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Candida albicans impacts carbohydrate metabolism of *Enterococcus faecalis* in interkingdom biofilms

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ABSTRACT

Objectives: This study investigated the transcriptional and physiological responses of *Enterococcus faecalis*, an opportunistic pathogen linked to endodontic infections, when cultivated in dual-species biofilms with *Candida albicans*, a yeast pathobiont found in the oral cavity.

Methods: Forty-eight-hour *E. faecalis* OG1RF biofilms were developed in BHI medium as mono- or dual-species with *C. albicans* SC5314. Biofilms were assessed for biomass, colony-forming units (CFUs), and architecture using confocal microscopy. RNA sequencing was performed on an Illumina platform. Mannose-PTS activity and glycerol quantification assays were conducted to investigate changes in carbohydrate metabolism.

Results: Transcriptomic analysis revealed 149 *E. faecalis* genes differentially expressed in dual-species biofilms. Genes linked to mannose-PTS and glycerol metabolism were notably upregulated. Mannose-PTS activity was significantly higher in dual-species biofilms. Mannose, as the sole carbohydrate source, increased *E. faecalis* CFUs and decreased *C. albicans* CFUs in co-culture, while glucose had no effect. As *C. albicans* is a glycerol net producer, glycerol levels were always higher when *C. albicans* was present, likely contributing to the upregulation of glycerol metabolism genes in *E. faecalis* when in co-cultures

Conclusions: The presence of *C. albicans* alters *E. faecalis* gene expression and metabolism, suggesting metabolic crosstalk that may influence their pathogenicity and role in oral infections.

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Introduction

Biofilm-related infections affect many aspects of human health due to the ability of bacteria and fungi to colonize a variety of human tissues and niches. In the oral cavity, environmental perturbations affect the composition, development and physiology of microbial biofilms and are intimately associated with many oral diseases such as dental caries, periodontal disease, and root canal infection [1]. Displaying high levels of microbial diversity, oral biofilms are known to harbor complex interactions among bacteria, fungi and even viruses [1–3]. For example, strong evidence supports that the dental pathogen *Streptococcus mutans* forms a powerful interkingdom synergism with the yeast pathobiont *Candida albicans*, contributing to more aggressive forms of caries [4,5]. However, another interkingdom interaction that occurs in the oral cavity as well as in the gastrointestinal tract, between the opportunistic bacterial pathogen *Enterococcus faecalis* and *C. albicans*, is much less understood.

A commensal of the gastrointestinal tract, *E. faecalis* is a Gram-positive facultative anaerobe that is also known for its capacity to enter a pathogenic state upon reaching extra-intestinal organs and tissues [6–8]. In humans, *E. faecalis* is predominantly associated with genitourinary and wound infections and is the 3rd leading cause of bacterial endocarditis and leading cause of secondary root canal infections [9,10]. The wide distribution of *E. faecalis* is generally attributed to its robust capacity to form biofilms along with a versatile metabolism and high adaptability, which allows it to survive prolonged starvation, and large fluctuations in pH, temperature, and osmotic pressure [10,11]. Moreover, *E. faecalis* also exhibits intrinsic resistance to several antibiotics and antiseptics, including cephalosporins, aminoglycosides, clindamycin, sodium hypochlorite, and chlorhexidine [12]. Collectively, its intrinsically tolerant nature contributes to the well-documented prevalence of *E. faecalis* in previously treated root canals.

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A dimorphic yeast, *C. albicans*, is a commensal of the human oral cavity, skin, and genitourinary tract [13]In the oral cavity, *C. albicans* can be found in different niches, including tongue, jugal mucosa, periodontal pockets, carious lesions, and root canal infections [14,15]. Importantly, *Candida* spp. can efficiently adhere and form biofilms on dentin and restorative materials [15,16]. In healthy individuals, colonization by *C. albicans* is limited by the salivary flow (shear force) and the natural shedding of epithelial cells. Additionally, some commensal bacteria play a role in inhibiting the development of fungal biofilms [15]. However, in cases of oral microbiome dysbiosis or host immunosuppression, *C. albicans* can overgrow and/or act synergistically with other microbes, resulting in oropharyngeal candidiasis and exacerbation of other oral diseases such as dental caries and root canal infections [17–19]. Regarding the latter, between 3% and 18% of root canals affected by periapical periodontitis are infected with *Candida spp*. [20].

Under certain clinical scenarios, both *E. faecalis* and *C. albicans* can become opportunistic pathogens, and are frequently co-isolated from blood and wound infections as well as genital secretions, urine and feces of hospitalized patients [7,21–25]. Of note, *E. faecalis* and *C. albicans* are co-isolated from about 10% of root canals of teeth affected by persistent periapical periodontitis [20,26–28]. The co-isolation of two or more microorganisms from the same site indicates that the environmental conditions favour each organism individually or, more likely, that they mutually benefit from each other's presence. In fact, clinical and animal studies have suggested that the co-infection of root canals with *E. faecalis* and *C. albicans* can increase severity of periapical periodontitis when compared to mono-infections [28,29].

Currently, there is no consensus in the literature regarding whether *E. faecalis* and *C. albicans* positively or negatively affect each other. Previous research has suggested that *E. faecalis* inhibits hyphal morphogenesis and biofilm formation of *C. albicans*, two important virulence factors of this dimorphic yeast [30–32]. However, other studies have suggested that fungal biofilm formation can be stimulated when exposed to *E. faecalis* and the co-inoculation of *E. faecalis* and *C. albicans* significantly increased the extent of *in vivo* periapical lesions in rats compared with mono-species infection [29]. Another report indicates that *E. faecalis* enhances *C. albicans* invasion of mucosal tissues [33]. The consequences of *C. albicans* proximity on *E. faecalis* physiology is even more poorly understood. In this study, we used a previously established two-species biofilm model to investigate the impact of *C. albicans* on *E. faecalis* global gene expression and metabolism. Among our main findings, we show that *C. albicans* stimulates transcription of genes required for mannose and glycerol utilization in *E. faecalis*. We validate this observation by showing that *E. faecalis* mannose and glycerol metabolism are indeed enhanced in the multi-species biofilm. Our study suggests a significant shift in *E. faecalis'* metabolism induced by the presence of *C. albicans*, which could be linked to higher resistance to nutritional stress and could have implications in oral health.

Materials and methods

Strains and routine culture conditions

The strains and routine culture conditions are listed in Table 1. The mutant strains were subjected to a two-step colony PCR screen (see primers on Table 2) to confirm the transposon was inserted in the correct location, as previously described [34] For biofilm quantification assays, *E. faecalis* and *C. albicans* colony forming units (CFUs) were estimated in Mitis Salivarius bacitracin (MSB) agar and Sabouraud Dextrose Chloramphenicol Agar (both from Sigma-Aldrich, USA), respectively.

Biofilm formation

Mono- and dual-species biofilms were formed as previously described, with minor modifications [35]. Briefly, colonies of *E. faecalis* and *C. albicans* were transferred to BHI broth. The cultures were incubated for 18 h at 37°C in an aerobic incubator. Cells were then harvested by centrifugation at 1,591 g for 5 min at 4°C and then washed twice in sterile phosphate buffered saline (PBS). Pellets were individually suspended in BHI supplemented with 20 mm glucose (BHIG), Chemically defined medium [36] (CDM) + 20 mm glucose or CDM +20 mm mannose. These suspensions were adjusted to OD_{600} of 0.5 for *E. faecalis* (corresponding to a cell density of 3 ×

Table 1. Strains and culture conditions.

