Inhibition of *Candida albicans* biofilm development by unencapsulated *Enterococcus faecalis* cps2

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**Abstract**

**Background/purpose:** In the oral environment, *Candida albicans* interacts with many bacteria, including *Enterococcus faecalis*. We investigated the susceptibility of *C. albicans* biofilm development to the presence of unencapsulated *E. faecalis* cps2 in comparison with reference strains (*E. faecalis* ATCC 29212) or their respective spent medium (collected at 6 hours).

**Material and methods:** Crystal violet stain was used to measure the total biofilm mass, whereas quantitative real-time polymerase chain reaction was used to analyze the change in expression of the mRNA of hypha morphology (ALS1 and ALS3) and biofilm maturation (EFB1).

**Results:** At the intermediate stage, *C. albicans* resisted the presence of each *E. faecalis* strain tested and their spent medium. However, at the maturation stage, the unencapsulated strain was stronger in reducing *C. albicans* biofilms than the reference strain (*P < 0.05*). At this maturation stage, the transcription levels of each gene tested decreased in the presence of either *E. faecalis* strains or their respective spent medium. The unencapsulated strain was more pronounced in reducing ALS1/ALS3 expression, whereas the respective spent medium had a similar capability to restrict the expression of EFB1.

**Conclusion:** This study showed, the unencapsulated strain is more effective in inhibiting *C. albicans* biofilm development compared with the reference strains. In contrast, the secreted molecules produced by each strain tested are necessary in controlling the growths of *C. albicans* biofilm.

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Introduction

Enterococcus faecalis is a Gram-positive bacterium that has the ability to colonize a variety of sites in humans, including the oral niche. Like E. faecalis, Candida albicans is a fungal species that exists in many niches in the human body, including the gastrointestinal tract and the oral cavity. Both organisms are often found as coisolates in samples collected from endodontic-related infections, especially those linked with chronic periodontitis, as well as from root-filled teeth with periapical lesions. This indicates that conditions in root canal teeth favoring infection with either are similar.

A report by Pinheiro et al. showed that the majority of E. faecalis isolates taken from root canal teeth were of the CPS (capsular polysaccharide) type 1 (cps1) genotype. However, a report by our group found that the dominant strain found in saliva and infected root canals is the E. faecalis cps2 genotype, a strain belonging to serotype C, and its virulent traits are associated with the presence of the surface CPS. This indicates that in infected root canal teeth, the E. faecalis cps2 strain may differ from those in other oral niches. Moreover, a previous report showed that E. faecalis QA29b (a nonstarter food isolate) is an incongruent cps2 strain. This strain carries the full-length cpsK promoter region. Thus, the presence of IS6770 suggests that at some points the nonencapsulated phenotype may be advantageous during some points in the E. faecalis life cycle and disadvantageous at others, suggesting a role in its adaptation. Because C. albicans and E. faecalis have shown antagonistic relationships in the Caenorhabditis elegans model, where E. faecalis inhibits hyphal formation of C. albicans, we hypothesized that the absence of the cps expression could enable the bacterium to communicate with C. albicans through antagonistic interactions while the fungus grows as biofilm.

A number of studies have also described changes in gene expression levels during biofilm development of C. albicans. Of these, ALS1 and ALS3, which belong to the ALS (agglutinin-like sequence) gene family, encodes cell surface glycoproteins. Another gene, EF81, has been reported to be constitutively expressed under most growth conditions of C. albicans. All genes have been demonstrated to be upregulated in C. albicans hyphae, which suggests that they may play a role in biofilm development by this organism. Considering the above-mentioned information, we used an unencapsulated E. faecalis cps2 strain, based on the presence of IS6770, to investigate its effect on C. albicans growth and biofilm formation in vitro. For this reason, we used crystal violet (CV) assays to measure the biofilm mass and the quantitative polymerase chain reaction (qPCR) method to evaluate the altered mRNA expression of the ALS1, ALS3, and EF81 genes. Analysis of the in vitro interaction of E. faecalis and C. albicans may contribute to the understanding of the behavior of the unencapsulated E. faecalis cps2 strain in the human body environment.

