Candida albicans and *Enterococcus faecalis* biofilm frenemies: When the relationship sours

Om Alkhir Alshanta [a,1], Khawlah Albashaireh [a,1], Emily McKloud [a], Christopher Delaney [a], Ryan Kean [b], William McLean [a], Gordon Ramage [a,*]

[a] *Glasgow Endodontology and Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, Glasgow, United Kingdom*
[b] *Department of Biological and Biomedical Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Glasgow, United Kingdom*

A B S T R A C T

The opportunistic yeast *Candida albicans* and lactic acid bacteria *Enterococcus faecalis* are frequently co-isolated from various infection sites on the human body, suggesting a common interkingdom interaction. While some reports suggest an antagonism, the reason for their co-isolation therefore remains unclear. The purpose of this study was to undertake a detailed characterisation of this dual-species interaction. We used standard biofilm characterisation methodologies alongside an RNASeq analysis to assess the response of *C. albicans* to *E. faecalis*. We evaluated the relevance of pH to dual-species biofilm interactions and demonstrated that *E. faecalis* rapidly and significantly impacted *C. albicans* morphogenesis and biofilm formation, which was mirrored by levels of gene expression. These transcripts were enriched in amino acids biosynthesis and metabolism pathways in co-cultures, a finding that guided our investigation into pH related mechanism. We were able to demonstrate the direct role of *E. faecalis* induced low pH, which inhibited *C. albicans* hyphal morphogenesis and biofilm formation. The results suggest that the anti-candidal effect of *E. faecalis* is not based solely on a single mechanism, instead it may involve various mechanisms, which collectively reflects the complexity of interaction between *C. albicans* and *E. faecalis* and impacts treatment outcomes.

1. Introduction

Candida albicans biofilm infections are often polymicrobial, highlighting the importance of interkingdom interactions. These relationships may take the form of synergistic, antagonistic, or simply coexistence. It is well documented that *C. albicans* has the capacity to interact with various bacterial species, including *Staphylococcus aureus*, *Streptococcus mutans* and *Pseudomonas aeruginosa* [1–3]. Beyond the basis of these interactions, *C. albicans* influences the composition, virulence and dysbiosis of the bacterial microbiome [4–6]. Reciprocally, it has also been shown that *C. albicans* is in turn influenced by bacteria or bacterial products in terms of growth, virulence, resistance and phenotypic switch [7–9]. The pleiomorphic fungus *C. albicans* and *Enterococcus faecalis* are opportunistic pathogens that colonize various niches of the human body, such as the skin, oral cavity, vagina and gastrointestinal tract [10–14].

Endogenous in nature, *C. albicans* and *E. faecalis* can become

pathogenetic due to impairment in host immunity or dysbiosis in microflora [15,16]. Infections caused by the opportunistic yeast *C. albicans* can range in severity from superficial oropharyngeal candidiasis to a serious life-threatening candidemia [17]. Likewise, translocation of *E. faecalis* from the gut to other body sites is associated with various infections, including endocarditis, meningitis, urinary tract infections and blood-stream fatal sepsis [16,18]. In particular, it has been observed that the oral microbiome of immunocompromised mice in oropharyngeal candidiasis model showed a shift towards *E. faecalis* dominance [19]. In the gut, *C. albicans* inoculated mice remained dominated by enterococci (mainly *E. faecalis*) compared with the non-infected candidal animals, in which the *Enterococcus* species were replaced by lactobacilli [20]. Therefore, it can be inferred that *C. albicans* and *E. faecalis* may favour the same ecological environment, or interact in a way that supports the survival of one another. Indeed, both microorganisms are frequently co-isolated from the root canals of the teeth and oral mucosa [21,22]. Notably, in endodontic literature

these two pathogens are often considered the paradigm fungal and bacterial model to study, test and evaluate the effectiveness of endodontic antimicrobials or techniques [23–26].

A variety of environmental conditions and microbial molecules have been shown to influence *C. albicans* hyphal morphogenesis and biofilm formation [27]. These include nutrient source, stressors, such as pH and temperature, and direct and indirect bacterial interactions [28]. RNASeq has been extensively used to analyse how *C. albicans* responds under these circumstances, which has dramatically enhanced our understanding of the transcriptional profiles of *C. albicans* response to weak organic acids [29], growth media, oxidative and nitrosative stresses [30], *Lactobacillus* [31], *Streptococcus gordonii* [7], *Pseudomonas aeruginosa* quorum sensing molecules [32], amongst others. The interaction between *C. albicans* and *E. faecalis*, and more specifically the effect of *E. faecalis* and its supernatant on *C. albicans* morphogenesis, biofilm formation and virulence has been the subject of several investigations [18,33–40]. It was proposed that *E. faecalis* secretes an anti-candidal protein [40], and that it is the EntV bacteriocin that is specifically linked to the observed *C. albicans* inhibition [34]. Nevertheless, despite the well documented adverse effect of low pH and acid producing bacteria, such as lactobacilli, on *C. albicans* hyphal formation [31], the influence of the acidifying potential of the lactic acid bacterium *E. faecalis* remains largely unexplored. Therefore, the present *in vitro* study aimed to examine the phenotypic and transcriptomic biofilm interaction of *C. albicans* when co-cultured with *E. faecalis*. To the best of our knowledge this the first study that reveals *E. faecalis* induced changes in *C. albicans* biofilm transcriptome using RNA sequencing, and the impact that low pH has on this phenomenon.

