

RESEARCH ARTICLE

Enterococcus faecalis Cell-Free Supernatant Inhibits Hyphal Morphogenesis and Biofilm Formation in *Candida albicans*

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Abstract

Enterococcus faecalis and *Candida albicans* are two of the most significant opportunistic and intricate nosocomial pathogens. They reside overlapping niches as major constituents of the gastrointestinal (GI) and oral microbiome. This study was designed to investigate the interaction between these two opportunistic pathogens that could affect treatment strategies and influence the interkingdom signaling and sensing in the microbiome conceptions. *E. faecalis* was isolated from 150 samples collected from human urine, cattle raw milk, milk products and chicken intestine and identified by both the routine microbiological and molecular identification methods based on 16S rRNA gene. Isolates susceptibility to nine antimicrobials was analyzed using the disk-diffusion method and *E. faecalis* isolates showed multidrug resistance. Most of *C. albicans* isolates were able to form biofilm with different intensities. Cell-free supernatant (CFS) of *E. faecalis* was purified and tested against *C. albicans* and/or biofilm formation. It inhibited the growth of *C. albicans* and their filamentation with regards to biofilm formation *in vitro*. The data presented here demonstrated the potential antifungal activity of *E. faecalis* CFS and could explain the biological interaction between these two microbes in one hand and the possibility of using *E. faecalis* CFS as a biological product to control yeast infection in the other hand.

Keywords: *C. albicans*, *E. faecalis*, 16S rRNA, Biofilm, Anti-Candida factor.

Introduction

Previous literatures are brimful with cases of specific interkingdom interactions, going from highly antagonistic (antibiotic production) to highly synergistic (bacterial-fungal symbiosis). *C. albicans* and *E. faecalis* are found as commensals in the normal mammalian microbiome, including the oral cavity, urogenital and gastrointestinal tract (GIT) [1,2]. These two opportunistic microbes can induce systemic diseases associated with very high morbidity and mortality that render them among the threatening microbes because they have considerably been deemed a co-constituents of polymicrobial infections [3,4]. Whereas, the third and the fourth most prevalent nosocomial agents are *Enterococcus* species and *Candida* species, respectively. *E. faecalis* and *E. faecium* were classified as the most common Enterococcal species [3], while *C. albicans* is the most repeatedly isolated *Candida* species. Co-isolation of *Enterococcus*

species and *C. albicans* from infection sites has been reported [5,6].

Biofilm formation is a common biological feature of *C. albicans* and *E. faecalis* and constitutes a major therapeutic complication [7,8]. *C. albicans* form a biofilm that is polymorphic in its structure and contains planktonic cells, pseudohyphae and true hyphae encased in extracellular polysaccharide matrix which is irreversibly attached to living or inert structures to provide a structural scaffold and protection for biofilm cells [8]. Hence, biofilm presents a barrier against antimicrobial drugs and immune surveillance *in vivo* [8].

E. faecalis can hinder *C. albicans* filamentation which is a significant element in *C. albicans* pathogenicity, raising the potential of a biological antifungal agent development. Graham *et al.* [9] reported that the

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extracellular filtrate of *E. faecalis* at the late logarithmic growth phase had antimicrobial activity. The antimicrobial activity of the *E. faecalis* was partially dependent on Fsr quorum-sensing system which is considered a major regulator of *E. faecalis* virulence associated factor. Other studies showed that the inhibitory substance characterization is between 3 and 10 kDa molecular size and this substance was found to be biologically stable after heat-treatment for 20 min at 90°C [10,11]. The anti-Candida protein (ACP) remained active after treatment with organic solvents, detergents, α -amylase and lipase indicating their proteineous nature. Nevertheless, complete inactivation was induced by proteinase K, while partial inactivation was by pronase E treatment.

This study aimed to investigate the effect of *E. faecalis* cell-free supernatant (CFS) on *C. albicans* biofilm formation. *E. faecalis* was isolated from different sources (human urine, chicken intestine, cattle raw milk and milk products) and then characterized by microbiological and molecular methods and tested against the most used antibiotics. Moreover, *E. faecalis* CFS was purified and tested against *C. albicans* clinical isolates. CFS activity was evaluated against biofilm formation either preformed (mature) or biofilm at zero time (after 90 min. adhesion).