Strains	Source	Culture conditions
E. faecalis OG1RF	ATCC (American-type culture collection)	MSB agar, BHI broth
E. faecalis manX::Tn	Kristich et al. [34]	MSB agar, BHI broth
E. faecalis manY::Tn	Kristich et al. [34]	MSB agar, BHI broth
E. faecalis manY2::Tn	Kristich et al. [34]	MSB agar, BHI broth
C. albicans SC5314	ATCC	Sabouraud dextrose agar, BHI broth

Table 2. Primers used for the transposon insertion confirmation.

Locus	*Primer forward*	*Primer reverse*
OG1RF_RS10019 *manX*	5′-GTCAGAATCTGATTT ATCTGATTTTTTTCA-3′	5′-GATTGCCCATTTGTCTTT ATGATCTTC –3′
OG1RF_RS10020 *manY*	5′-CGCGTAACTCGC CAAATAC –3′	5′-GAACAAACCTGCAAC CGC-3′
OG1RF_RS10021 *manY2*	5′-GGCGTTCAACAT TTA TCC AAG G-3′	5′-CACCCATTGCTAAGG AAGCA-3′
Transposon	5′-CTTCTCCTTTACTCATATGTATATCTCCGG-3′	

10^9 CFU ml^{-1}) and OD_{600} of 0.3 for *C. albicans* (corresponding to a cell density of 2.5×10^6 CFU ml^{-1}). Then, 200 µl of each culture was used to inoculate mono-species biofilms in 96-well-flat-bottomed polystyrene microtiter plates. For the dual-species biofilms, 100 µl aliquots of each microbial suspension (1:1 ratio volume) were used to inoculate the 96-well microtiter plates. Plates were incubated at 37°C for 48 h in a 5% CO_2 atmosphere, with the culture medium renewed after 24 h incubation.

For biomass analysis, the biofilms were stained with 200 µl of 0.1% crystal violet (CV) (Sigma-Aldrich, USA) for 20 min. After removal of CV solution, the wells were washed twice with 200 µl distilled water. To extract the dye, 200 µl of acetic acid (33%) (Sigma-Aldrich, USA) was added to each well. Absorbance was read at 575 nm using a microtiter plate reader.

For cell viability, *E. faecalis* and *C. albicans* colony forming units (CFUs) of adherent cells were determined by plating on selective media. After removing the culture supernatant, biofilms were scraped using a pipette tip, transferred to sterile microcentrifuge tubes, serially diluted in sterile 0.9% saline, and 10 µl of the diluted cells plated on MSB agar and Sabouraud Dextrose Chloramphenicol agar, for *E. faecalis* and *C. albicans* recovery, respectively. The plates were incubated in a 5% CO_2 incubator at 37°C for 24 h before colonies were counted.

For confocal laser scanning microscopy (CLSM) analysis, biofilms were formed on IbidiTreat™ 8-well plates (Ibidi, Germany). Biofilms were stained with SYTO 9 obtained from the LIVE/DEAD Bacterial Viability Kit (Invitrogen, USA) and Calcofluor MR2 (Molecular Probes, Inc, USA) (438/405 nm). Images were acquired at a magnification of 600X with a series of optical sections with a thickness of 1 µm with intervals of 0.5 µm throughout the biofilm depth in a confocal microscope Nikon C2plus (Nikon, Japan). After image acquisition, three-dimensional images were synthesized using the software NIS ELEMENTS v. 5.30.01 (Nikon, Japan).

Transcriptome analysis of E. faecalis *in dual-species biofilm with* C. albicans

E. faecalis and *C. albicans* mono-species and dual-species biofilms grown in BHIG were formed in 6-well plates, in biological triplicates and 3 technical replicates. Each well of the mono-species *E. faecalis* biofilm received 5 ml of bacterial suspension prepared as described above; the wells inoculated with both-species received 2.5 mL of the *E. faecalis* suspension and 2.5 ml of the *C. albicans* suspension. Plates were incubated at 37°C for 48 h in a 5% CO_2 atmosphere and the culture medium renewed after 24 h. Following 48 h of biofilm growth, the media was gently removed, and 1 mL of RNA Protect® Bacterial Reagent (Qiagen, Germany) was added to each well. The biofilms were mechanically removed using a cell scraper, homogenized, and incubated for 5 min at room temperature. Next, technical triplicates were combined in conical tubes and centrifuged at 1,500 g, for 10 min at 4°C. The supernatants were discarded, and pellets were suspended in 1 ml of RNA Protect Bacterial Reagent and transferred to 1.5 mL microcentrifuge tubes that were subsequently centrifuged at 6,000 g for 5 min at 4°C. Next, the supernatants were discarded and the pellets stored at –80°C for further use.

For mRNA extraction, cell pellets were gently allowed to thaw and suspended in 1 ml of RNA Protect Bacterial Reagent supplemented with 10 mg ml^1 lysozyme (Sigma-Aldrich) for 30 min at 37°C to weaken the cell wall. Then, samples were centrifuged at 1,200 g for 5 min at 4°C, the supernatants were discarded and total RNA extracted from the lysates using the RNA-Purelink kit (Thermo-fisher, USA) following the manufacturer's instructions. The quantification of the RNA obtained from the samples was performed using Nanodrop One (Thermo-fisher, USA). Samples with A_{260}/A_{280} >2 were diluted to obtain a solution of 100 ng μl^{-1} of RNA and stored at –80°C.

For RNA-Seq analysis, samples were further processed and analysed by SeqCenter (Pittsburg, USA). First, samples were treated with DNase I (Invitrogen, USA) to remove contaminating genomic DNA. The preparation of the libraries was performed using Illumina's Stranded Total RNA prep Ligation with Ribo-zero plus kit and 10-bp IDT to Illumina's index. Additionally, customized probes for ribodepletion of *E. faecalis* and *C. albicans* rRNA were prepared and used in all samples. For *E. faecalis* mono-species biofilms, 12 million reads were generated and for the *E. faecalis* dual-species biofilms, 25 million

reads were generated. The sequencing was performed in NextSeq 2000, providing 2 × 50base readings. Demultiplexing, quality control, and trimming were performed using the software bcl-convert (v.3.9.3). Mapping of the reads was performed by the software HISAT2. Read quantification was done by the software feature Countings™, and the counts were normalized using edgeR's Trimmed Mean of *M* values algorithm. The values were then converted to counts per million (CPM).

Data availability

Gene expression data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE290415.

Measurement of biofilm metabolites

Mono- and dual-species biofilm of *E. faecalis* and *C. albicans* formed as described above were disrupted with a cell scraper and homogenized. The pH was measured immediately using an Accumet™ bench top pH meter (Fisherbrand, USA) and the supernatants collected and stored at −80° C. The Glucose-HK-assay kit (Sigma-Aldrich) and Glycerol GK assay kit (Megazyme, USA) were used to quantify the glucose and glycerol levels from the supernatants, respectively.

