum* after separate treatment with both cell and supernatant solutions of single and dual-species biofilms were compared with each other [15].

2.6. Metabolomic analysis

As mentioned above, the biofilms (single and dual) were formed in 96-well micro plates with minor revisions. Shortly, *C. albicans* (10^6 cfu/mL) were attached for 4 hours individually. After transferring *E. faecalis* (10^6 cfu/mL) the culture medium at the end of 4 hours, the co-culture were incubated at 37 ^{0}C for 24 hours [16].

Previously reported studies were taken as references for preparation of samples and GC-MS-dependent conditions [16.]

2.7. Freeze substitution TEM analysis

TEM analysis was applied as described previously [17]. Briefly, *C. albicans* biofilm cells were harvested by sonication and centrifugation as described above. Briefly, the cell pellets were mixed in 1% agarose and moved to the sample carriers. After freeze-substitution of the cells in liquid nitrogen, the samples were embedded in epoxy resin. Ultra-thin sections were obtained (100 nm thickness). Samples were visualized with a Hitachi HT7800 TEM.

2.8. Statistics

SPSS program (version 23, SPSS, Chicago, IL, USA) were used for the statistical analysis. Comparisons of the groups were performed by Student's t-test. P values <0.05 were statistically significant. Each test were performed at least three times.

Ethics Committee Approval

The authors declared that there is no need an ethics committee approval for this study.

3. Results

3.1. Impacts of *E. faecalis* supernatant and biofilm cells on *C. albicans* hyphal morphogenesis and biofilm development

When grown in the common medium, *E. faecalis* biofilm cells prevented the growth of *C. albicans* cells. However, no significant change was seen in the growth of *E. faecalis* (Figure 1). Although it was not statistically significant a decrease in *C. albicans* biofilm cell counts treated with biofilm culture supernatant of *E. faecalis* was observed (Figure 1)

To analyze the impact of both *E. faecalis* cells and factors released by *E. faecalis* on *C. albicans* hyphal cell formation, *C. albicans* biofilms were formed on the slides. At 48 hours of mature *C. albicans* single-species biofilm formation, a significant amount of hyphal cells were observed (Figure 1b). However, the hyphal formation of *C. albicans* cells was inhibited by both *E. faecalis* biofilm cells and its supernatant when they were incubated together (Figures 1c and 1d respectively).

3.2. *EFG1* gene expression profile in *C. albicans*

To research the inhibitory activity of the biofilm cells and supernatant of *E. faecalis* on *C. albicans* hyphae formation, *Efg1* expression in *C. albicans* was determined by RT-qPCR. The expression of *Efg1* gene in *C. albicans* was found to be significantly downregulated for both treatment ($p < 0.05$) (Figure 2). The results were shown in Figure 2.

3.3. The changed metabolite levels in the single and dual-species biofilms.

In this study, GC-MS based metabolomic analyses were performed to understand how the presence of one microbial species in the dual biofilm environment developed by *E. faecalis* and *C. albicans* affects the other at the metabolic level. A total of 172 different metabolites were determined and 112 of them were identified by the index library. PLS-DA methods were used for both multivariate statistical analysis of GC-MS metabolomic results and the determination of the differences in metabolomic profiles between single and dual-species biofilms (Figure 3). First, the statistical analysis of the models was determined using R2 and Q2 values. The values > 0.7 for all biofilms show us that the method is valid and the models are stable.

The changed metabolite levels determined in the biofilms (single and dual) are shown separately in Table 1. There was no significant difference in the amounts of the rest of the tricarboxylic acid (TCA) cycle intermediates except for succinate and citric acid in both biofilms of *E. faecalis* (Table 1). This result is not surprising considering that *E. faecalis* lacks the tricarboxylic acid cycle. *C. albicans* has lower concentrations of TCA intermediates in the dual-species biofilm when compared to its single-species biofilm (Table 1). Maltose, glucose and leucrose were in high levels in *C. albicans* biofilm alone. The existence of *E. faecalis* in the same environment caused a significant decline in the amounts of these metabolites.

In comparing the both biofilms (single and dual), the concentrations of valine, leucine, glycine, methionine, threonine, and phenylalanine were significantly reduced specifically for *E. faecalis*, and a decrease in the level of tyrosine was also notable for *C. albicans*. Putrescine and pipecolic acid concentrations in the dual biofilm remained significantly which are the most promising results of this study.