Materials and Methods

Sampling

One hundred and fifty samples were collected and examined for the presence of enterococci. The samples included human urine (n= 30) from patients with impaired kidney function attending the outpatient clinic of Al-Ahrar General Hospital, Sharkia Governorate, Egypt; intestinal contents (n= 45) from fresh carcasses of apparently healthy chicken; milk products (n= 45) (Cheddar, kariesh cheese and yoghurt, 15 sample of each) and cattle raw milk (n= 30) from retail outlets.

Isolation and identification of enterococci

For the isolation of enterococci, Slanetz and Bartley media and Bile-Esculin Agar (BEA) (Oxoid B.V., Landsmeer, Netherlands) were used. After inoculation of agar media for

48 h at 37°C, the suspected colonies of pure cultures were selected for phenotypic characterization. Sodium chloride 6.5% tolerance test using Salt Broth, Modified (Himedia Laboratories M1290) was performed for differentiation of the enterococcal from non enterococcal group D streptococci [12].

Molecular identification

Genomic DNA from the suspected isolates was extracted from 25 representative Enterococci isolates including 5 from milk samples, 3 from milk products, 11 from chicken intestine and 6 from human urine samples using QIAamp DNA Mini Kit (Sigma- Aldrich, St. Louis, USA, catalogue No.51304) according to the manufacture instructions. . The 16S rRNA gene specific for *Enterococcus* species was amplified using the primers 5'-ATC AGA GGG GGA TAA CAC TT-3', and 5'-ACT CTC ATC CTT GTT CTT CTC-3' [13]. Further, the *E. faecalis*-specific primers 5'-GTT TAT GCC GCA TGG CAT AAG AG-3' and 5'-CCG TCA GGG GAC GTT CAG-3' and *E. faecium* -specific primers 5'-TTG AGG CAG ACC AGA TTG ACG-3', 5'-TAT GAC AGC GAC TCC GAT TCC-3' targeting 16S rRNA gene were used for the identification of the isolates [14,15]. The amplification reaction was performed in a final volume of 25 μ L per sample consisting of 12.5 μ L of Dream Taq™ Green master mix (2x), 1 μ L (20 pmol) from each primer, 5 μ L template DNA and 5.5 μ L of nuclease free water. DNA amplification was carried out with cycling condition as previously described [13-15] in a GeneAmp PCR system 9700 (PE Biosystems, Tokyo, Japan).

Antibiotic sensitivity testing

The sensitivity of *E. faecalis* isolates was tested against the following discs (BBL Microbiology Systems, Cockeysville, MD): vancomycin (VA, 30 μ g), gentamicin (CN 10, 120 μ g), doxycyclin (DO, 30 μ g), ciprofloxacin (CIP, 5 μ g), erythromycin (E, 15 μ g), ampicillin (AM, 10 μ g), ceftriaxone (CFT, 30 μ g) rifampicin (RA, 5 μ g) and fosfomycin (FOS, 200 μ g) using Kirby-Bauer standard disc diffusion method [16]. The interpretation of the inhibition zones around the discs was evaluated according to Clinical

and Laboratory Standards Institute (CLSI) [17].

Preparation and purification of CFS from *E. faecalis*

Purification of CFS from *E. faecalis* was performed as described previously [18] with minor modifications. Briefly, *E. faecalis* was cultured in Brain Heart Infusion (BHI) broth for 48 h at 37°C then centrifuged at 12,000 rpm for 30 min at 4°C for harvesting of the cells. After that the CFS was saturated in up to 85% (w/v) pulverized ammonium sulphate. The supernatants were transferred to sterile tubes then filtrated through 0.45 µm membrane filter. Sequential ammonium sulphate precipitation was done to the culture supernatant to achieve 75-85% saturation at 4°C. By centrifugation at 12,000 rpm for 30 min, the precipitated proteins (CFS) were pelleted and dissolved in sterile 20 mmol sodium phosphate buffer (pH 8.0), dialysed against the same buffer overnight at 4°C by using a dialysis membrane-60 (HiMedia), a 12,000-Da cut-off. In the next day, the precipitated protein was dialyzed against Poly Ethylene Glycol (10% PEG) for 6 h. The CFS was then stored at -80°C for further use.