Growth of E. faecalis during carbohydrate limitation

The capacity of *E. faecalis* strains to grow using glucose, mannose, or fructose as sole carbohydrate sources was investigated using CDM [36] as the base medium and supplemented with 20 mm of the desired carbohydrate. Briefly, *E. faecalis* OG1RF, *manX:Tn, manY:Tn*, and *manY2:Tn* strains were grown overnight in BHI. The cell pellets were harvested by centrifugation, washed in sterile PBS and resuspended in CDM adjusted to OD_{600} of 0.25. These cultures were used to inoculate the desired media at a 1:100 dilution. The plates were incubated at 37°C in a Bioscreen C plate reader (Growth Curves USA, USA), where optical density at 600 nm was monitored every 30 min for 24 h with 5 seconds agitation before each reading.

Phosphotransferase assays

The PTS assay was performed as described previously [37]. Briefly, mono-species biofilms of *E. faecalis* OG1RF and *E. faecalis manX:Tn* were formed in BHIG in 24-well plates where each well-received 1 ml of bacterial suspension. Dual-species biofilms with *E. faecalis* OG1RF or *E. faecalis manX:Tn* and *C. albicans* SC5314 were formed as described above in 24-well plates where each well received 500 µl of *E. faecalis* suspension and 500 µl of *C. albicans* suspension. Biofilms were grown for 48 h in a 5% CO_2 atmosphere with daily media refreshment. After removal of the medium, the biofilm cells were scraped from the wells in 1 ml sterile saline, transferred to a microcentrifuge tube, dispersed by vortexing and centrifuged at 4,000 g for 10 min. The supernatants were discarded, and the pellets were washed twice with 0.1 M $NaPO_4$/5 mm $MgCl_2$ buffer and then suspended in 1 ml of the same buffer. Permeabilization of the cells was achieved by addition of 50 µl 1:9 toluene:acetone solution and 2 cycles of 2 minutes vortexing. The PTS activity assay reaction was performed by mixing, in a 1-ml reaction, 50 µl of permeabilized cells, 100 µl NADH (β- nicotinamide adenine dinucleotide, Thermo Fisher, USA) 10 µl sodium fluoride (Fisher Scientific, USA), 10 mm (final) mannose, fructose or glucose, 10 mm L-LDL (L-dehydrogenase, Sigma-Aldrich), 0.1 M $NaPO_4$ buffer and 5 mm PEP (phosphoenolpyruvate acid monopotassium salt, Sigma-Aldrich, USA). The reaction was incubated at 37°C and the oxidation of NADH was monitored by recording the OD_{340} every 10 seconds for 2.5 min. The PTS activity was calculated based on the NADH oxidation rate of PEP (ΔOD_{340}/min) and normalized to the protein concentration determined by bicinchoninic acid (BCA) assay. The oxidation levels of NADH-dependent PEP of *C. albicans* mono-species biofilms were calculated and subtracted from the oxidation levels of NADH dependent on PEP of the dual-species biofilms.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Statistical differences of biofilm formation, PTS activity and glucose and glycerol assays were assessed by one-way analysis of variance (ANOVA) for multiple-comparison corrections with Tukey's multiple comparison test or unpaired t-test applying a p value of < 0.05. All experiments were repeated at least twice, each with a minimum of three independent biological replicates. The CPM files of *E. faecalis*' transcriptome in mono-species and dual-species biofilm were analysed using Degust-Interactive RNA-seq analysis (56) using Voom/Limma methodology.

Results

C. albicans induced significant changes in the transcriptome of E. faecalis

We assessed the capacity of *E. faecalis* and *C. albicans* to form biofilms in mono- and dual-species cultures by determining their respective biomass and recovered colonies (CFUs), and by

observing cell morphology and biofilm architecture via CLSM. When *E. faecalis* was grown in a dual-species biofilm along with *C. albicans*, the recovered CFU or biomass were not statistically different as compared to growth in mono-species biofilms (Figure S1A, C). For *C. albicans*, higher CFUs were recovered from mono-species biofilm as compared to dual-species biofilms, though we did not observe a difference in biomass (Figure S1B, C). CLSM analysis of *C. albicans* (purple) and *E. faecalis* (green) revealed that *C. albicans* displayed a mixture of yeast, pseudo-hyphal and hyphal morphologies in both mono-species and dual-species biofilms (Figure S1D).

While global transcriptional responses of *C. albicans* in the presence of *E. faecalis* have been reported [32], the effects of *C. albicans* on the *E. faecalis* transcriptome are as yet unknown. Here, we compare the transcriptome of 48 h *E. faecalis* biofilms grown as a mono-species or in a dual-species biofilm with *C. albicans*. Applying a False Discovery Rate (FDR) of 0.05 and a 1.5-fold cutoff, 115 *E. faecalis* genes were found to be upregulated, and 34 genes downregulated when grown in the dual-species biofilms (Figure 1, Table S1). Gene Ontology (GO) and KEGG analyses revealed an enrichment for genes involved in sugar metabolism, including sugar alcohol glycerol, pyrimidine metabolism, ABC-type transporters, and folate biosynthesis as well as genes associated with virulence and stress responses (Table 3). Among the most upregulated genes, five belonged to the phosphoenolpyruvate: sugar phosphotransferase system (PTS), including genes from the mannose-fructose PTS operon (*manX*, *manY* and *manY2*), and cellobiose/lactose PTS (*celA2* and *celA3*). In addition, genes associated with glycerol transport (*ugpC*) and metabolism (*gldA*, *gldA2*, *glpK*, *glpO*). These findings suggest that growth in proximity to *C. albicans* might reprogram carbohydrate metabolism in *E. faecalis*.

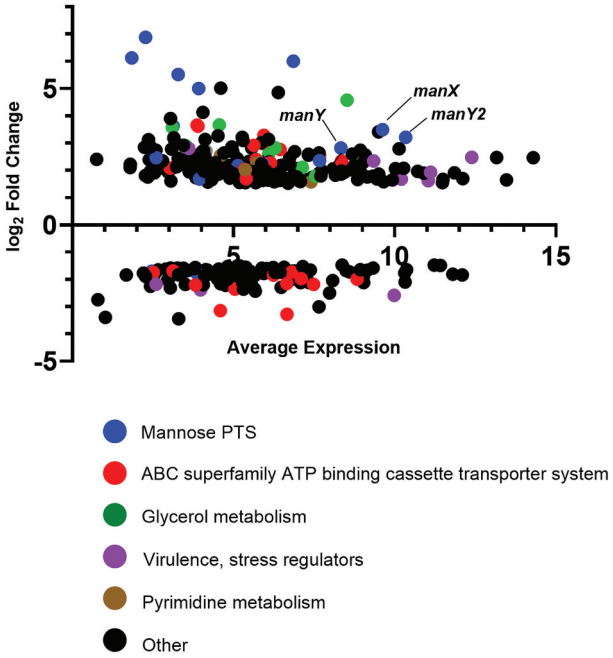

Figure 1. Impact of *E. faecalis* and *C. albicans* coculture on the *E. faecalis* transcriptome. Minus average plot of differently expressed genes of *E. faecalis* in dual-species biofilm compared to mono-species biofilm categorized using gene ontology (GO) classification. Differently expressed genes versus mean levels normalized by gene expression FDR = 0.05, LogFC = 1.5.