2. Materials and methods

2.1. Microbial growth conditions and standardisation

All *C. albicans* and *E. faecalis* strains used in this study are listed in Table 1. *C. albicans* strains were incubated at 30 °C for 48 h on Sabouraud dextrose agar (SAB [Sigma–Aldrich, Dorset, UK]). *E. faecalis* strains were maintained on 5% v/v horse blood Columbia agar plate in 5% CO_2 at 37 °C for 24 h. *C. albicans* overnight cultures were grown in yeast peptone dextrose ([YPD - Sigma-Aldrich, Dorset, UK]) with shaking at 120 rpm in an orbital shaker (IKA KS 4000 i control, Berlin, Germany) at 30 °C. For *E. faecalis*, Brain Heart Infusion ([BHI] Sigma–Aldrich, Dorset, UK) with incubation in 5% CO_2 for 18 h at 37 °C was used. Cells were then harvested by centrifugation for 5 min at 3000 rpm and washed twice with PBS. Yeast cells were counted using a

Table 1
List of used *C. albicans* and *E. faecalis* strains.

C. albicans		E. Faecalis	
Name	Reference	Name	Reference
SC5314	[41]	ATCC-29212	[42]
BC023 (LBF) BC146 (HBF)	[43]	NCTCC 5957	National Collection of Type Cultures, Public Health Laboratory Service (www.phe-culturecollections.org.uk).
		E1, E2, E3	[44]
		ER5/1	[45]
		ER35	GDS culture collection
		OS-16	[44]
		V583	[46]
		OGX-1	[47]
		J 42-7	GDS culture collection
		AA-OR 34	[48]

HBF = high biofilm former; LBF = low biofilm former; GDS = Glasgow Dental School.

hemocytometer and standardized to (1×10^6 yeasts/mL). Bacterial cells were standardized using a spectrophotometer at an OD_{600nm} of 0.3 ($\sim 2 \times 10^8$ cells/mL).

2.2. Assessment of Candida albicans characteristics when co-cultured with Enterococcus faecalis

For phenotypic evaluation, *C. albicans* SC5314, high biofilm formers (HBF) and low biofilm formers (LBF) [49,50], and *E. faecalis* ER5/1, were standardized to 1×10^6 cells/mL for *C. albicans* and 1×10^7 cells/mL for *E. faecalis* in Todd-Hewitt broth (THB; Merck UK) supplemented with 10 mM menadione and 10 mg/mL hemin (Thermo Fisher). These were subsequently mixed 1:1 v/v with Roswell Park Memorial Institute (RPMI-1640 [Sigma–Aldrich, Dorset, UK]) (THB:RPMI), a media which has been shown to support the co-culture of *C. albicans* and bacterial species [51]. Mono-cultures of *C. albicans* and co-cultures of *C. albicans* with *E. faecalis* were created in a 24 well microtiter plates (Corning Incorporated, Corning, NY, USA) for 24 h in 5% CO_2 at 37 °C. After incubation, biofilms were washed with PBS and stained with 5 μM calcofluor white (Invitrogen, Paisley, UK), a stain which specifically stains the chitin and beta-glucans of fungal cell wall [52]. Biofilms were incubated in the dark for 20 min and excess stain was washed with sterile water. 2% paraformaldehyde was used for 1 h to fix the stained biofilms which were then imaged using EVOS FL Cell Imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Biofilm biomass of the same biofilms was also quantified using crystal violet stain as previously described [53].

2.3. Quantitative analysis using quantitative PCR

Mono- and dual-species biofilms of *C. albicans* SC5314 and *E. faecalis* ER5/1 and E2 were grown as described above in 6 well microtiter plates for 4, 6, 8 and 24 h in 5% CO_2 at 37 °C. Biofilms were then removed by scraping into 1 mL of PBS before DNA extraction using QIAamp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). For qPCR, a mastermix containing Fast SYBR GreenER™ (Thermo Fisher Scientific, Paisley, UK), forward/reverse primers (Table 2), and UV treated RNase-free water was prepared, to which extracted DNA was added. Standard curves for each strain were also included. The used thermal cycles were 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 3 s and 60 °C for 30 s using Step-One plus real time PCR machine and StepOne software V2.3 (Life Technologies, Paisley, UK). Colony forming equivalents (CFE) was calculated in relation to each species standard curve, as previously described [54]. Data obtained is from triplicates from three independent experiments.