3.4. Changes in *Candida* cell wall architecture in single and dual-species biofilms

The cell wall biomass significantly differed in dual biofilm including the thicker β-glucan-chitin layer and the more dense and narrower fibrillar layer of mannan than the cells in biofilm alone (Figure 4).

3.5. Measurement of violacein in single and dual-species biofilms

The amount of violacein produced by *C. violaceum* was determined in single and dual biofilms formed by *E. faecalis* and/or *C. albicans* (Figure 5). Compared with untreated media containing only *C. violaceum* (control), it was observed that *C. violaceum* produced less violacein after separate treatment of *E. faecalis* and *C. albicans* single and dual-species biofilms with both supernatant and cell culture suspensions. When single and dual biofilms of both microorganisms were compared, it was determined that *C. violaceum*, which was treated

with both cell and supernatant suspensions of the dual-species biofilms, produced less violacein for all test conditions except for the supernatant of *E. faecalis*.

4. Discussion

Infections are often considered and treated as a condition caused by a single microorganism, however, in plenty of them, coexistence of multiple human microbiome members is observed. These microorganisms live together in a balance under physiological conditions. Many environmental factors may disrupt this balance consequently single or several species become dominant in the environment [18].

In this study, the effect of interaction between *E. faecalis* and *C. albicans* on biofilm formation was investigated based on microscopy and metabolomics. The results revealed that in dual biofilm, the proliferation of *E faecalis* is not affected by the presence of *C. albicans* however existence of these species in the same environment has an antagonistic impact on the growth of *C. albicans* (Figure 1). Compared to control, the reduction of the production of violacein, which provides QS signal communication in *C. violaceum* treated by single-biofilm cells of *C. albicans* also indicates the presence of a molecule that provides *C. albicans*-induced anti-QS activity in the environment.

In this study, compared to the untreated *C. albicans* cells, the number of *C. albicans* hyphal cells decreased when treated with cell suspension or supernatant of *E. faecalis* biofilm (Figure 2). Therefore, both *E. faecalis* cells and the factors released into the medium have been found to inhibit the hyphal development of *Candida*. Similar to this finding, in recent studies, bacterial-fungal cooccurrence has been reported to have an antagonizing effect on *Candida* cell growth. A study that investigates the interference between *C. albicans* and *Lactobacillus* species showed that *C. albicans* did not grow on the surface of the vaginal mucosa due to the lactic acid produced by the *Lactobacillus* species [19]. The coexistence of *S. aureus* and *C. albicans* in the biofilm environment leads to a substantial increase in the attachment and colonization abilitity of *S. aureus*. Thus, *S. aureus* can use *C. albicans* hyphal cells as a scaffold to development a biofilm [20].

The coexistence of *E. faecalis* and *C. albicans* in the biofilm model developed in our study may have supported the formation of an anaerobic environment due to more oxygen consumption. Under this condition, *Candida* relies on the glycolytic pathway to produce energy. There was not significant difference in the single and dual-species biofilms of *E. faecalis* in glucose consumption. The elevated levels of maltose and leucrose in the dual biofilm are thought to be caused by the existence of *C. albicans*. The bacteria within the biofilm are exposed to a variety of environmental conditions, causing the population to be highly heterogeneous in terms of oxygen content [21]. Fox et al. showed that the hypoxic nature of *C. albicans* biofilms supports the growth of anaerobic bacteria that share the same environment [22].

Compared to *C. albicans* alone, reduced amounts of citric acid, fumaric acid and oxalic acid in the dual biofilm are indicative that *C. albicans* need more energy in the presence of *E. faecalis*. It was reported in a study that □-ketoglutarate dehydrogenase, TCA cycle enzyme, is suppressed by *Efg1* which is a crucial factor for the hyphal development of *C. albicans* [23]. The downregulation of *Efgl* in *C. albicans* obtained in our study may have led to the suppression of a-ketoglutarate dehydrogenase, which may lead to the transition of *C. albicans* into the glyoxylate cycle. Thus, it can be explained as a reason for the accumulation of large amounts of ketoglutaric acid and malic acid in the dual biofilm.