E. faecalis-C. albicans *co-culture resulted in significant changes in pH and in glucose and glycerol availability*

As the dual-species biofilm transcriptome results pointed to changes in expression of genes involved in carbohydrate metabolism in *E. faecalis*, we next compared the ability of *E. faecalis* to consume glucose and to acidify the growth media in either mono- or dual-species biofilms. As glucose is the main carbohydrate in BHI, we measured the remaining glucose levels and final pH of supernatants from 48 h mono- or dual-species biofilms. When compared to *E. faecalis* mono-species biofilms, dual-species biofilms showed a significant decrease (~20%) in the amount of glucose still available in the media with no significant differences observed when compared to *C. albicans* mono-species biofilms (Figure 2a). The resting pH of *E. faecalis* mono-species biofilm was significantly more acidic (mean = 4.18) than of *C. albicans* mono-species (mean = 6.65) or dual-species biofilm (mean = 5.28) (Figure 2b). Because *C. albicans* is a net producer of glycerol [38] and the transcriptome analysis revealed the upregulation of *E. faecalis* genes associated with glycerol metabolism, we next determined glycerol concentration in mono- and dual-species 48 h biofilms. Both dual species and *C. albicans* mono-species biofilms displayed significantly higher amounts of glycerol when compared to *E. faecalis* mono-species biofilms (Figure 2c).

PTS activity of mono-species and dual-species biofilms

The upregulation of fructose-mannose-PTS *manXYY2* operon in *E. faecalis*, a high-affinity sugar transport system, in the presence of *C. albicans* leads us to hypothesize that these two microorganisms compete for carbon sources. In this system, *manX* encodes the EIIA and EIIB subunits that ultimately phosphorylate the incoming sugar while *manY* and

Table 3. *E. faecalis* upregulated genes in dual-species biofilms compared to mono-species.

	Locustag	Gene	Description	P value	Linear FC
PTS	**OG1RF_10018**		PTS family mannose/fructose/sorbose porter component IIB	2.01E–08	5.11746596
	OG1RF_10019	*manX*	PTS family mannose porter, IIAB component	1.58E–11	11.2529639
	OG1RF_10020	*manY*	PTS family mannose/fructose/sorbose porter component IIC	1.16E–10	7.02737383
	OG1RF_10021	*manY2*	PTS family mannose/fructose/sorbose porter component IID	1.01E–10	9.24236651
	OG1RF_10456	*lacR*	lactose PTS family porter repressor	8.34E–08	4.50840447
	OG1RF_10745	*celA2*	PTS family porter, enzyme I	2.31E–10	117.519709
	OG1RF_10746		PTS family lactose/cellobiose porter component IIC	2.32E–06	5.51093983
	OG1RF_10750	*celA3*	PTS family porter, enzyme I	2.35E–09	69.4467177
	OG1RF_10751	*lacF*	PTS family lactose/cellobiose porter component IIA	4.65E–10	45.6217579
	OG1RF_10752		PTS family lactose/cellobiose (lac) porter component IIC	1.45E–12	63.8802754
	OG1RF_11552		PTS family porter, mannose/fructose-specific component IIA	2.58E–07	5.23763446
	OG1RF_11981		PTS family glucitol/sorbitol porter component IIA	6.01E–08	5.0982978
	OG1RF_12476		PTS family fructose/mannitol (fru) porter component IIA	2.81E–08	12.322933
	OG1RF_12477		PTS family ascorbate porter, IIB component	2.25E–09	31.9491491
	OG1RF_12478		PTS family fructose/mannitol (fru) porter component IIC	6.08E–08	8.20888814
	OG1RF_12479		PTS family porter component II	7.63E–07	5.71767068
ABC superfamily ATP binding transporter	**OG1RF_10426**	*pptA*	ABC superfamily ATP binding cassette transporter, ABC protein	2.15E–08	4.101271831
	OG1RF_10427	*pptB*	ABC superfamily ATP binding cassette transporter, membrane protein	2.81E–09	4.827308324
	OG1RF_10665	*ugpC*	ABC superfamily ATP binding cassette transporter, ABC protein	1.51E–09	6.76880992
	OG1RF_10835		ABC superfamily ATP binding cassette transporter, ABC protein	1.05E–08	7.53644882
	OG1RF_11131		ABC superfamily ATP binding cassette transporter, ABC protein	2.10E–08	0.19523452
	OG1RF_11470	*pstS2*	Phosphate ABC superfamily ATP binding cassette transporter, binding protein	4.19E–08	5.25453317
	OG1RF_11761		ABC superfamily ATP binding cassette transporter, binding protein	9.93E–11	7.86172917
	OG1RF_11763		ABC superfamily ATP binding cassette transporter, membrane protein	1.58E–10	12.5929416
	OG1RF_11828	*sufD*	Iron-sulfur ABC superfamily ATP binding cassette transporter, membrane protein	2.81E–10	5.78219459
	OG1RF_11829	*sufC*	ABC superfamily ATP binding cassette transporter, ABC protein	5.42E–11	9.71629728
	OG1RF_12354	*fitA*	ABC superfamily ATP binding cassette transporter, membrane protein	5.86E–10	0.1128755
	OG1RF_12370	*opp2D*	ABC superfamily ATP binding cassette transporter, ABC protein	6.23E–10	0.10276066
Glycerol	**OG1RF_10085**		Diacylglycerol kinase catalytic domain protein	3.59E–10	7.06157763
	OG1RF_11146	*gldA*	Glycerol dehydrogenase	6.75E–13	23.7886456
	OG1RF_11147	*dhaM*	glycerone kinase PTS family porter component IIA	8.34E–09	6.01860644
	OG1RF_11148	*dhaK*	Dihydroxyacetone kinase	5.01E–06	4.30268429
	OG1RF_11591	*glpO*	Glycerol-3-phosphate oxidase	1.52E–06	4.51750439
	OG1RF_11592	*glpK*	Glycerol kinase	3.80E–09	12.6638567
	OG1RF_12403	*gldA2*	Putative glycerol dehydrogenase	1.42E–06	6.99669396
Virulence and regulation	**OG1RF_10072**	*gls24*	Stress response regulator Gls24	3.36E–08	5.55942524
	OG1RF_10653	*rr09*	Response regulator	1.25E–08	6.01749821
	OG1RF_10954	*luxS*	S-ribosylhomocysteine lyase	2.62E–08	5.07136315
	OG1RF_11529	*fsrA*	FsrA response regulator	4.61E–07	6.98620499
	OG1RF_11943		Flavodoxin	8.74E–08	8.71187803
Folic acid biosynthesis	**OG1RF_12228**	*folC*	Tetrahydrofolate synthase	5.81E–09	4.86382143
	OG1RF_12514	*folP*	Dihydropteroate synthase	8.12E–08	0.23735884
Pyrimidine metabolism	**OG1RF_11428**	*carA*	Carbamoyl-phosphate synthase, small subunit	2.24E–06	4.38190634
	OG1RF_11429	*pyrC*	Dihydroorotase	1.76E–06	4.8408032
	OG1RF_11431	*pyrP*	NCS family uracil:cation symporter	9.44E–07	4.5677657
	OG1RF_11432	*upp*	Uracil phosphoribosyltransferase	7.34E–08	5.84119425

manY2 act together to transport the sugar across the cell membrane [39].