2.4. Gene expression analysis of Candida albicans genes and Enterococcus faecalis EntV gene

For assessment of *C. albicans* related genes, biofilms of *C. albicans* SC5314 alone or in co-culture with *E. faecalis* ER5/1 were developed for 2 h, as described above. For *E. faecalis EntV* gene expression, biofilms of 12 *E. faecalis* strains were grown for 4 h in THB:RPMI 5% CO_2 at 37 °C. After incubation, biofilms were removed by scraping, and RNA extracted using the MasterPure™ Yeast RNA Purification Kit (Cambio, Cambridge, UK) for yeast, as per the manufacturer's protocol, and with a TRIzol extraction for bacteria (Thermo Fisher Scientific, Paisley, UK). Extracted RNA was converted to cDNA with a high-capacity cDNA reverse transcription kit (Thermo Scientific, Loughborough, UK). Real-time PCR was carried as described above. The list of primers for genes assessed are located in Table 2. Gene expression was analysed for *C. albicans* using ΔΔCT method, after normalising CT values to ACT1 housekeeping gene [55]. *EntV* gene expression in *E. faecalis* strains was calculated as a percentage of expression in relation to ΔΔCT of the housekeeping gene. No-reverse transcription samples were included. Data obtained is from triplicates from three independent experiments.

Table 2

C. albicans and *E. faecalis* primers used for quantitative and real time qPCR.

Primer	Gene name	Sequence (5′–3′)	Function
C. albicans	ITS	ITS3 - GCATCGATGAAGAACGCAGC	
		ITS4 - TCCTCCGCTTATTGATATGC	
	ACT1	F - AAGAATTGATTTGGCTGGTAGAGA	Housekeeping
		R - TGGCAGAAGATTGAGAAGAAGTTT	
	HWP1	F- GCTCAACTTATTGCTATCGCTTATTACA	Hyphal wall protein
		R - GACCGTCTACCTGTGGGACAGT	
	ALS3	F - CAACTTGGGTTATTGAAACAAAAACA	Adhesion
		R - AGAAACAGAAACCCAAGAACAACCT	
	ECE1	F - GCTGGTATCATTGCTGATAT	Hyphae specific protein
		R - TTCGATGGATTGTTGAACAC	
	YWP	F - TCCGGTTCTGGTTCTGATTC	Yeast wall protein
		R - TACCGTGGACCGTAGTGACA	
	SAP2	F- GAATTAAGAATTAGTTTGGGTTCAGTTGA	Secreted aspartyl proteases
		R - CCACAAGAACATCGACATTATCAGT	
	SAP5	F - CCAGCATCTTCCCGCACTT	Secreted aspartyl proteases
		R - GCGTAAGAACCGTCACCATATTTAA	
	PLB1	F - GGTGGAGAAGATGGCCAAAA	Phospholipase
		R - AGCACTTACGTTACGATGCAACA	
	HSP90	F - GGTTGCTGATCACGTCCAAGTT	Heat shock protein
		R - AACTTACCACCAGCGTTAGATTCC	
	CDR1	F - GTACTATCCATCAACCATCAGCACTT	Efflux pump
		R - GCCGTTCTTCCACCTTTTTGTA	
	MDR1	F - TCAGTCCGATGTCAGAAAATGC	Efflux pump
		R - GCAGTGGGAATTTGTAGTATGACAA	
	ARG1	F - CTTTGGTTTGTGCCACTGGG	Arginine synthesis
		R - TGCCGTTTCTCACGGTTGTA	
	ARG3	F - TGCGTCTTCACAAACACCAC	Arginine and citrulline biosynthesis
		R - ATGATGCCGCTCCTTCAGTA	
	CPA1	F - TGAAATGGTGCCTTGGTGGT	Arginine synthesis
		R - AAACGTTCTGGTGTTGCTGC	
	CPA2	F - TGCTCAAGGTGTGGTGGTTT	Arginine synthesis
		R - TGGCATCACCGGAATGAACA	
	GPT1	F - CAGTTCGGCTGGTACCACTT	Transmembrane transporter activity
		R - CGATACTGACATCACCCCCG	
	AAP1	F - CTCGTCAACGGTCAACCAGA	Transmembrane transport
		R - TGCGCTATTGGGGCATTACA	
E. faecalis	DDL	F - CAAACTG TTGGCATTCCACAA	housekeeping
		R - TGGATTTCCTTTCCAGTC ACTTC	
	EntV	F - AGCTGCACAAAAGAAAGCCTG	Secreted bacteriocin
		R- GCTTAGCCCACATTGAACTGC	