Materials and methods

The unencapsulated E. faecalis cps2 strain used in this study was a clinical isolate. This strain was isolated from one of the endodontic patients in our previous study. PCR- and qPCR methods were used to determine CPS genotyping characterization and to determine the encapsulated or unencapsulated strain by detecting the presence of the insertion sequence (IS6770). To visualize the presence or absence of the CPS, expressed by this strain, we used the staining method, stain-Ail (Sigma-Aldrich, St. Louis, MO, USA) (Figure 1). E. faecalis strain ATCC 29212 was used as a control during testing. All E. faecalis strains were maintained in brain–heart infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom) with 30% (v/v) glycerol at −80 °C until testing. C. albicans used in this study was C. albicans ATCC 10231 that was routinely propagated in yeast extract–peptone–dextrose (YPD; 1% yeast extract, 2% peptone, 2% glucose) agar plates or, when indicated, in yeast nitrogen base (YNB; Difco Laboratories, Detroit, MI, USA) medium (pH 7), supplemented with 50 mM glucose.

To obtain spent medium samples, we used a method as described previously. E. faecalis was grown in 20 mL BHI broth. Then, 10 mL of the medium was taken from overnight cultures at a middle exponential stage of growth (6 hours) and centrifuged at 5000 g, 10 minutes (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was filter sterilized through a 0.22-μm filter (Millipore, Billerica, MA, USA). Protein concentration in the spent medium was determined using the Bradford method. Spent medium was diluted in phosphate buffer saline (PBS; Sigma-Aldrich) to yield 10 µg/mL and 100 µg/mL concentrations and used immediately or stored for short periods at −20 °C. The pH of the spent medium was adjusted to pH 7.

Biofilm staining with CV and the colony-forming unit assay

For the biofilm assay, yeast cells of C. albicans were grown overnight at 35 °C. After incubation for 16 hours at 37 °C with...
E. faecalis cps2 inhibits C. albicans biofilm

In this study, the E. faecalis cps2 type was determined as an unencapsulated strain by using qPCR,7 and the result was visually confirmed by All-stain (Sigma–Aldrich, St. Louis, MO, USA) (Figure 1). The unencapsulated strain was further tested for its effect on the susceptibility of C. albicans biofilm growth in intermediate (24-hour) and mature development (48-hour) phases, whereas E. faecalis ATCC 29212 was used as a reference strain. As shown in Figures 2A and 2B, in comparison to the control, neither E. faecalis strains tested nor their spent medium had any inhibitory effect on preadhered C. albicans biofilms after the intermediate phase (P > 0.05). After a prolonged incubation time (48 hours), only the unencapsulated strain was sufficient to reduce biofilm mass (P < 0.001). At this maturation stage, the number of viable cells detected within this biofilm was significantly reduced by approximately 50%, compared with the control (P < 0.005), because of the addition of spent medium produced by either unencapsulated E. faecalis cps2 or the reference strain ATCC 29212. Our data also found that 10 µg/mL protein concentration of the spent medium was sufficient to reduce biofilm mass, and a similar trend was observed when using a higher protein concentration (100 µg/mL; not shown). We also
observed that untreated 48-hour biofilms (controls) of *C. albicans* generated a higher biomass than their 24-hour counterparts; however, this increase was not significant ($P > 0.05$). Furthermore, comparisons between 24-hour and 48-hour biofilms treated with the *E. faecalis* strains tested or their spent medium did show a significant difference in reducing total biomass ($P < 0.05$). In parallel, the viability of cells remaining in the biofilm measured using the CFU assay showed that, in general, cell viability decreased in proportion to the elimination of biofilm biomass for each time point measurement (Figure 2B).