To verify the contribution of the *manX*, *manY*, and *manY2* genes to the growth of *E. faecalis* using glucose, mannose, or fructose as sole carbohydrate, we took advantage of the availability of the *E. faecalis* OG1RF transposon mutant arrayed library [34] to obtain *E. faecalis manX::Tn*, *manY:Tn*, and *manY2:Tn* mutants. To verify if the *E. faecalis manXYY2* operon was annotated correctly, we aligned the genes from the well characterized mannose-glucose PTS *Streptococcus mutans* operon [40,41] finding that they share substantial (~60–67%) amino acid similarity with each other. Upon confirmation by PCR that the transposon element was inserted at the location originally indicated (Figure S2) and considering that in *S. mutans* this operon is associated with uptake of mannose, glucose and fructose [40,41], we compared growth of *E. faecalis* OG1RF, *manX:Tn*, *manY:Tn*, and *manY2:Tn* in media containing glucose, fructose or mannose as the single sole source of carbohydrate. While all mutant stains grew as well as the parent in fructose, the *E. faecalis manX:Tn* mutant displayed reduced growth defect when grown in glucose. Moreover, all mutants showed more substantial defects in growth yields as compared to *E. faecalis* OG1RF, in particular the *E. faecalis manX:Tn*, *manY:Tn* strains, when mannose was provided as the sole carbohydrate (Figure 3a–c).

To follow up on the phenotype that we observed for the *E. faecalis manX:Tn* mutant strain, we next wanted to study the behaviour of this strain in a biofilm. Here,

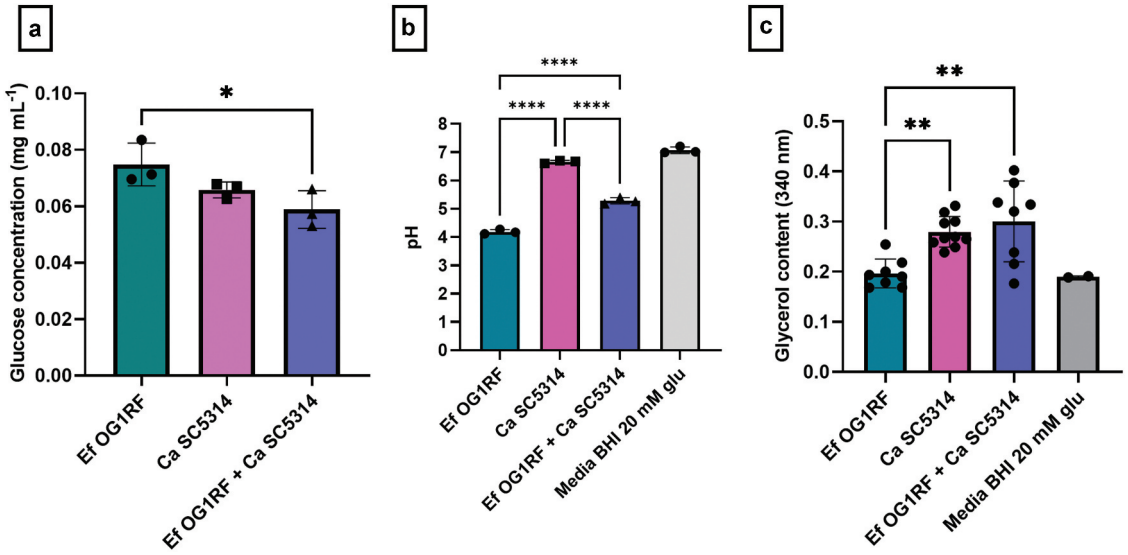

Figure 2. Co-culture of *E. faecalis* and *C. albicans* impacts glucose, glycerol and pH levels. (A) Glucose, (B) pH, and (C) glycerol levels of mono-species and dual-species biofilm supernatants. Mono-species biofilms were formed with either *E. faecalis* OG1RF (green) or *C. albicans* SC5314 (pink) in BHI +20 mm of glucose (BHIG) in 5% CO_2 at 37°C for 48-h. The dual-species biofilms (ef OG1RF+ Ca SC5314) were formed with *E. faecalis* OG1RF and *C. albicans* SC5314 inoculated in a 1:1 ratio of the two inocula in BHIG. Data expressed in mean ± standard deviation. (*) represents $p < 0.05$; (**) represents $p < 0.01$; (****) represents $p < 0.0001$.

E. faecalis OG1RF or *E. faecalis manX:Tn* were each grown in a dual-species biofilm with *C. albicans* for 48 h in BHIG (Figure S3). In this case, we did not observe that cultivation in dual-species biofilms had an impact upon recovered colonies or on biomass (Figure S3 A-C). The final pH values of the *E. faecalis manX::Tn* biofilms were similar to those of the parent strain, for both mono- and dual-species (Figure S3D). Despite the reduced growth yields observed when *E. faecalis manX:Tn* was grown in glucose, we found no statistically significant differences in glucose- PTS activity of *E. faecalis* parent or mutant strains in either mono- or dual-species biofilms (Figure 3d). In contrast, when compared to *E. faecalis* OG1RF mono-species biofilms, the dual-species biofilms displayed ~50% increase in mannose-PTS activity (Figure 3e). As expected, this elevated mannose-PTS activity was lost when *C. albicans* was co-cultured with the *E. faecalis manX:Tn* mutant. While fructose-PTS activity was unaltered in *E. faecalis* OG1RF mono- or dual-species biofilms, the activity was significantly increased in the *E. faecalis manX:Tn* dual-species biofilm (Figure 3f).

Influence of glucose and mannose on E. faecalis-C. albicans *cross-kingdom biofilm formation*

We next determined whether inactivation of *manX*, the gene encoding for the EIIA and EIIB subunits, altered the ability of *E. faecalis* to form dual-species biofilms with *C. albicans* in media containing either glucose or mannose as the carbohydrate source. Here we focused on glucose and mannose as we observed that mutations in the PTS mannose operon did not impact growth or PTS activity in fructose. First, we compared biofilm biomass and CFUs recovered from *E. faecalis* mono-species biofilms (OG1RF and *manX:Tn*) to those cultivated in dual-species biofilms with *C. albicans* in BHI +20 mm glucose (BHIG) or BHI +20 mm mannose (BHIM). We observed that loss of *manX* did not affect biofilm formation or resting pH after 48 h of *E. faecalis* in mono-species biofilms (Figure S3). Moreover, no significant differences in biofilm biomass, CFUs or pH were observed among *E. faecalis* OG1RF-*C. albicans* SC5314 and *E. faecalis manX:Tn-C. albicans* SC5314 dual species biofilms (Figure S3).