2.5. Assessing the transcriptional response of Candida albicans to Enterococcus faecalis

Transcriptional analysis of *C. albicans* SC5314 in response to co-culture with *E. faecalis* ER5/1 was performed using RNA sequencing as previously described [31,56]. Briefly, *C. albicans* mono-cultures were grown in THB:RPMI media in T-75 cell culture flasks (Corning, USA) at a cellular density of 10^6 cells/mL for 4 h in 5% CO_2 at 37 °C. Next, biofilms were washed with PBS and *E. faecalis* in cellular density of 10^7 cells/mL was added for additional 2, 4, 20 h. After each time point, biofilms washed and scraped in 1 mL of RNAlater (Thermo Scientific, Loughborough, UK). RNA was extracted using RiboPure™ RNA purification kit for yeast (Thermo Scientific, Loughborough, UK). The quantity and quality of extracted RNA were evaluated using Bioanalyzer system. RNA samples were submitted to Edinburgh Genomics facility where RNA sequencing was performed using Illumina NOVASeq6000 sequencing platform. The obtained raw fastq reads were trimmed to remove adaptors and poor-quality reads using Trimmomatic (V0.38). Reads were then aligned to a reference *C. albicans* genome (http://www.candidagenome.org) and the number of aligned reads per gene were counted. Differential expression analysis was then performed using DESeq2 in RStudio which also used to generate principal component analysis (PCA), volcano and heatmap plots.

To further evaluate transcriptional changes based on key findings, amino acid biosynthesis/metabolism related gene expression were analysed using RT-PCR. In this experiment, *C. albicans* SC5314 was grown in THB:RPMI in 6 well plates in 5% CO_2 for 4 h. After incubation, supernatant was removed, and biofilms were washed with PBS.

E. faecalis ER5/1 suspended in 3 different media conditions was then added to *C. albicans* biofilms. The media conditions used where THB: RPMI, THB:RPMI supplemented with amino acids (Sigma-Aldrich, Dorset, UK) and THB:RPMI supplemented with glucose (Sigma-Aldrich, Dorset, UK). Amino acids (L-glutamine, L-arginine and L-histidine) were added to THB:RPMI to reach a final concentration of 0.03, 0.4 and 0.6 g/ L respectively. The final glucose concentration in THB:RPMI with glucose was 5 g/L. Supplemented media without *E. faecalis* was also added to *C. albicans* biofilms for mono-species biofilms. After incubation for an additional 2 h in 5% CO_2 at 37 °C, biofilms washed, removed by scraping, RNA extracted and RT-PCR performed as described above using primers specific for ARG1, ARG3, CPA1, CPA2, GPT1 and AAP1 genes (Table 2).

2.6. Assessment of Candida albicans biofilm biomass with Enterococcus faecalis supernatant

Cell free supernatant from 12 *E. faecalis* strains was obtained by growing *E. faecalis* to a cellular density of 1×10^7 cells/mL in THB:RPMI in T-15 cell culture flasks (Corning, USA) for 24 h in 5% CO_2 at 37 °C. Supernatant was centrifuged at 4000 rpm for 5 min before filtering with 0.2 µm syringe filter (Sartorius™ Minisart®, Fisher Scientific, Loughborough, UK). Next, a 9:1 v/v of supernatant to 10 times concentrated RPMI (10 × RPMI) media was then used to grow *C. albicans* SC5314, HBF and LBF in 24 well plate for 24 h in 5% CO_2 at 37 °C (10 × RPMI was used to ensure any effects were not a result of nutrient depletion). Following incubation, biofilms were washed with PBS and dried at room temperature overnight. The biofilm biomass of the dry biofilms assessed

with crystal violet stain.

Next, *E. faecalis* E2, ER5/1 and V583 were selected based on their differential level of *EntV* expression. *E. faecalis* supernatant from these strains was ultrafiltered using Amicon Ultra-0.5 Centrifugal Filter Unit with 3 kDa NMWCO pore size (Merck Life science, Gillingham, UK). As *E. faecalis* EntV was indicated to be larger than 3–10 kDa [34], the 3 kDa pore size was selected to ensure removal of any proteins larger than 3 kDa NMWCO from the supernatant. Following ultrafiltration, both normal and ultrafiltered supernatant were boiled for 10 min at 100 °C to heat inactivate any active proteins in the supernatant. Afterwards, the supernatant from each condition was mixed with 10 × RPMI at 9:1 ratio and used to grow *C. albicans* SC5314, HBF and LBF, as described above. The pH of each supernatant was also measured using pH meter (Fisher Scientific, Loughborough, UK). *C. albicans* biofilm biomass was assessed as described above.