**Different effects of unencapsulated *E. faecalis* cps2 and its spent medium on the expression of ALS1, ALS3, and EFB1 in *C. albicans* biofilm**

Based on the data of measuring *C. albicans* biofilm mass, we sought to determine whether the capacity of the bacterium tested and its spent medium in inhibiting biofilm formation took place through the regulation of certain biofilm-related genes. As shown in Figure 3, when each *E. faecalis* strain tested was added to *C. albicans* biofilm for 24 hours, the expression of each gene had greater than onefold upregulation in comparison with biofilm control without *E. faecalis* ($P < 0.05$). Interestingly, a similar trend in the expression of ALS1 and ALS3 genes was observed against the 48-hour biofilm in our system. By comparing to the intermediate stage (24 hours), the transcription level of these hyphae-specific genes was decreased progressively ($P < 0.05$). For EFB1, our data showed that the unencapsulated *E. faecalis* cps2 strain had a stronger capacity in reducing this gene (>60%) after the 48-hour incubation time, compared with the reference strain (ATCC 29212) that showed <10% ability ($P < 0.05$).

To uncover whether the spent medium from each of these strains had an effect in reducing the ability of *C.
organisms. A previous study reported that often present in root canal infections as persistent micro-

from clinical oral samples. In our study, we also found that Enterococcus faecalis

growth as biofilms, we assessed the expression of all three genes mentioned above of C. albicans biofilm grown in YPD medium containing spent medium from each E. faecalis strain tested. We observed that after 24 hours, irrespective of the source of the spent medium, more than twofold increases in ALS1, ALS3, and EFB1 expression levels were recorded, compared with that observed in the controls (P < 0.05). However, a 48-hour exposure of the candidal biofilm to the spent medium resulted in minimal effects on induction of the expression of ALS1 and ALS3 (Figure 4). Their transcription levels were comparable with those of the negative control. The expression of EFB1 was also modulated by spent medium. Figure 4 shows that the added spent medium led to more than a 40% decrease in the transcription level of EFB1 mRNA. Statistical analysis showed that either spent medium derived from unencapsulated or reference strain had a stronger effect in down-regulating the EFB1 gene in C. albicans biofilm cells, compared with the control (P < 0.05).

Discussion

Biofilm formation and the inhibition of C. albicans growth by oral bacteria are a general phenomenon in the oral ecosystem, and together with E. faecalis, this fungus is often present in root canal infections as persistent microorganisms. A previous study reported that E. faecalis cps1 strain, a non-CPS phenotype, is the more common isolate found in canals of root-filled teeth with periapical lesions. This result appears to contradict our findings, wherein we found that E. faecalis cps2 is a dominant strain isolated from clinical oral samples. In our study, we also found that genetic diversity within E. faecalis isolates, as revealed with Enterobacterial Repetitive Intergenic consensus (ERIC)–PCR was not only found among different cps types, but also among isolates belonging to the same cps type, including cps2. In the current study, we demonstrated that the silencing of cps2 genes in our E. faecalis clinical isolate was attributable to the presence of IS6770. This corroborates earlier findings that the CPS phenotype may not necessarily be expressed in an E. faecalis cps2 genotype.

Because E. faecalis–C. albicans interactions have potential implications in the oral ecosystem, we assumed that the observed difference in CPS phenotype within E. faecalis strains tested may have a different effect on C. albicans, when the fungus is growing as biofilm. Therefore, in the current study, we assessed the effect of the presence of the clinical isolates of E. faecalis cps2, unencapsulated strain or its spent medium, on the formation of C. albicans biofilms on microtiter plate in vitro. The E. faecalis reference strain (ATCC 29212), which shows encapsulated phenotype (Figure 1), is needed for the evaluation and validation of the assay.

When taken individually, each type of E. faecalis strain tested here had significant effects, either inhibitory or stimulatory, on C. albicans biofilm formation. When the phase of biofilm development was evaluated, both E. faecalis strains tested showed a similar stimulating effect at the intermediate phase (24 hours), with no apparent antagonism on C. albicans biofilm, indicating the ability of the bacteria and yeasts to coinhabit. It seems that at the 24-hour interval of biofilm development, the promoting effect could be attributed to the presence of E. faecalis as a source of peptidoglycan, which in turn triggers C. albicans hyphal growth. Additionally, because E. faecalis produces large amounts of extracellular superoxide, the presence of this bacterium might induce a reactive oxidative stress-stimulated filamentation of C. albicans. Thus, it is important to emphasize that, in the presence of unencapsulated E. faecalis cps2, the number of C. albicans cells increased in specific time points during the development of the biofilm comparatively to control. However, at some point, the relationship between these microorganisms becomes antagonistic. As shown in the current study, a reduction on C. albicans cell viability was found after 48 hours following inoculation of the unencapsulated E. faecalis cps2 strain. This result highlights the capacity of the unencapsulated E. faecalis cps2 strain to interfere with C. albicans biofilm formation in a time-dependent manner. Indeed, this result is indicative that the presence of IS6770 in the cps locus of unencapsulated E. faecalis cps2 strains resulted in a greater ability to adhere to C. albicans, as occurs when the bacterium interacts with epithelial cells.