Because BHI is a complex media containing different nutrient sources and glucose, we considered observing biofilm formation in a semi-defined medium, and repeated the experiments in CDM supplemented with either 20 mm glucose or 20 mm mannose as sole sources of carbohydrate (Figure 4a–f). We found that supplementation of CDM medium with 20 mm of the desired sugars resulted in phenotypic differences between our *E. faecalis* mutant strains and the wild-type OG1RF (Figure 3). Since this concentration of sugars supported growth of our strains while simultaneously allowing us to observe differences, we found that 20 mm was relevant for our investigation. *C. albicans* mono-species biofilms produced greater biomass than all the other biofilms either when

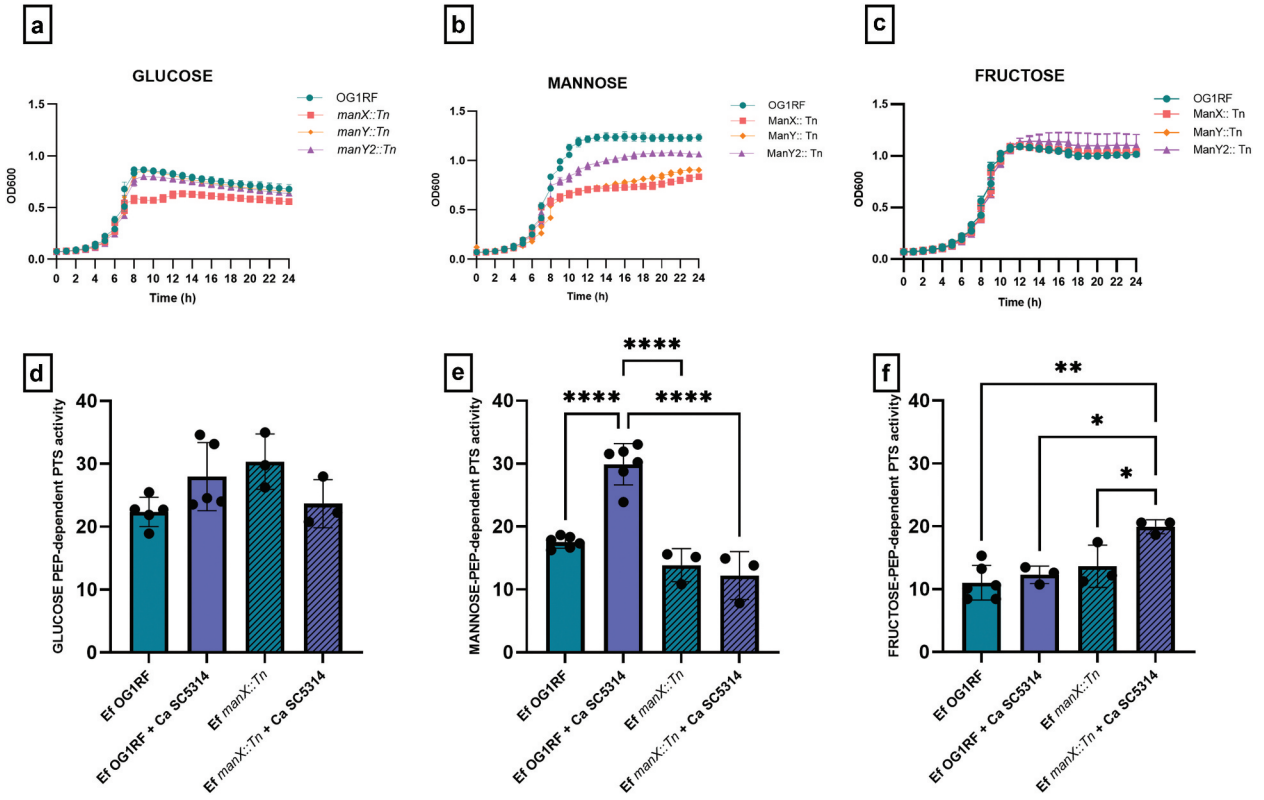

Figure 3. The *E. faecalis* mannose-PTS systems are activated by *C. albicans* and promotes *E. faecalis* growth in the presence of mannose. Growth curves of *E. faecalis* OG1RF, *manX:Tn, manY:Tn* and *manY2:tn* in CDM medium containing 20 mm glucose (a), mannose (b), or fructose (c) as the only source of carbohydrate during incubation at 37°C for 24 h. Dots represent the median of three experimental replicates. Error bars represent the standard deviation. Values expressed in absorbance (OD_{600}). (d–f) Phosphoenolpyruvate (PEP) dependent on PTS porter activity of the mono-species and dual-species biofilms. Mono-species biofilms were formed with either *E. faecalis* OG1RF (green) or *E. faecalis manX:Tn* (striped green) in BHI containing 20 mm glucose (d), mannose (e), or fructose (f) as the only source of carbohydrate in 5% CO_2 at 37°C for 48-h. The dual-species biofilms (Ef OG1RF+ Ca SC5314 in blue) or (or *E. faecalis manX:Tn* + Ca SC5314 in striped blue) were formed with *microorganisms* inoculated in a 1:1 ratio in BHIG. Data expressed in mean ± standard deviation of the oxidation levels of NADH dependent on PEP and normalized by the protein concentration determined by the BCA assay. The oxidation levels of NADH-dependent PEP of *C. albicans* mono-species biofilms were calculated and subtracted from the oxidation levels of NADH dependent on PEP of the dual-species biofilms. (*) represents $p < 0.05$; (**) represents $p < 0.01$ (****) represents $p < 0.0001$. (one-way ANOVA followed by Tukey's multiple comparison tests).

cultivated in CDM +20 mm glucose or CDM +20 mm mannose (Figure 4a,d). Mono-species *E. faecalis* OG1RF and *E. faecalis manX:Tn* biofilms had similar biomass formation in either glucose or mannose. Similarly, dual-species *E. faecalis* OG1RF and *E. faecalis manX:Tn* biofilms showed no statistical difference in biomass levels in either carbohydrate source (Figure 4a,d). Both dual-species biofilms had higher biomass than the *E. faecalis* mono-species biofilms in CDM +20 mm glucose or CDM +20 mm mannose but lower biomass than *C. albicans* alone (Figure 4a,d). Also, *C. albicans* CFU in mono-species biofilms were similar to *E. faecalis* OG1RF and *E. faecalis manX:Tn* dual-species biofilms (Figure 4c). However, in CDM +20 mm mannose grown biofilms, *C. albicans* CFU were reduced in the *E. faecalis* OG1RF dual-species biofilm (Figure 4f), which was not observed for *E. faecalis manX:Tn* dual-species. When grown in CDM +20 mm glucose, the *E. faecalis* CFU counts were not affected by the presence of the mannose PTS operon or the presence of *C. albicans*. However, when grown in CDM +20 mm mannose, the CFU counts of OG1RF dual-species was slightly higher than mono-species, which was not observed when we compared *E. faecalis manX:Tn* mono-species and *E. faecalis manX:Tn* dual-species (Figure 4e). These findings suggest that the availability of different sugars directly impacts the ability of *E. faecalis* to grow in biofilms.