2.7. pH modification of Enterococcus faecalis supernatant and growth media

The pH of *E. faecalis* E2, ER5/1 and V583 supernatant was quantified and shown to be ~5 for all three strains. The pH of the supernatant was then adjusted to 6.0 and 7.0 using 1 M sodium hydroxide. The pH of THB:RPMI (normally has pH of 7.0) was also adjusted to 5.0 and 6.0 using 35% hydrochloric acid (Sigma–Aldrich, Dorset, UK) and 90% lactic acid (Sigma–Aldrich, Dorset, UK). pH modified supernatant was mixed with 10 × RPMI and used along with pH modified media to grow *C. albicans* SC5314, HBF and LBF. *C. albicans* biofilm biomass was assessed as described above.

2.8. Assessment of Enterococcus faecalis inhibition of Candida albicans hyphal morphogenesis

Mono- and dual-cultures of *C. albicans* SC5314, HBF, LBF and *E. faecalis* E2, ER5/1 and V583 were prepared, as described above. Biofilms were washed with PBS and stained and imaged with calcofluor white at 2, 4, 6, 8 and 24 h, as described above. The pH of supernatant from each biofilm at the indicated time were also measured.

2.9. Statistical analysis

Figures and statistical analysis were performed using GraphPad Prism (version 7.0 d, GraphPad, La Jolla, CA, USA). To compare data sets and calculate significance, non-parametric Kruskal–Wallis test with Dunn's multiple comparison test was used. The ClueGO application in Cytoscape (Version: 3.8.0) software was used to generate gene ontology networks.

3. Results

3.1. Enterococcus faecalis impacts biomarkers of Candida albicans biofilms

First, we investigated the general effect of *E. faecalis* on *C. albicans* hyphal morphogenesis, biofilm formation, growth, and expression of key genes related to biofilm formation, virulence and drug resistance. *C. albicans* SC5314, HBF and LBF were grown in mono-species biofilms or co-cultured with *E. faecalis* ER5/1 for 24 h. As reported by various studies [33,34,36,38,39], *C. albicans* hyphal morphogenesis was shown to be clearly inhibited in co-cultures, as shown in calcofluor stained images (Fig. 1a). *C. albicans* SC5314 (panel a [i]) and HBF (panel a [iii])
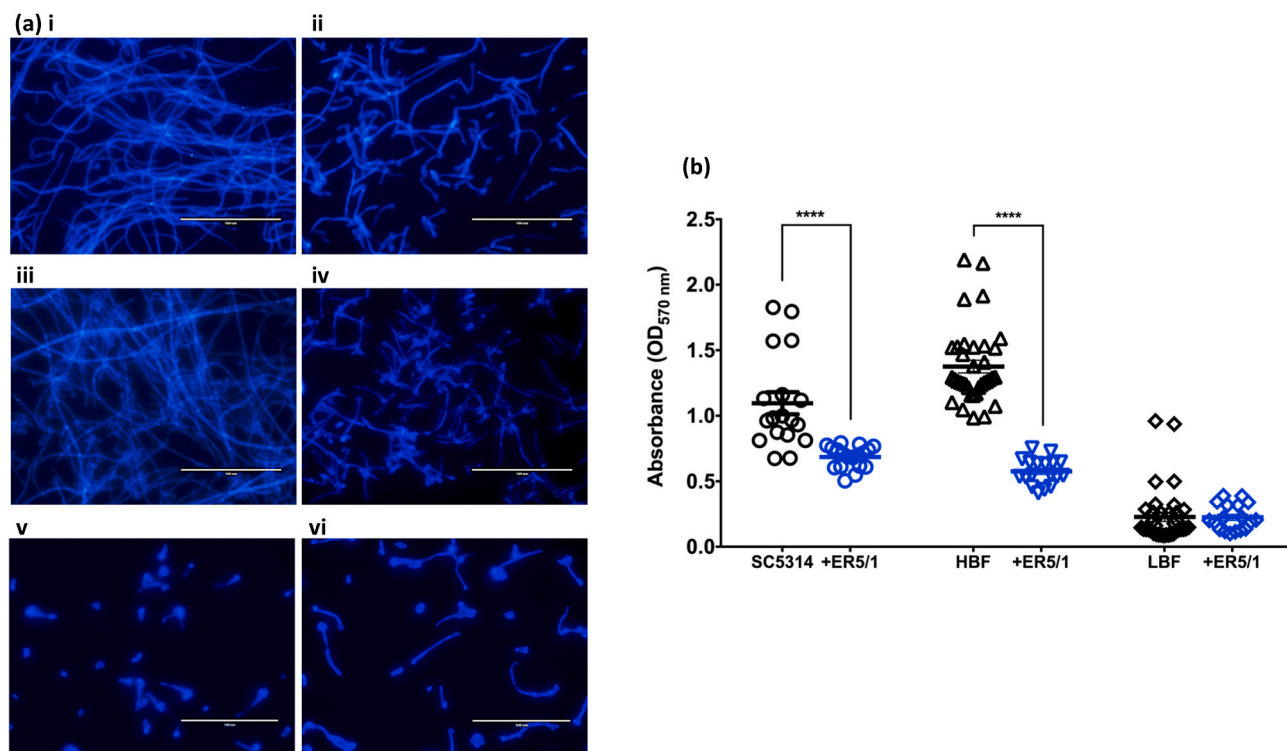
Fig. 1. *E. faecalis* inhibits *C. albicans* hyphal morphogenesis and biofilm formation in *in vitro* coculture. (a) *C. albicans* laboratory strain SC5314 (i, ii), HBF clinical isolates BC146 (iii, iv) and LBF clinical isolate BC023 (v, vi) were grown in THB:RPMI media in mono-culture (I, iii, v) or in co-culture with *E. faecalis* strain ER5/1 (ii, iv, vi) for 24 h. Biofilms were stained with calcofluor white. Scale bars are 100 μm. **(b)** *C. albicans* biofilm biomass measured using crystal violet stain after 24 h of incubation in mono-culture and with ER5/1 supernatant supplemented with 10x RPMI. Statistical significance was presented as ****p < 0.0001. Data obtained from triplicates of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

