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ORIGINAL ARTICLE

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

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KEYWORDS
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genes

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.[1] 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.[2] This indicates that conditions in root canal teeth favoring infection with either are similar.

A report by Pinheiro et al[3] 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[4] found that the dominant strain found in saliva and infected root canals is the *E. faecalis* cps2 genotype, a strain belonging to serotype C,[5] and its virulent traits are associated with the presence of the surface CPS.[6] 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 *cps*2 locus, but its *cps* gene is not expressed because of an insertion sequence, IS6770, inserted in the cpsC—cpsK promoter region.[7] 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,[8] 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*.[9–11] Of these, *ALS1* and *ALS3*, which belong to the ALS (agglutinin-like sequence) gene family, encodes cell surface glycoproteins.[12,13] Another gene, *EFB1*, has been reported to be constitutively expressed under most growth conditions of *C. albicans*.[14] 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.[13,15,16] 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 *EFB1* 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.[4] PCR[5] 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).[7] To visualize the presence or absence of the CPS, expressed by this strain, we used the staining method, stain-All (Sigma-Aldrich, St. Louis, MO, USA) (Figure 1).[17] *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 50mM glucose.

To obtain spent medium samples, we used a method as described previously.[18] *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

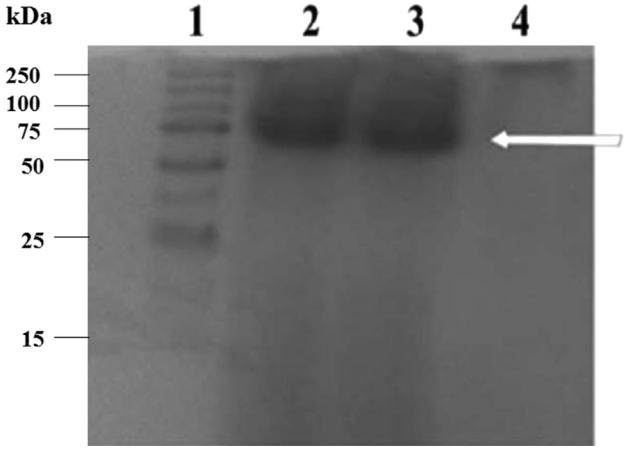

Figure 1 Stain-all staining of heat extracts of different *Enterococcus faecalis* strains after electrophoresis in an acrylamide gel. Lanes: 1 = prestained protein markers (New England BioLabs); 2 = reference strain (ATCC 29212); 3 = encapsulated *E. faecalis* cps2 (strain that showed no positive result after qPCR); 4 = unencapsulated cps2 (strain that showed positive result after qPCR). The arrow indicates locations of CPS. CPS = capsular polysaccharide; qPCR = quantitative polymerase chain reaction.

aeration in YNB, cells were harvested via centrifugation (5000*g*, 5 minutes) and then suspended in YNB adjusted to an optical density at 600 nm of 0.2 ($OD_{600} = 0.2$). *C. albicans* biofilm was grown on 96-well microtiter plates (Iwaki, Tokyo, Japan) that were previously coated with human salivary proteins (1 hour at 37°C).[19] Plates were first incubated for 90 minutes with a cell suspension containing 1.8×10^6 cells/mL, counted using a hemacytometer, and then washed twice with PBS to remove nonadherent yeast cells. For mature biofilms to develop, 100 μL fresh YNB medium was added and the plates were incubated at 37°C for 24 hours and 48 hours at 30°C. Following incubation, media containing dispersal cells were discarded and the wells were washed three times with 150 μL sterile PBS. The biofilms were then stained with 100 μL (1%) CV (Sigma-Alrich) for 15 minutes. They were then washed three times with PBS to remove unbound CV dye and dried at room temperature. The stains were released from the biofilms by adding 1 mL absolute ethanol, and the wells were incubated on a rocking platform for 20 minutes at room temperature. Absorbance was recorded at 590 nm. Each biofilm assay was run in triplicate, and the means ± standard deviations of three separate experiments were calculated and plotted.