In contrast, bacteria cell products appear to have a similar capacity in reducing C. albicans biofilm formation, also in time-dependent fashion. As shown in Figure 2, each bacterium spent medium substantially reduced the density of candidal biofilm at the maturation stage along with the amount of live cells, compared with the biofilm at the intermediate phase. This suggests that unlike their CPS phenotypes, spent medium (or active components therein) produced by E. faecalis cps2 is inhibitory toward Candida biofilm. This result is consistent with previously reported experiments that the inhibitory effect is secreted rather than cell associated. We further noted that the 10-μg/mL protein concentration in spent medium produced by each was enough to reduce the biofilm formation of C. albicans. A

Figure 4  Spent medium, containing 10 μg/mL protein, from unencapsulated Enterococcus faecalis cps2 strain or E. faecalis ATCC 29212 that was added into Candida albicans biofilm for 24 hours and 48 hours, respectively. Means of ALS1, ALS3, and EFB1 transcription level were deduced from three separate experiments is presented with SD. * Indicates statistically significant (P < 0.05) difference between transcription level of the EFB1 genes in C. albicans biofilm, compared with the negative control. SD = standard deviation; UEs = unencapsulated E. faecalis strain.
similar trend was found at the higher protein concentrations (100 μg/mL, not shown), indicating the spent medium effect was not dependent on the protein concentration used.

To complement all of these data, we also aimed to determine how these bacteria affect the differential expression of \textit{C. albicans} biofilm-related genes (ALS1, ALS3, and EFB1) in biofilms. The products produced by ALS1 and ALS3 genes are highly expressed \textit{in vitro}. Both ALS1 and ALS3 are key adhesins for biofilm formation by \textit{C. albicans}, and they play important roles during biofilm development. The \textit{EFB1} gene is constitutively expressed under most growth conditions and is frequently used as a normalization gene in the real-time PCR quantification of other \textit{Candida} genes. This gene is appropriate to quantitatively measure the damaging effects of antifungal agents against mature biofilms.

In this study, we observed consistent results between the expression of the cell wall adhesins ALS1/ALS3, which were upregulated in the intermediate stage (24 hours), and stimulating effect for biofilm formation, an adhesin-dependent phenotype of \textit{C. albicans}. The stimulating effect decreased significantly after prolonged incubation (48 hours; \(P < 0.05\)). However, at this maturation phase, the transcription levels of ALS1 and ALS3 mRNA were comparable with the control (Figure 3). This reveals that, at the intermediate stage, hypha formation, which is favorable for \textit{C. albicans} adhesion, and biofilm formation are not specifically sensitive to the bacteria. However, at the maturation stage, the fungus failed to offset the biofilm formation inhibition caused by the presence of \textit{E. faecalis} strains used in this study. Our data further show (Figure 3), at the maturation stage, that the unencapsulated strain had a stronger capacity to reduce the transcription level of \textit{EFB1} mRNA, compared with the reference strain that showed less ability. In this case, we cannot yet say whether the reductions observed at this maturation stage—the time factor—are a general phenomenon in \textit{C. albicans} biofilm formation, as suggested by Nailis et al.

Collectively, these results indicate that the intermediate biofilm phase of \textit{C. albicans} proved resistant to the presence of each tested strains of \textit{E. faecalis}. Neither the presence of the bacteria strains nor their spent mediums inhibit the expression of genes encoding ALS1 and ALS3, which are proteins for adhesive properties. Thus, at this point, increased expression of the hypha-related genes are associated with increased adherence to the microtiter plate and increased biofilm mass and cell counts. Additionally, the stimulating effects of both encapsulated and unencapsulated \textit{E. faecalis} strains tested on \textit{Candida} biofilms were proven to depend on the application time. In contrast to regulation in the intermediate biofilm phase, the stimulating effects as well as regulation of ALS1 and ALS3 genes were decreased significantly after 48 hours. This means that in the presence of unencapsulated \textit{E. faecalis} bios2, \textit{C. albicans} has a decreased ability to maintain the hyphal phenotype and grow into a mature biofilm, which is indicated by the significantly decreased \textit{EFB1} gene expression after the biofilm maturation stage (Figure 3).