are known for their ability to form dense biofilm with tangled networks of hyphae [57,58]. This was obvious in mono–species biofilms, though in the presence of *E. faecalis*, less abundant and shorter hyphae are evident (panel a [ii], [iv]). The phenotype of *C. albicans* LBF (panel a [v], [vi]) remained unaltered. This phenotype of *C. albicans* usually grows in yeast form with occasional hyphae, therefore, it was not influenced by *E. faecalis*. To assess the effect of *E. faecalis* on *C. albicans* biofilm biomass, cell free *E. faecalis* supernatant supplemented with $10 \times$ RPMI was used instead of live *E. faecalis* to ensure that the measured biofilm biomass is purely *C. albicans* biomass. *E. faecalis* supernatant was reported to have similar inhibitory effect on *C. albicans* as live *E. faecalis* [33]. The biofilm biomass, assessed using crystal violet assay, showed that *E. faecalis* supernatant significantly inhibited biofilm formation of *C. albicans* SC5314 and HBF compared with *C. albicans* only biofilms. Again, LBF remained unaffected (Fig. 1b).

Next, qPCR analysis was used to further quantify cell numbers during these interactions. *E. faecalis* was confirmed to inhibit the growth of *C. albicans* SC5314 compared with mono-species (Fig. 2a). This inhibition was notably more significant with *E. faecalis* ER5/1 than with that of E2. Whereas, the growth of *E. faecalis* remained unaffected by the presence of *C. albicans*, as there was no significant difference in *E. faecalis* CFE/mL between mono- and dual-species cultures at all time points for both strains, although there was a trend toward increased growth of ER5/1 in co-cultures at 24 h. Similar growth inhibition was observed in the kinetic growth curve of *C. albicans* grown in *E. faecalis* supernatant compared with *C. albicans* only at 24 h (Fig. 2b). The growth kinetics of C. *albicans* was characterised by steadily increasing growth up to 8 h, followed by a plateau where the growth remained stable over the

remaining hours. C. *albicans* grown with *E. faecalis* supernatant showed a higher growth rate between the 8 and 20 h, after which the growth decreases sharply at 24 h compared with C. *albicans* only control. This matches the growth inhibition observed in qPCR analysis at 24 h in Fig. 2a(i).