***C. albicans* strains**

For assaying the anti-Candida activity of *E. faecalis* CFS against *C. albicans*, ATCC® 90028™ reference strain and *C. albicans* clinical isolate were used.

Determination of CFS anti-Candida activity

The agar-well diffusion method [11] was used for assaying the CFS anti-Candida activity against *C. albicans* reference strain (ATCC® 90028™) and *C. albicans* clinical isolates. An aliquot (100 µL) of appropriately diluted, freshly grown *C. albicans* was spread uniformly on yeast extract peptone dextrose (YPD) agar plates and wells were done into agar by using cork borer. CFS (100 µL) was added to each well, and the plates were incubated at 37°C for 48-72 h. To test the strength and CFS effect on *C. albicans*, different double fold serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) of CFS was performed and the inhibition zone diameter was measured and graded as strong + + +,

moderate + +, weak +, or negative - with reference to their zone diameter.

***In vitro* hyphal morphogenesis assay**

In vitro hyphal morphogenesis assay was performed as described by Cruz *et al.* [10]. Briefly, YPD broth containing fresh culture of *C. albicans* strain was centrifuged at 4,000 rpm for 22 min and the harvested cells were washed once with water. Fungal cells (0.1 mL) at 600 nm optical density (OD600) were inoculated into Roswell Park Memorial Institute (RPMI1640) (Sigma Chemical Co., St. Louis, MO) supplemented with l-glutamine and buffered with morpholine propane sulfonic acid (Angus Buffers and Chemicals, Niagara Falls, N.Y.) with or without CFS. Hyphal morphology was assessed using the light microscope after 2 h.

Evaluating the effect of CFS on *C. albicans* developed and preformed biofilm

Clinical *C. albicans* strain and the reference strain (ATCC® 90028™) were selected for subsequent assays with strong and moderate biofilm-forming phenotype, respectively. The effects of CFS on the initial stages of biofilm formation and on the preformed biofilm were evaluated. *C. albicans* biofilms were prepared on commercially available, flat-bottomed, pre-sterilized 96-well polystyrene microtitre plates (Iwaki®Cell biology, I.C.T, S.L.) as previously described [19,20]. An aliquot (100 µL) of standard *C. albicans* cell suspension was transported to the wells and incubated for 1.5 h (adhesion phase) at 37°C with agitation. Aspiration of the liquid was performed and the wells were washed twice with sterile phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride [pH 7.4] [Sigma, St. Louis, Mo.]) to remove loosely attached yeast cells. RPMI1640 (200 µL) containing different concentrations of CFS was added to each well followed by incubation of the plate at 37°C for 24 h. To examine the impact of CFS on pre-formed biofilms, *C. albicans* biofilms were set up for 24 h at 37°C. After washing the wells twice with PBS, 200 µL of fresh RPMI 1640 medium containing different concentrations of CFS (1024- 128 µg/mL) were added and the plate was incubated at 37°C for 24 h. Wells

containing antifungal agent only and biofilm wells were included and served as negative and positive control wells, respectively. Crystal Violet (CV) assay was used to quantify the resultant biofilm biomass in comparison with untreated controls. This experiment was performed on two independent occasions using three replicates of each strain.

Statistical analysis

The differences in means between effects of CFS on *C. albicans* biofilm were investigated according to orthogonal comparisons (Proc GLM through SAS, 2012)

Results and Discussion

The biological interaction between *E. faecalis* and *C. albicans* was investigated. We focused to isolate *E. faecalis* from different sources and their cell free supernatant (CFS) antimicrobial properties (anti-candida) were evaluated. Out of 150 examined samples, 45 Enterococci were identified as, non-motile Gram-positive cocci, acid producing, catalase

and oxidase negative. The isolates were positive for esculin hydrolysis and revealed good growth pattern at 6.5% NaCl confirming that they belong to enterococcal species. Based on PCR amplification of 16S rRNA gene targeting Enterococci-specific primers for 25 representative Enterococci isolates, an amplicon of approximately 337 bp was observed. By using *E. faecalis* and *E. faecium*-specific primers, a PCR amplicons of 310 bp and 658 bp were detected (Figure 1). *E. faecalis* ($n = 9$), *E. faecium* ($n = 11$), and 5 *Enterococcus sp.* were identified. Most of the studies performed on Enterococci support the same findings; for instance, Nováková and Kačániová [21] reported that the most isolated Enterococci from the various GIT segments of broilers were *E. faecium* and *E. faecalis*. Moreover, Enterococci specifically *E. faecalis* and *E. faecium* were the most predominant isolates from raw milk and urine samples [22,23].