Discussion

Recent studies have highlighted the importance of interkingdom interactions between species of clinical interest and the repercussions these interactions can have on disease establishment and outcomes [15,27,33,42–44]. For example, in oral infections, there is significant evidence of the importance of *C. albicans* interaction with bacterial species in the development of dental caries, periodontitis, and endodontic infections [45–47]. Although isolation of

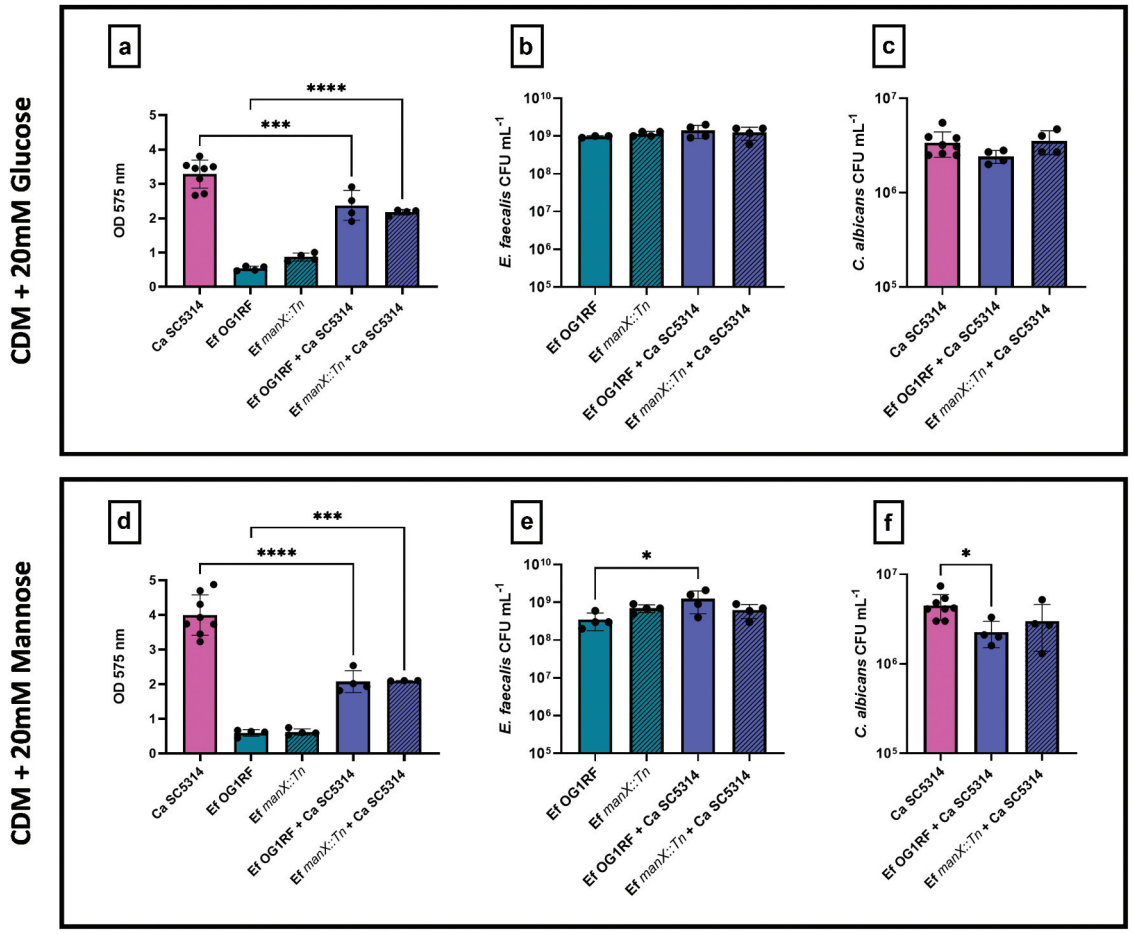

Figure 4. Colony forming units and biomass of *E. faecalis* and *C. albicans* in mono-species and dual-species biofilms when grown with glucose or mannose as the sole carbohydrate. Colony forming units for biomass (a, d), *E. faecalis* (b, e) and *C. albicans* (c, f) counts of *E. faecalis* OG1RF (in green) and *E. faecalis manX:Tn* (in striped green) in mono-species and dual-species biofilms with *C. albicans* SC5314 (in pink). The mono-species or dual species biofilms were grown in CDM +20 mm glucose (a, b, c) or were grown in CDM +20 mm mannose (d, e, f) in a 5% CO_2 atmosphere at 37°C for 48-h. Data expressed in mean ± standard deviation. (*) represents $p < 0.05$; (***) represents $p < 0.005$; (****) represents $p < 0.0001$ (one-way ANOVA followed by followed by Tukey's multiple comparison tests).

fungi from endodontic primary infections has previously been reported, the literature suggests that fungi are more commonly isolated in obturated root canals in which treatment has failed [48–50]. This failure in endodontic treatment is frequently associated with microleakage in coronal sealing of the teeth, which leads to prolonged exposure of the root canal system to the environment [49,51]. An *in vitro* study reported by Miletic et al. suggested that over time, root canals filled with gutta-percha endodontic sealers experienced a slightly higher penetration of *Candida albicans* as compared to bacteria [48]. These findings reinforce the clinical importance of *C. albicans* in persistent endodontic infections, a habitat that is also inhabited by *E. faecalis*.

Previously, *C. albicans* was shown to influence carbohydrate metabolism of *S. mutans* through upregulation of genes involved in nutrient acquisition including PTS genes and other genes involved in the catabolism of carbohydrates [52]. While *E. faecalis* is one of the bacterial species most frequently co-isolated with *C. albicans* from clinical samples of root canal and periapical infections [20,24,27,53], there are conflicting reports available in the literature regarding the nature of the interactions between these microorganisms, with some studies reporting mutualism [29,54–56] and others claiming competition [30–33]. Recently, Alshanta et al. [32] described the effects of *E. faecalis* on the transcriptome of *C. albicans* and proposed that bacterial metabolites decrease the environmental pH which is crucial for inhibition of yeast filamentation. Here, we addressed the impact of *C. albicans* on the transcriptome of *E. faecalis* and validated our findings by conducting a series of biofilm and physiological assays.

Unlike the biofilm enhancement observed following the co-culture of *S. mutans* and *C. albicans* [57], we observed that when biofilms of *E. faecalis* were co-cultivated with *C. albicans* in BHIG, there were no increases in *E. faecalis* biofilm CFUs or total biomass when compared to mono-species biofilms. Interestingly,

as compared to mono-species biofilms, we observed a decrease in *C. albicans* CFUs when co-cultivated with *E. faecalis* mono-species biofilms. When biofilms were cultivated in CDM supplemented with either glucose or mannose, dual-species biofilms showed decreased biofilm biomass and a slight decrease in *C. albicans* CFUs when compared to *C. albicans* mono-species biofilms. Thus, our findings suggest that under the tested conditions, *E. faecalis* and *C. albicans* are likely to compete for nutrients.