At the single transcript level, co-culturing *C. albicans* SC5314 with *E. faecalis* ER5/1 resulted in down-regulation of the hyphal related genes *HWP1* and *ECE1* in relation to *C. albicans* only at 2 h. Conversely, the adhesion gene *ALS3* was upregulated, which may indicate that *E. faecalis* affects hyphal formation of *C. albicans* without affecting its adhesion to surfaces (Fig. 2c). *SAP5* encoding SAP5 enzyme, which are expressed upon hyphal formation [59], is also downregulated in dual-species biofilms. In contrast, *SAP2* and *YWP*, yeast typical genes, were upregulated. The gene expression patterns support the above-mentioned inhibition levels observed microscopically and by crystal violet assay. In addition, the effect of *E. faecalis* on *C. albicans* virulence was assessed using genes encoding proteolytic enzymes, *PLB1* and *SAP2* and those associated with drug resistance *CDR1*, *MDR1* and *HSP90*. The upregulation of the genes *PLB1* and *SAP2* indicates enhanced virulence of *C. albicans* via the increase in proteolytic enzymes activities in the dual-species biofilms. Similarly, the efflux pump genes, *CDR1*, *MDR1* and *HSP90*, which are directly linked to drug resistance were also upregulated. This upregulation in efflux pumps expression may refers to *C. albicans* attempt to remove intracellular toxic substances produced by *E. faecalis*, such as quorum sensing molecules, bacteriocins or metabolites. Overall, the above analysis indicates that *E. faecalis* inhibits *C. albicans* hyphal morphogenesis, biofilm formation and growth, while positively affecting enzymatic activity and drug resistance.
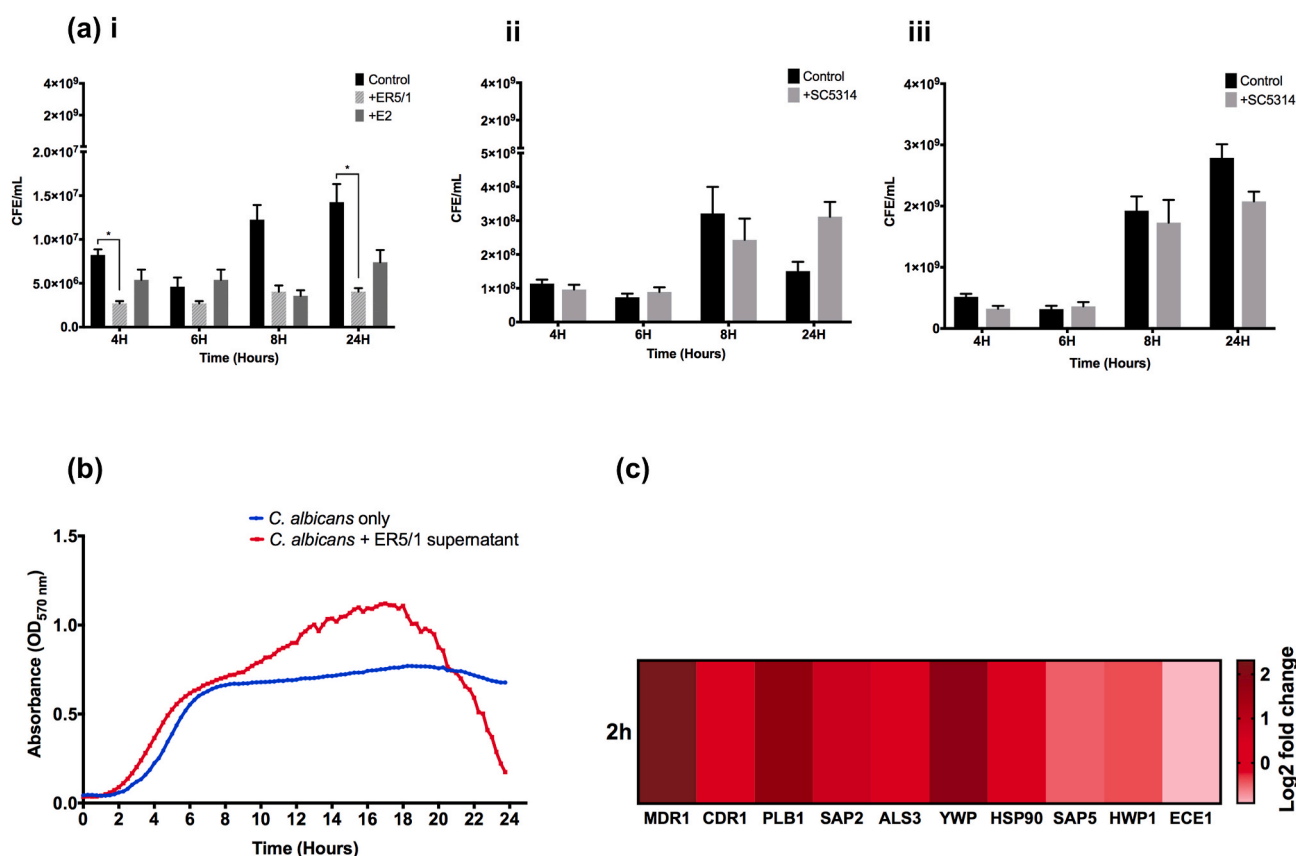
Fig. 2. *E. faecalis* **inhibits** *C. albicans* **growth and induce change in** *C. albicans* **SC5314 genes associated with biofilm, virulence and resistance. (a)** The growth of *C. albicans* SC5314 (i) *E. faecalis* ER5/1 (ii) and *E. faecalis* E2 (iii) in mono- and dual-species was assessed using qPCR at 4 h, 6 h 8 h and 24 h. Statistical significance was presented as * p < 0.05, ****p < 0.0001. **(b)** 24 h kinetic growth curve of *C. albicans* SC5314 only (blue) and *C. albicans* cultured in *E. faecalis* ER5/1 supernatant (red) at 570 nm absorbance **(c)** Heatmap of *C. albicans* SC5314 genes associated with biofilm, virulence and resistance was measured in mono-culture and in presence of *E. faecalis* at 2 h. Control = *C. albicans* only biofilms. Data shown is the mean log fold change relative to mono-species biofilms. Results represent data from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Transcriptomic analysis of Candida albicans response to Enterococcus faecalis demonstrated the importance of early interactions