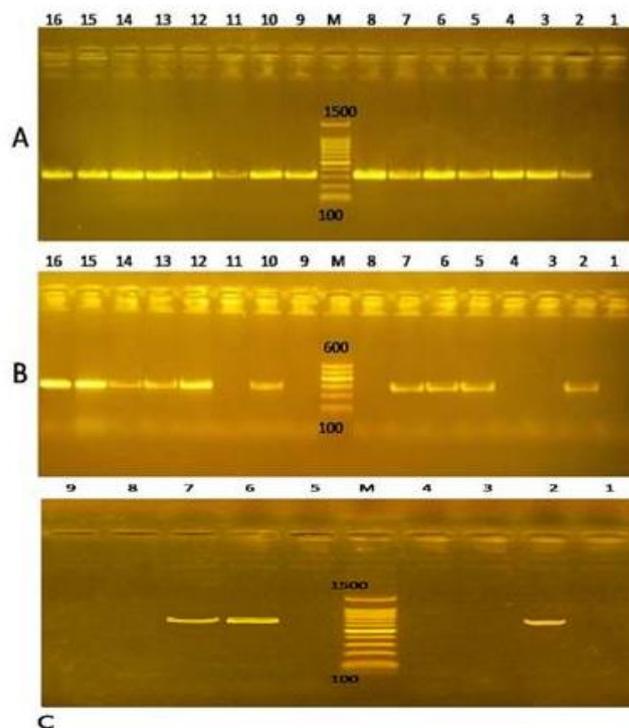


Figure 1: Agarose gel electrophoresis for the amplicons of 16S rRNA genes of Enterococcus genus, *E. faecalis* and *E. faecium*. A: Lane M, 100-bp ladder; lane 1, negative control; lane 2, positive control; lanes 3–16, positive isolates at 337bp amplicons for Enterococcus genus. B: Lane M, 100-bp ladder; lane 1, negative control; lane 2, positive control; lanes 5-7,10, 12-16 positive isolates at 310 bp amplicons for *E. faecalis*. C: Lane M, 100-bp ladder; lane 1, negative control; lane 2, positive control; lanes 6,7 positive isolates at 658 bp amplicons for *E. faecium*.

Table 1: Antibiogram of *E. faecalis* isolates and their cell-free supernatant inhibition zones` diameters on *C. albicans* by agar- well diffusion method

<i>E. faecalis</i> isolates code	Antibiotic (concentration µg)										<i>E. faecalis</i> CFS Zone diameter and Grade	
	VA (30)	CN (10)	CN (120)	AM (10)	RA (5)	CIP (5)	DO (30)	FOS (200)	E (15)	CFT (30)	mm±SD	Grade
Ch9	S	S	S	S	S	S	S	S	R	S	32± 0.84	+++
Ch23	S	S	S	S	I	S	S	S	R	R	14± 0.21	++
C3	S	R	S	S	R	I	S	S	R	R	15± 0.23	++
C1	S	R	S	S	R	R	R	S	R	R	14± 0.20	++
M2	S	S	S	S	S	S	S	S	I	S	15± 0.20	++
M4	S	S	S	S	R	S	S	S	S	S	13± 0.19	++
Y11	S	R	S	S	R	S	R	S	R	R	15± 0.22	++
U2	S	R	S	S	I	R	R	S	R	S	13± 0.20	++
U21	S	S	S	S	R	S	R	S	I	S	11± 0.19	+

Source of *E. faecalis* producing ACF: Ch; Cheese, C; Chicken, M; milk; Y; yougart; U, urine. VA, vancomycin; CN, gentamicin; DO, doxycyclin; CIP, ciprofloxacin; E, Erythromycin; AM, ampicillin; CFT, ceftriaxone; RA, rifampicin; FOS, fosfomycin. S: sensitive, R: resistant, I: intermediate.