E. faecalis from culture stock was grown in BHI agar (Oxoid) overnight at 37°C, and was harvested via centrifugation (5000*g*, 10 minutes). The bacterium was subsequently grown aerobically in liquid medium containing 70% YNB and 30% BHI (vol/vol) for 16 hours. To test the capability of *E. faecalis* to inhibit biofilm mass and the expression of *ALS1*, *ALS3*, and *EFB1* genes expressed by *C. albicans* biofilm, 100 μL of a suspension-containing bacterium [2×10^7 colony-forming units (CFU)/mL] was added into microtiter plates containing *C. albicans* biofilm. The CV assay was performed as described above after each incubation period (24 hours and 48 hours). Alternatively, for assays in which only *E. faecalis* spent medium was used, the 30% BHI fraction was replaced by sterile spent medium prepared as mentioned above. *C. albicans* biofilms added to PBS (pH 7.2) instead of *E. faecalis* cells or its spent media were included to serve as negative controls. Each assay was performed in triplicate and repeated in two independent experiments.

In addition to the CV assay, the CFU assay was used to estimate the viable number of *C. albicans* cells. For this assay, 10-fold serial dilutions in PBS were made, and the number of CFU was determined by plating the yeast cells from 100-μL aliquots onto YPD agar, which contained gentamicin sulfate (50 μg/mL), to kill the bacterium. The number of colonies (CFU/mL) was counted after the cells were incubated for 48 hours at 37°C under aerobic conditions.

Quantitative real-time PCR

The mRNA expression changes of *ALS1*/*ALS3* and *EFB1* in response to the tested *E. faecalis* strain or its respective spent medium were assessed using qPCR. To do this, *C. albicans* 24-hour and 48-hour biofilms were used for the isolation of total RNA (RNeasy Mini kit; Qiagen, Valencia, CA, USA). Then, 3 μg RNA was treated with DNase I (TaKaRa Bio, Tokyo, Japan) and used for cDNA synthesis using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was further diluted 1:5 with sterile MilliQ water. The cDNA was conserved at −80°C until used.

To quantify cDNAs (which had been synthesized from RNA as described above), we used primers that previous studies showed to correspond to *C. albicans ALS1*, *ALS3*,[20] and *EFB1*.[14] The PCR mix contained 5 mL template-diluted cDNA, 100nM of forward and reverse primers, Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Life Technologies, Carlsbad, CA, USA), and passive reference (ROX; Invitrogen). The final volume was 25 mL. PCR reactions were run on The ABI StepOnePlus™ Real-Time PCR Systems with conditions including a 10-minute denaturation step at 95°C followed by 40 cycles of 95°C (15 seconds) and 60°C (1 minute). The melt curve profile was as follows: 95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds. In this study, the *ACT1* was used for the normalization of qPCR data.[21] The formula, fold change $= 2^{-\Delta\Delta Ct}$, was used to calculate, *ALS1*, *ALS3*, and *EFB1* expression levels,[22] at the different time intervals set in this study during biofilm formation relative to the expression in the negative control (*C. albicans* ATCC 10231). The C_t values were provided from qPCR instrumentation and were imported into a Microsoft Excel 2010 spreadsheet. The experiment was carried out in triplicate, and three independent experiments were performed. The collected data were expressed as mean ± standard deviation, and the mean quantitative gene expression patterns were compared via Student *t* test using the SPSS Win 12.0 program (SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

Results

Different effects of unencapsulated E. faecalis cps2 and its spent medium on the biomass and viability of C. albicans as 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