To address the changes in gene expression that are triggered by growth in the presence of C. albicans, we performed transcriptomic analysis. Our RNA-Seq analysis revealed that biofilm co-culture with *C. albicans* induced broad changes in gene expression in *E. faecalis*, with genes coding for mannose-PTS and glycerol uptake and metabolism being among the most upregulated. We validated this observation by showing that the mannose-PTS activity was significantly increased when *E. faecalis* was co-cultured with *C. albicans* in comparison to mono-species *E. faecalis* biofilm. This suggests that in a glucose-rich media such as BHI, *C. albicans* likely induces alleviation of carbon catabolite repression (CCR) in *E. faecalis* as the residual glucose levels in the media were significantly lower in the co-culture than in the *E. faecalis* monoculture. Importantly, changes in carbohydrate uptake and metabolism have been shown to impact bacterial virulence and environmental adaptation [58], such that it is possible that this interkingdom interaction affects *E. faecalis* pathophysiological behaviour. We propose that these two microorganisms are likely competing for glucose, based on alleviation of genes known to be under CCR in *E. faecalis*, such as PTS porter (*manX, manY, manY2*) and glycerol metabolism genes (*gldA*, *glpO*, *glpK*).When comparing the growth kinetics of *E. faecalis* OG1RF and three different *man*::Tn mutants in media containing a single carbohydrate source, our results clearly indicate that the *man* operon mediates mannose, and to a lesser extent, glucose uptake. Yet, due to the notoriously promiscuous behaviour of PTS transporters [39], other systems might be partially compensating for the loss of the *man* genes, hence the growth differences in mannose being restricted to final growth yields. In many oral streptococcal species, the *man* operon genes were shown to participate in CCR by competing with other PTS importers for phosphoenolpyruvate (PEP), creating a hierarchy in PEP distribution [41,59–61]. Previous work investigated the role of other genes annotated as mannose and fructose PTS, including OG1RF_RS07760-OG1RF _RS07770 in *E. faecalis* OG1RF. Surprisingly, in that work, the authors detected an improved growth of the *E. faecalis* RS07760 and RS07770 mutants when compared to the parent strain for all carbohydrates tested [62], indicating that the affected genes, while not critical for growth in glucose, mannose or fructose, somehow affect the activity of other transport systems. Of note, we did not find significant differences in the expression of OG1RF_RS07760 and OG1RF_RS07770 in our transcriptome analysis.

Another interesting finding from the RNA-seq study was the upregulation of genes related to glycerol transport and metabolism such as *glpK, glpO, gldA* and *dhaK*. Different than many other lactic acid bacteria, *E. faecalis* can efficiently metabolize glycerol either aerobically or anaerobically [63]. It has been reported that *C. albicans* is a net producer of glycerol, a molecule that can function as a regulatory signal for expression of adhesins that are directly related to fungal virulence and filamentation. Further, the production of glycerol by *C. albicans* has been shown to be elevated in biofilms when compared to planktonic culture [64]. It has been shown that root canal-derived *E. faecalis* strains can utilize glycerol as an alternative energy source to form biofilms and maintain metabolic activity in nutrient-deprived alkaline environments [65]. While this is the first study to investigate the effects of *C. albicans* co-culture on the *E. faecalis* transcriptome, a previous study has shown that the metabolic profiles of *C. albicans-E. faecalis* grown in dual-species biofilm is markedly different than their corresponding mono-species counterparts [42]. Specifically, the dual-species biofilm displayed a different metabolic profile for amino acids, polyamine, nitrogen-containing compounds, and carbohydrates, including enhanced lactic acid and glycerol production. Some of these metabolic changes in the dual-species biofilm, such as glycerol and mannose metabolism, are reflected in the present study. Here, we showed that mannose promotes the growth of *E. faecalis* in co-cultures with *C. albicans* in a *manX*-dependent manner. This finding suggests that in environments where non-preferred sugars such as mannose are more abundant than glucose, the *manX* system might be an important player in the adaptation to a glucose-depleted environment. The relevance of mannose metabolism in *E. faecalis* is underscored by recent studies linking mannose availability and activity of the mannose PTS to increased virulence in wound infection models, as well as enhanced alkaline resistance [66,67]. Moreover, mannan, a mannose-derived polysaccharide, also holds vital importance in fungal physiology, as it constitutes an essential component of *C. albicans* cell wall playing critical roles in structural integrity and immune interactions [38]. Specifically, mannans are linked to cell wall proteins (mannoproteins) and are displayed in the outermost layer of *C. albicans* cell wall, hiding the glucan and chitin layers [68]. Interestingly, at low pH, the *C. albicans* cell wall undergoes substantial remodeling [69] whereby the mannan layer is

partially lost and the glucan/chitin inner layer becomes exposed [70]. Thus, it is possible that the low pH of the dual-species biofilms with *E. faecalis* induces cell wall remodeling in *C. albicans*, that contribute to shedding of mannans or even mannose (through production of mannan-targeting mannosidases) into the environment. Although not mutually exclusive, it is also possible that dead *C. albicans* cells release mannans. Of note, some oral *Streptococcus* species such as *S. mitis, S. parasanguinis*, and *S. sanguinis* have been shown to degrade fungal mannans and use the released mannose as a nutrient source [71]. Studies are underway to address whether mannans and/or mannose are released by *C. albicans* in dual-species biofilms with *E. faecalis* and whether *E. faecalis* can utilize these mannans as a nutrient source.

Our findings here describe a drastic change in *E. faecalis* metabolism when in the presence of *C. albicans*. We can speculate that such metabolic shifts are relevant factors in the lifestyle of *E. faecalis* as a pathobiont, influencing the switch from a health-associated organism to a pathogen. Metabolic plasticity, as reflected in the transcriptomic data reported here, is likely to contribute to the ability of *E. faecalis* to cope with stressors common to the oral environment such as nutritional deprivation, oxidative stress and pH fluctuation [72,73]. How this organism responds to stress at the gene expression level and the subsequent metabolic shifts are both likely to have important implications in the dynamics of *E. faecalis* as it colonizes the root canal.

We acknowledge the limitations of the present study. For instance, the biofilm formation assays were conducted in plastic plate wells. While it is possible that gene expression dynamics and organism interactions could differ on a different substrate, such as dentin, our study nonetheless provides valuable insights into the dynamics of interkingdom interactions [74]. Another limitation of this study is that root canal infections usually have a polymicrobial nature and here we tested only two culprits. Future studies will address gene expression though metatranscriptomic analyses of *ex vivo* biofilms derived from samples collected from endodontic infections where *E. faecalis* and *C. albicans* are present. Importantly, reproducing our transcriptomics assays using *in vivo* models would reveal even more significant insights to *E. faecalis* and *C. albicans* interactions in a clinical scenario.

In conclusion, we showed that when growing in biofilms together, *C. albicans* induces alterations in *E. faecalis*' metabolism, resulting in alleviation of CCR through upregulation of PTS activity and glycerol metabolism. Moreover, we characterized the mannose-PTS of *E. faecalis* and showed that it plays a role in mannose uptake and may have a small role in glucose uptake but not fructose. We also showed that mannose supports a robust biofilm growth and development in favour of *E. faecalis* in a mannose-PTS-dependent manner.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

CRediT: **Nicole de Mello Fiallos:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft; **Iriana J. Zanin dos Santos:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft; **Debra N. Brunson:** Formal analysis, Methodology, Supervision, Writing – review & editing; **Jessica K. Kajfasz:** Investigation, Methodology, Supervision, Writing – review & editing; **Lin Zeng:** Conceptualization, Investigation, Methodology, Validation, Writing – review & editing; **Rossana de Aguiar Cordeiro:** Conceptualization, Data curation, Supervision, Writing – review & editing; **José A. Lemos:** Investigation, Resources, Supervision, Writing – review & editing; **Jacqueline Abranches:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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