As depicted in Table 1, all *E. faecalis* isolates were sensitive to vancomycin, ampicillin, gentamicin (120 µg) and fosomycin. While resistance to erythromycin, cefotaxime, ciprofloxacin, rifampicin and gentamicin (10 µg) was observed. This was in concordance with the results of previous studies [21-24]. This resistance pattern may be attributed to the unconstrained regulations of antibiotics use or due to resistance gene transfer among species and genera.

After ammonium sulphate precipitation and dialysis, the antifungal activity of *E. faecalis* CFS was evaluated. As demonstrated in Table 1 and Figure 2, CFS of *E. faecalis* isolated from chicken, yoghurt and human urine samples have antifungal activity against *C. albicans*. The impact was concentration-dependent, as reflected by the inhibition zone

diameters and CFS from *E. faecalis* isolated from Cheddar cheese (Ch9) have the highest effect. This is in context with Roy *et al.* [25] who have shown that *E. faecalis* isolated from Cheddar cheese produce effective antifungal protein. These data are in agreement with others [11, 18,25,26] who found that the identified *E. faecalis* non-haemolytic anti-candida protein (ACP), might be developed as an effective treatment for candidiasis and its complications especially in immunocompromised individuals. The CFS is protein in nature as confirmed by boiling the culture supernatant and testing its ability as antimicrobial and anti-biofilm formation. No biological activities were determined after boiling (at 100°C for 30 min) either by using the concentrated or diluted CFS (data not shown).

Table 2: Effect of *E. faecalis* CFS on *C. albicans* preformed biofilm and initiation of biofilm formation (at zero time) by Crystal violet method

<i>C. albicans</i> biofilm	Preformed biofilm after 24h		Biofilm at zero time after 1.5h adhesion	
	ATCC® 90028™	Clinical isolate	ATCC® 90028™	Clinical isolate
Control (without CFS)	0.239± 0.005 ^{a***}	0.355±0.005 ^{b***}	0.200±0.004 ^{a***}	0.320±0.012 ^{b***}
With CFS (µg/mL)				
1024	0.207±0.031	0.314±0.056	0.110±0.0106	0.130±0.013
512	0.209±0.0	0.317±0.057	0.112±0.0112	0.133±0.0133
256	0.212±0.032	0.321±0.058	0.114±0.0114	0.136±0.0136
128	0.214±0.032	0.325±0.059	0.114±0.0116	0.139±0.0139

Results shown were the average of optical density (OD) of three independent experiments ± SD.

Means± Standard Deviation in the same raw carrying different superscripts are significantly different ($p < 0.001$).