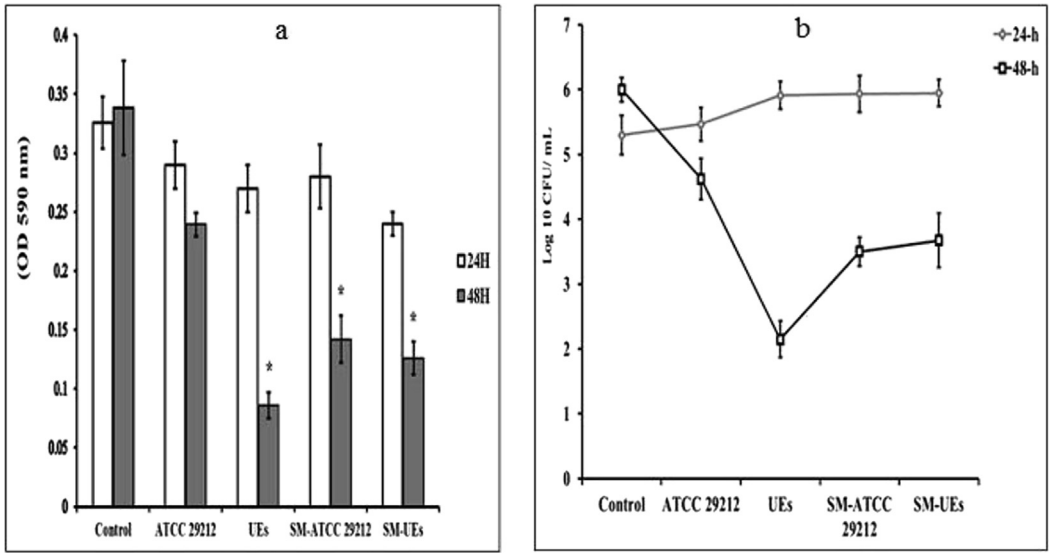

Figure 2 Effects of unencapsulated *Enterococcus faecalis* cps2 or *E. faecalis* reference strain (ATCC 29212) or their respective spent medium, which contains 10 μg/mL protein, on established *Candida albicans* ATCC 10231 biofilms and cell viability within the biofilms. After each time point (24 hours and 48 hours). (A) Biofilm masses were quantified using crystal violet staining and (B) viability cell within the remaining biofilms were assessed using CFU evaluation. Data are means ± standard deviations of three independent experiments performed in triplicate. * Statistical significance ($P < 0.05$) of observed differences between each time point. CFU = colony-forming unit; OD = optical density; SM-ATCC 29212 = spent medium of the reference strain; SM-UEs = spent medium of unencapsulated *E. faecalis* strain; UEs = unencapsulated *E. faecalis* strain.

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.*

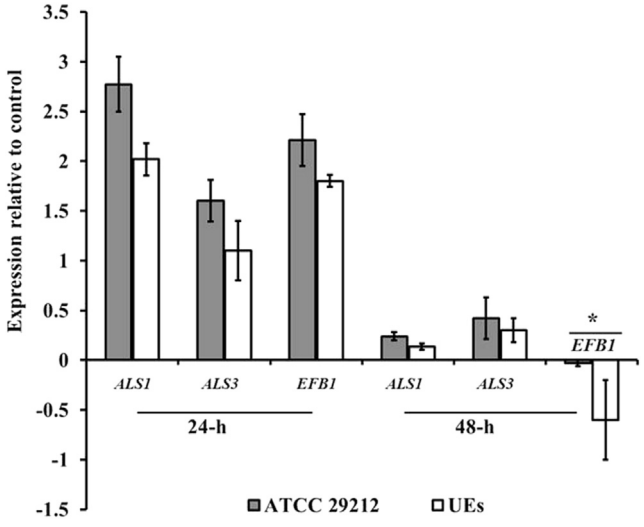

Figure 3 *ALS1*, *ALS3*, and *EFB1* gene expression levels at different time points (24 hours and 48 hours) during *Candida albicans* biofilm development in the presence of unencapsulated *Enterococcus faecalis* cps2 or *E. faecalis* ATCC 29212. Data are shown as the fold regulation calculated using the comparative C_t method. The tested genes were normalized to the *ACT1* gene level. * Indicates a statistically significant difference ($P < 0.05$) in reducing *EFB1* gene between the *E. faecalis* strains tested after 48 hours. UEs = unencapsulated *E. faecalis* strain.

albicans to grow 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,[23] and together with *E. faecalis*, this fungus is often present in root canal infections as persistent microorganisms.[24] 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.[3] This result appears to contradict our findings,[4] 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,

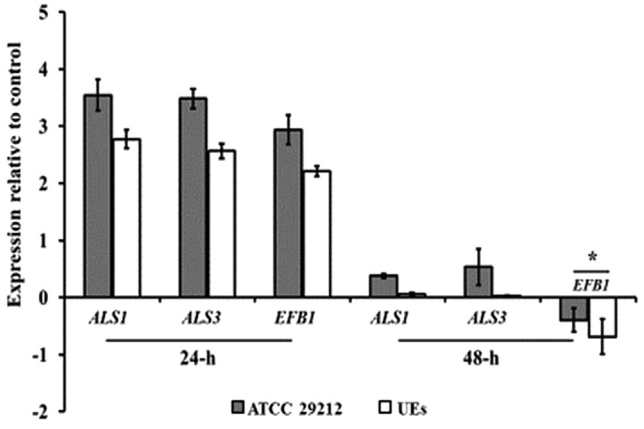

Figure 4 Spent medium, containing 10 μg/mL protein, from unencapsulated *Enterococcus faecalis* cps2 strain or *E. faecalis* ATCC 29212 that was added into *Candida albicans* biofilm for 24 hours and 48 hours, respectively. Means of *ALS1*, *ALS3*, and *EFB1* transcription level were deduced from three 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.