Glycerol metabolism is an important pathway for the synthesis of lipids and (lipo) teichoic acids in *E. faecalis*. Lipids, one of the main membrane components, are needed for energy accumulation [24]. *E. faecalis* has increased lipid-related metabolite synthesis when grown with *C. albicans*. This increase indicates that there is a greater need for lipid-related cell

membrane products such as phospholipids and/or lipoteichoic acids in *E. facealis*.
Putrescine, an important polyamine in cellular survival, does not support cell proliferation in low amounts, on the other hand, overabundant quantity inside cells lead to the inhibition of cell proliferation [25, 26]. In the present study, one of the most important difference was the concentration of putrescine. Compared to *C.* albicans biofilm alone, it enhanced approximately by 10 and 3.4 fold in dual biofilm and *E. faecalis*, respectively. In our previous study, the high level of putrescine detected in the dual biofilms formed by *C. albicans* and *P. mirabilis* supports our current data [16].
Another interesting result of our study was that the pipecolic acid level increased by 24 and 14 fold for *C. albicans* and *E. faecalis* in dual biofilm environment when compared to the both *C. albicans* and *E. faecalis* single biofilms, respectively. The naturally occurring alkyl derivatives of pipecolic acid (piperidine-2-carboxylic acid) are structural components of many biologically active compounds [27]. Also, detailed studies have shown that the organic compound pipecolic acid is an osmoprotectant and plays a role in protecting macromolecules from denaturation. In the osmoregulation stages, which are generally the same in all living organisms, the first stage is the accumulation of potassium and glutamate, followed by the accumulation of small organic compounds by intracellular synthesis or uptake by external media [28]. In our study, higher levels of sugars such as maltose and leucrose in the dual biofilm than *E.* faecalis biofilm alone may have been a threat for *E. faecalis* due to increased osmolarity. *E. faecalis* may have synthesized pipecolic acid known to be an osmoprotectant to deal with this threat. It is known that the synthesis of bacterial pipecolic acid occurs as a byproduct during the catalysis of the proline amino acid, which may explain the low level of proline in the dual-species biofilm obtained from our study.
The alterations in the yeast cell wall as an adaptation to osmotic stress have been highlighted in the literature [29]. We detected the more dense and shorter mannan layer and thicker β-glucan-chitin layer in *Candida* cell wall grown in dual biofilm than the single biofilm. In both cases, the alterations in the cell wall of *C. albicans* are similar to those in cells with and without salt-induced osmotic stress in the study of Ene et al. This strengthens the possibility of increased osmotic stress in the dual biofilm environment [29].
Compared to the single biofilm of both microorganisms significant decrease was observed in many amino acids levels in the dual biofilm. This reduction in amino acid levels in the dual biofilm shows that anabolic reactions are dominant for both species to grow, develop and multiply. It has been clearly defined that amino acid synthesis is required for *C. albicans* biofilm development
The metabolite diversity of both microorganism was affected by each other by increasing the cellular stress due to high carbohydrate consumption, more energy needs, etc was demonstrated in our results. The high levels of putrescine and pipecolic acid synthesized as osmoprotectant by both species may have suppressed the growth of *Candida*. The presented study provided preliminary data for a detailed investigation of the possible role of putrescine and pipecolic acid in the prevention of *C. albicans* via bacterial species.

Conflicts of interest:
The authors declare that they have no conflicts of interest.
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Table 1. Relative metabolite amounts in the biofilms of *E. faecalis* or *C.albicans.*

Metabolites	Ef-Ca/Ca	Ef-Ca/Ef	Pathways
Tricarboxylic acid cycle			
Citric acid	0.09*** ↓	3.95** ↑	
Fumaric acid	0.49* ↓	1.58** ↑	
Lactic Acid	0.38** ↓	-	
Malic acid	2.1** ↑	-	
Ketoglutaric Acid	5.42** ↑	-	
Oxalic Acid	0.4** ↓	-	**Carbohydrate Metabolism**
Pyruvic Acid	-	-	
Succinate	0.45* ↓	0.3** ↓	
Maltose	0.49* ↓	5.11** ↑	
Glucose	0.29** ↓	-	
Leucrose	0.49* ↓	5.43** ↑	
Amino acid metabolism			
Cysteine	0.21** ↓	0.44* ↓	
Serine	0.15*** ↓	0.4** ↓	
Threonine	-	0.33** ↓	
Aspartate	2.05** ↑	-	
Glutamic Acid	2.37** ↑	1.63** ↑	
Proline	0.40** ↓	0.41** ↓	
Tyrosine	0.06*** ↓	-	
Valine	-	0.36** ↓	**Amino Acid Metabolism**
Leucine	-	0.33** ↓	
Alanine	0.44** ↓	0.45* ↓	
Glysine	-	0.40** ↓	
Methionine	-	0.37** ↓	
Lysine	-	-	
Tryptofan	-	-	
Phenylalanine	-	0.43** ↓	
Metabolism of nitrogen containing compounds			
Urea	-	0.45* ↓	
Ornithine	8.74*** ↑	-	
Ornithine-Arginine	7.33*** ↑	-	**Nitrogen Metabolism**
Creatine	-	0.43** ↓	
Other Metabolisms			
Putrescine	9.99*** ↑	3.38*** ↑	**Polyamine metabolism**
Pipecolic Acid	24.2*** ↑	14.10*** ↑	
Ethanolamine	-	3.09** ↑	
Glycerol-1-phosphate	-	9.37*** ↑	**Lipid metabolism**
Glycerol	-	2.53** ↑	