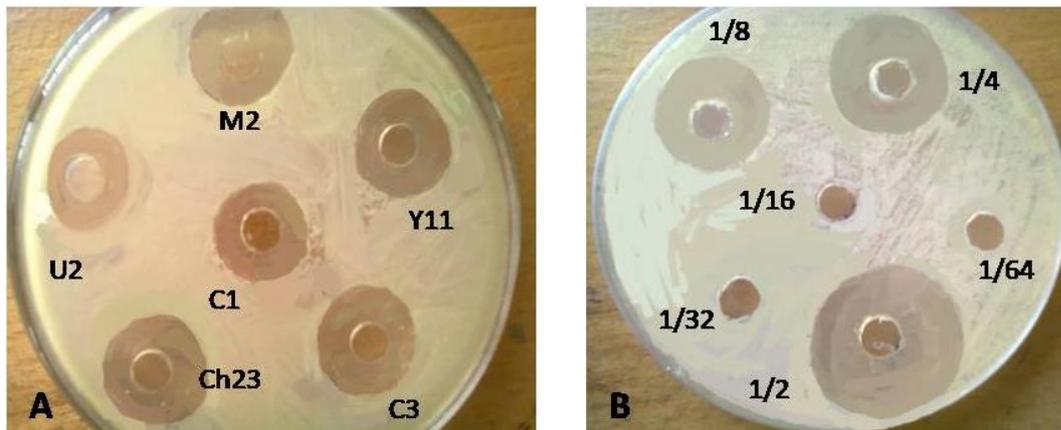


Figure 2: Biological activity of *E. faecalis* CFS against *C. albicans*. The zone of inhibition was detected in CFS of *E. faecalis* isolated from chicken (C), cheese (Ch), yoghurt(Y), milk (M) and urine (U) (A), Different concentration of dialyzed concentrate of *E. faecalis* ACF of cheese origin (Ch9) showing zone of inhibition against a lawn of *C. albicans* (B).

The antifungal activity of *E. faecalis* CFS on *C. albicans* biofilm was investigated using CV assay. As reflected by OD values in Table 2, the preformed *C. albicans* biofilm was more resistant to anti-Candida factor (ACF) versus biofilm after 1.5 h adhesion phase in comparison to untreated control OD. Therefore, we hypothesized that the stage of growth on which the *C. albicans* cells were exposed to ACF may affect overall biofilm development. We showed that ACF displayed reduced overall biofilm formation either at zero time or mature biofilm. This result was compatible with previous studies that both species antagonize each other's virulence in both nematode infection and *in vitro* biofilm models through the inhibition of hyphal formation, raising the potential that it may be utilized as an efficient antifungal agent [9,10]. Although in comparison to control levels, biofilm formation was generally inhibited, there was as yet noteworthy biofilm remaining confirming that early preventative intervention is generally useful in Candidiasis control [27]. Nevertheless, when we observe the data in its entirety, we suggest that CFS exhibits positive biological anti-Candida effects, though further detailed studies are required to confirm our observations as a hope to develop biological anti-candida therapy to manage biofilm formation and treat the associated complications.

Conclusion

E. faecalis CFS after ammonium sulphate precipitation and dialysis has a potential as an antifungal agent and it was able to affect one of the significant pathogenesis elements, biofilm formation. However, further studies should be designed to investigate the possibilities of using purified CFS as a novel anti-Candida therapeutics.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

خلاصة الانتيروكوكس فيكالز الخالية من الخلايا تثبط التشكل الخيطي وتكوين بيوفيلم الكانديدا ألبياكنز

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اثنين من أهم الممرضات الانتهازية، المثيرة للجدل هي بكتيريا الانتيروكوكس فيكالز وخميرة الكانديدا ألبياكنز. تحتل الانتيروكوكس فيكالز و الكانديدا ألبياكنز محاور متداخلة كمكونات الميكروبيوم في كل مكان من الجهاز الهضمي والفم. هذه الدراسة تم تصميمها لتحري التفاعل ذو الصلة البيولوجية بين اثنين من الميكروبات المهمة سريريا والتي يمكن أن تؤثر على استراتيجيات العلاج وكذلك تأثير فهمنا للإشارات والاستشعار في الميكروبيوم. لذلك تم عزل الانتيروكوكس فيكالز في هذه الدراسة من عدد 150 عينة من البول البشري وعينات اللبن ومنتجات اللبن الماشية وعينات الأمعاء من الدجاج والتي تم تصنيفها بواسطة الخصائص البيوكيميائية والطرق الجزيئية على أساس جين 16S rRNA. وقد تم تحليل قابليتها لعدد 9 من المضادات الحيوية باستخدام تقنية الانتشار عبر القرص و قد أظهرت بعض عزلات الانتيروكوكس فيكالز مقاومة للأدوية المتعددة. بالإضافة الى استخلاص المركب المضاد للكانديدا ألبياكنز الذي تنتجه الانتيروكوكس فيكالز و اختباره ضد الكانديدا ألبياكنز بطريقة الانتشار في الأجار وعليه تم تقييم تأثيره على تكوين بيوفيلم الكانديدا ألبياكنز. كان مستخلص الانتيروكوكس فيكالز الخالي من الخلايا يحول دون نمو الكانديدا ألبياكنز كما انه كان قادرا على تثبيط تكوين البيوفيلم في المعمل. البيانات المعروضة هنا تثبت ان هذا المستخلص لديه إمكانات كعامل مضاد للفطريات.