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.[7]

Because *E. faecalis*—*C. albicans* interactions have potential implications in the oral ecosystem,[25] 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.[26] Additionally, because *E. faecalis* produces large amounts of extracellular superoxide,[27] the presence of this bacterium might induce a reactive oxidative stress-stimulated filamentation of *C. albicans*.[28] 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.[8]

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.[8] 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

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 *C. albicans* biofilm-related genes (*ALS1*, *ALS3*, and *EFB1*) in biofilms. The proteins produced by *ALS1* and *ALS3* genes are highly expressed *in vitro*.[13] Both ALS1 and ALS3 are key adhesins for biofilm formation by *C. albicans*,[11] and they play important roles during biofilm development.[10,29] The *EFB1* gene is constitutively expressed under most growth conditions and is frequently used as a normalization gene in the real-time PCR quantification[30,31] of other *Candida* genes. This gene is appropriate to quantitatively measure the damaging effects of antifungal agents against mature biofilms.[14]

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 *C. albicans*.[11,15] 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 *C. albicans* adhesion, and biofilm formation[32,33] are not specifically sensitive to the bacteria.[8] However, at the maturation stage, the fungus failed to offset the biofilm formation inhibition caused by the presence of *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 *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—thus the time factor—are a general phenomenon in *C. albicans* biofilm formation, as suggested by Nailis et al.[13]

Collectively, these results indicate that the intermediate biofilm phase of *C. albicans* proved resistant to the presence of each tested strains of *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.[11,34] Thus, at this time 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 *E. faecalis* strains tested on *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 *E. faecalis* cps2, *C. albicans* has a decreased ability to maintain the hyphal phenotype and grow into a mature biofilm, which is indicated by the significantly decreased *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 *EFB1* gene expression levels were recorded when adhered *C. albicans* was treated with spent medium derived from the tested *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 *EFB1* gene was also modulated by spent medium. The added spent medium derived from either *E. faecalis* strain led to a significant decrease in the transcription level of *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 *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 *EFB1* gene.[14] Interestingly, at the maturation phase, significantly reduced *C. albicans* biofilms occurred, which is evident from the reduction of biofilm mass and cell's viability by unencapsulated *E. faecalis* cps2 strain and of hypha-related genes (*ALS1* and *ALS3*) expression by both strains. At this maturation phase, the presence of *C. albicans* appeared to protect *E. faecalis* from cell death.[8] Thus, the survival *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 *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,[8] and which we did not identify in this study. However, as both yeast and hyphal forms of *C. albicans* are capable of biofilm formation,[9] our data are not able to explain whether the existence of *E. faecalis* strains tested inhibited the growth of yeast or hypha forms of *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 *C. albicans* strain was examined.[35] From a clinical perspective, our data may suggest a potentiation effect of antifungals seen with unencapsulated *E. faecalis* cps2. However, we do not know if the antagonistic effect on *C. albicans* biofilm formation is a common character of unencapsulated *E. faecalis* cps2 in oral infection, because the present study used only the clinical strain isolated from one patient requiring endodontic treatment. Additionally, the exact role of *cps2* gene and CPS on the biofilm formation of *C. albicans* is not clearly identified in the current study. Further investigation is necessary to compare the influence on the biofilm formation of *C. albicans* between unencapsulated *E. faecalis*, which has the *cps2* gene in the presence of IS6770, and the encapsulated isogenic strain, which has the *cps2* gene in the absence of IS6770.

Irrespective of the mechanism involved, the results of the present work suggest that *E. faecalis* cps2 carrying the IS6770 sequence is an unencapsulated phenotype. When the bacterium interacts with *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 *E. faecalis* cps2 and *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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