*Compared to dual-species biofilm, the metabolite level was significantly changed in single-species biofilm (*P < 0.5, **P < 0.05, ***P < 0.001)

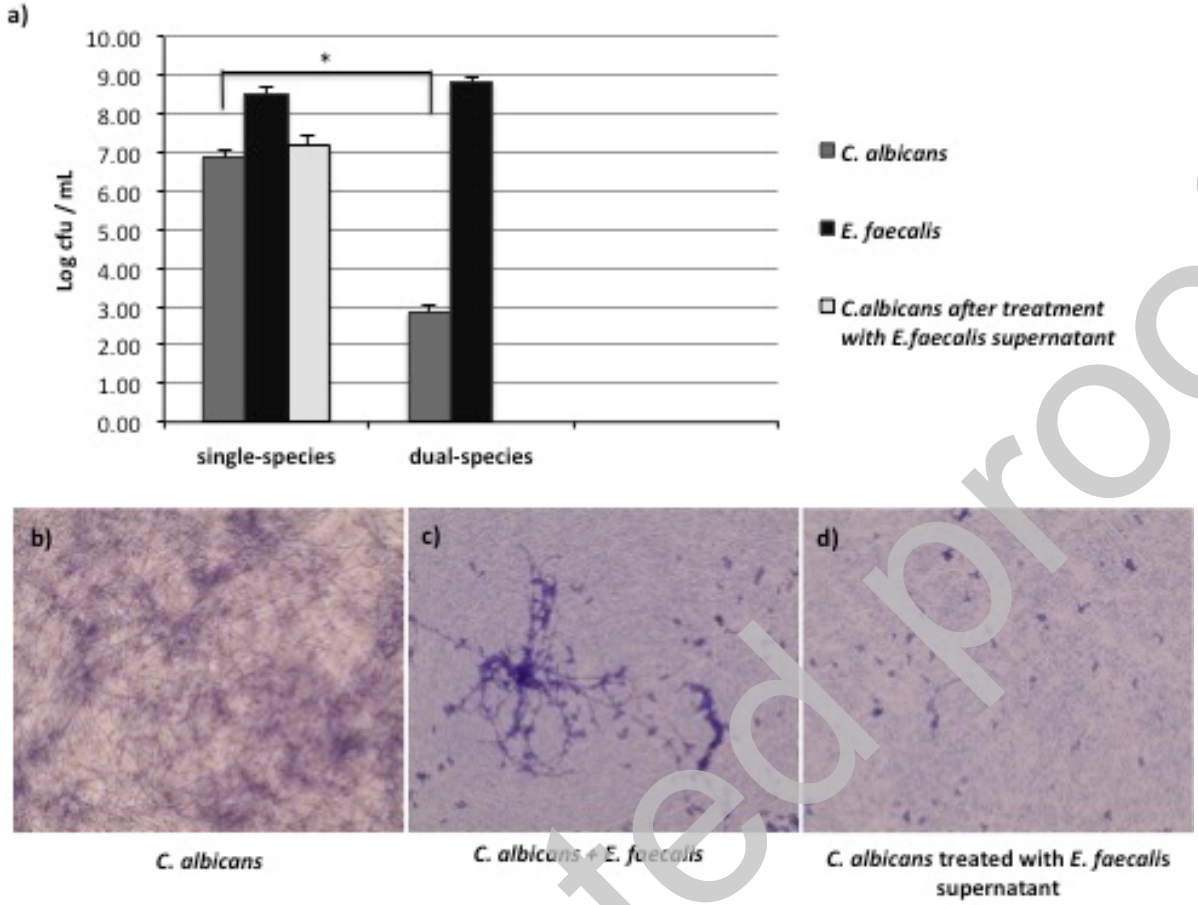

Figure 1. *E. faecalis* biofilm cells prevent the proliferation and hyphal development of *C. albicans*. a) The proliferation of cells in single and dual biofilms (cfu/mL). Compared to *C. albicans* single biofilm, *E. faecalis* prevented the proliferation of *C.albicans cells* in dual biofilm (*, $P < 0.05$). Optical microscope images of b) *C. albicans* biofilm cells formed in 6 well cell culture plate, c) *C. albicans* biofilms in the existence of *E. faecalis* cells and d) *C. albicans* biofilms exposed to the supernatant of biofilm culture of *E. faecalis.*