In this study, we observed that after 24 hours, greater than twofold increases in ALS1, ALS3, and \textit{EFB1} gene expression levels were recorded when adhered \textit{C. albicans} was treated with spent medium derived from the tested \textit{E. faecalis} strains, compared with those observed in the controls. However, exposing the candidal biofilm to the spent medium for a longer incubation time (48 hours) resulted in minimal effects on inducing the expression of ALS1 and ALS3 genes. Their transcription levels were comparable with those of the negative control (Figure 4). The \textit{EFB1} gene was also modulated by spent medium. The added spent medium derived from either \textit{E. faecalis} strain led to a significant decrease in the transcription level of \textit{EFB1} mRNA, compared with the control.

Based on the results described above, if the transcription levels were positively associated with protein production, our data might suggest that ALS1 and ALS3 proteins function in response to the presence of \textit{E. faecalis} tested or their respective spent medium at intermediate stages of biofilm development. This was consistent with the strain’s ability to induce the expression of biofilm maturity marker, the \textit{EFB1} gene. Interestingly, at the maturation phase, significantly reduced \textit{C. albicans} biofilms occurred, which is evident from the reduction of biofilm mass and cell’s viability by unencapsulated \textit{E. faecalis} cps2 strain and of hypha-related genes (ALS1 and ALS3) expression by both strains. At this maturation phase, the presence of \textit{C. albicans} appeared to protect \textit{E. faecalis} from cell death. Thus, the survival \textit{E. faecalis} strains may have the ability to produce secreted molecules, and changed the environment. This resulted in retarding the hypha formation, thus leading to the defect of biofilm maturation as shown in this study by the reduction in biofilm metabolic activity as indicated by decreased \textit{EFB1} gene transcription levels. In this case, the biofilm inhibition effect was not dependent on the bacteria CPS phenotype. It could be attributable to the secreted substances as reported by Cruz et al., and which we did not identify in this study. However, as both yeast and hyphal forms of \textit{C. albicans} are capable of biofilm formation, our data are not able to explain whether the existence of \textit{E. faecalis} strains tested inhibited the growth of yeast or hypha forms of \textit{C. albicans} after the 48-hour incubation period on the microtiter plate. In this matter, we are not ruling out the possibility that the expression of hypha-related genes is varied. It depends on which \textit{C. albicans} strain was examined. From a clinical perspective, our data may suggest a potentiation effect of antifungals seen with unencapsulated \textit{E. faecalis} cps2. However, we do not know if the antagonistic effect on \textit{C. albicans} biofilm formation is a common character of unencapsulated \textit{E. faecalis} cps2. Alternatively, the exact role of \textit{cps2} gene and CPS on the biofilm formation of \textit{C. albicans} is not clearly identified in the current study. Further investigation is necessary to compare the influence on the biofilm formation of \textit{C. albicans} between unencapsulated \textit{E. faecalis}, which has the \textit{cps2} gene in the presence of IS6770, and the encapsulated isogenic strain, which has the \textit{cps2} gene in the absence of IS6770.

Irrespective of the mechanism involved, the results of the present work suggest that \textit{E. faecalis} cps2 carrying the IS6770 sequence is an unencapsulated phenotype. When the bacterium interacts with \textit{C. albicans}, it has a capacity to inhibit the fungus as it develops as biofilm. The
antagonistic effect relates to the process of biofilm maturation, when the secreted molecules were produced. Additional studies are necessary to fully understand the complex relationship between unencapsulated \textit{E. faecalis} cps2 and \textit{C. albicans} while the fungus grows as biofilm.

**Conflicts of interest**

The authors have no conflicts of interest relevant to this article.

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