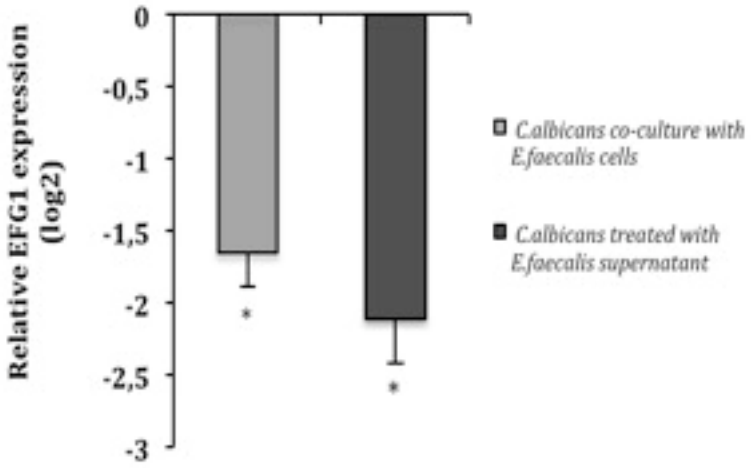

Figure 2. The expression of *Efg1* gene in *C. albicans.* It was significantly downregulated both in the existence of *E. faecalis* cells and in treatment with biofilm culture supernatant of *E. faecalis.* The statistical significance (*, $P < 0.05$) relative to untreated *C. albicans* single biofilm cells.

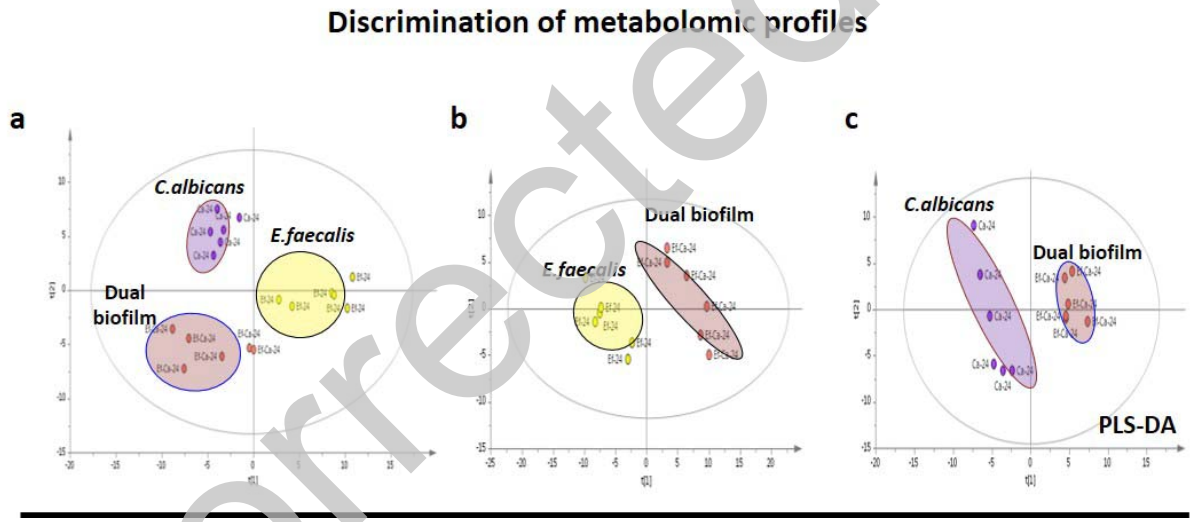

Figure 3. a) PLS-DA score graphs of single and dual biofilm of C. albicans. for metabolomic profile comparison. **b)** PLS-DA score plots show clear separation between *E. faecalis* and its dual-species biofilm. **c)** PLS-DA score plots demonstrate apparent distinction with *C. albicans* and its dual biofilm. Each circle represents the sharp metabolomic distinction in the biofilms.

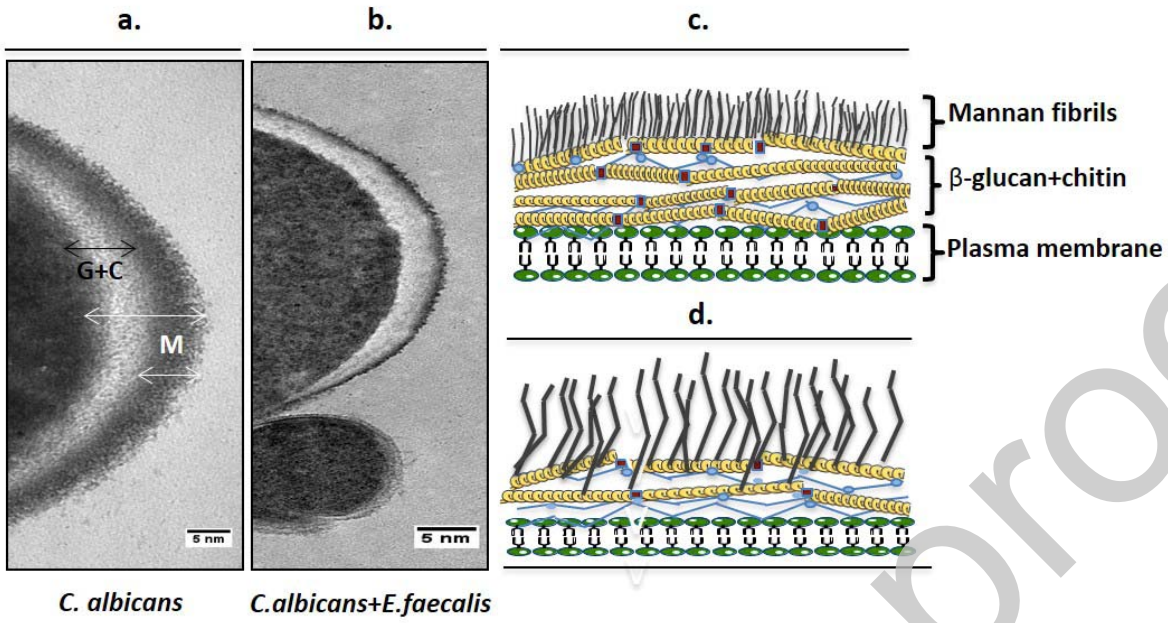

Figure 4. The visualizaton of *C. albicans* cell walls grown in the single (a) and dual biofilms (b). (Presented figures were consisted of ≈100 cells images). Bar, 5 nm. G+C, β-glucan and chitin; M, mannan. Drawings representing the possible structural changes are shown in c (for the cell wall of *C. albicans* in dual biofilm) and d (for the cell wall of *C. albicans* in single biofilm).

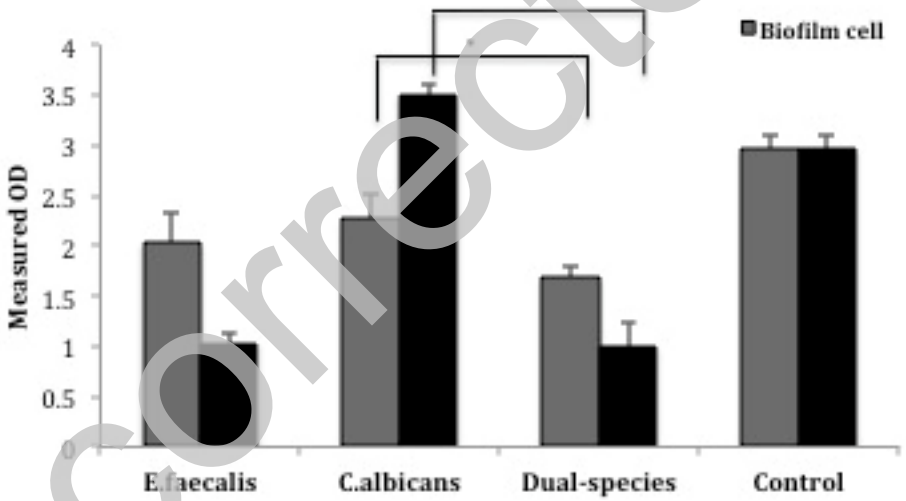

Figure 5. Quantitative measurement of violacein in both single and dual biofilms. Asterisks indicate the statistical significance ($P < 0.05$). A statistically significant decrease was shown for all test conditions compared to the control.