We have shown *E. faecalis* to have a significant effect on various biological functions of *C. albicans*. In order to gain a more comprehensive overview of the molecular mechanisms of this interaction, a whole transcriptome sequencing (RNA-Seq) approach was employed. For this purpose, *C. albicans* SC5314 as mono-cultures and as co-cultures with *E. faecalis* ER5/1 were prepared at 2, 4 and 20 h, after the addition of *E. faecalis* to 4 h *C. albicans* biofilms, to enable a temporal analysis. This approach enabled us to capture gene expression levels at early, intermediate and late time points of microorganisms' interaction.

The principal component analysis demonstrates the level of variance between *C. albicans* mono- and co-cultures at the different time points (Fig. 3a). The variance in clustering was the highest at 6 h (*E. faecalis* was added to 4 h pre-established *C. albicans* biofilms and samples were harvested after 2 h of further incubation). This indicates that *C. albicans* shows a quick transcriptional response to *E. faecalis*. The degree of variance then decreased at 8 and 24 h. Therefore, the subsequent analysis was focused on 6 h time point. In total, 186 genes were significantly and differentially expressed between mono- and dual-species biofilms at 6 h (with cut-off of 1.5 for fold change and 10e-3 for p-adjusted value). Of these, 112 genes were upregulated in dual-species and 74 were downregulated (Fig. 3b). The top 50 differentially expressed genes are shown in Fig. 3c, accompanied by a list of the top 10 upregulated genes in *C. albicans* mono-and co-cultures, and their function (if known) are shown in Table 3. However, most of these genes are of unknown function and were not involved in the enriched biological pathways within the subsequent enrichment analysis. The full list of all significantly differentially expressed genes in *C. albicans* mono- and dual-species biofilm are available in Table S1.

Table 3

List of the top 10 upregulated genes in *C. albicans* mono– and dual-species biofilms at 6 h.

	Gene ID	Gene Name	P value (adj)	Log2FC
Up in *C. albicans* mono-species biofilms	CR_03580C_A	CR_03580C_A	0.00140825	−5.9714945
	CR_04510W_A	HXK2	4.07E-15	−5.4994791
	C2_01000W_A	HGT7	4.44E-05	−5.436272
	C3_01540W_A	C3_01540W_A	8.47E-05	−4.7324912
	C4_00450C_A	PGA10	4.08E-07	−4.3971164
	C4_04720W_A	C4_04720W_A	9.05E-09	−4.1599839
	C1_07160C_A	C1_07160C_A	0.00283371	−4.1157133
	CR_07170W_A	CR_07170W_A	2.39E-06	−4.0613455
	C6_00150W_A	ARD	2.09E-09	−4.0025723
	CR_01910C_A	CR_01910C_A	0.02841275	−3.8845479
Up in *C. albicans - faecalis* dual-species biofilms	C4_05580C_A	C4_05580C_A	1.65E-13	14.967598
	C1_02520W_A	SCW4	2.64E-22	14.6848791
	C1_13080W_A	OP4	5.11E-18	14.2265626
	C1_07580C_A	PRY1	4.27E-13	12.9814901
	C1_05890W_A	C1_05890W_A	2.05E-18	12.4438686
	C2_09800C_A	C2_09800C_A	2.21E-13	12.1176629
	CR_10320W_A	CR_10320W_A	1.21E-07	12.052965
	C2_02230C_A	C2_02230C_A	6.16E-19	11.4784176
	C6_00070C_A	PGA25	8.75E-26	10.9038636
	C1_09580C_A	LIP1	0.04085374	10.6107591

3.3. Amino acid biosynthesis and metabolism genes are upregulated by Enterococcus faecalis, regardless of glucose and amino acids concentration in the media

Gene ontology (GO) term analysis was then carried out to investigate the biological functions of the differentially expressed genes in mono- and dual-species biofilms. The upregulated genes in the dual-species
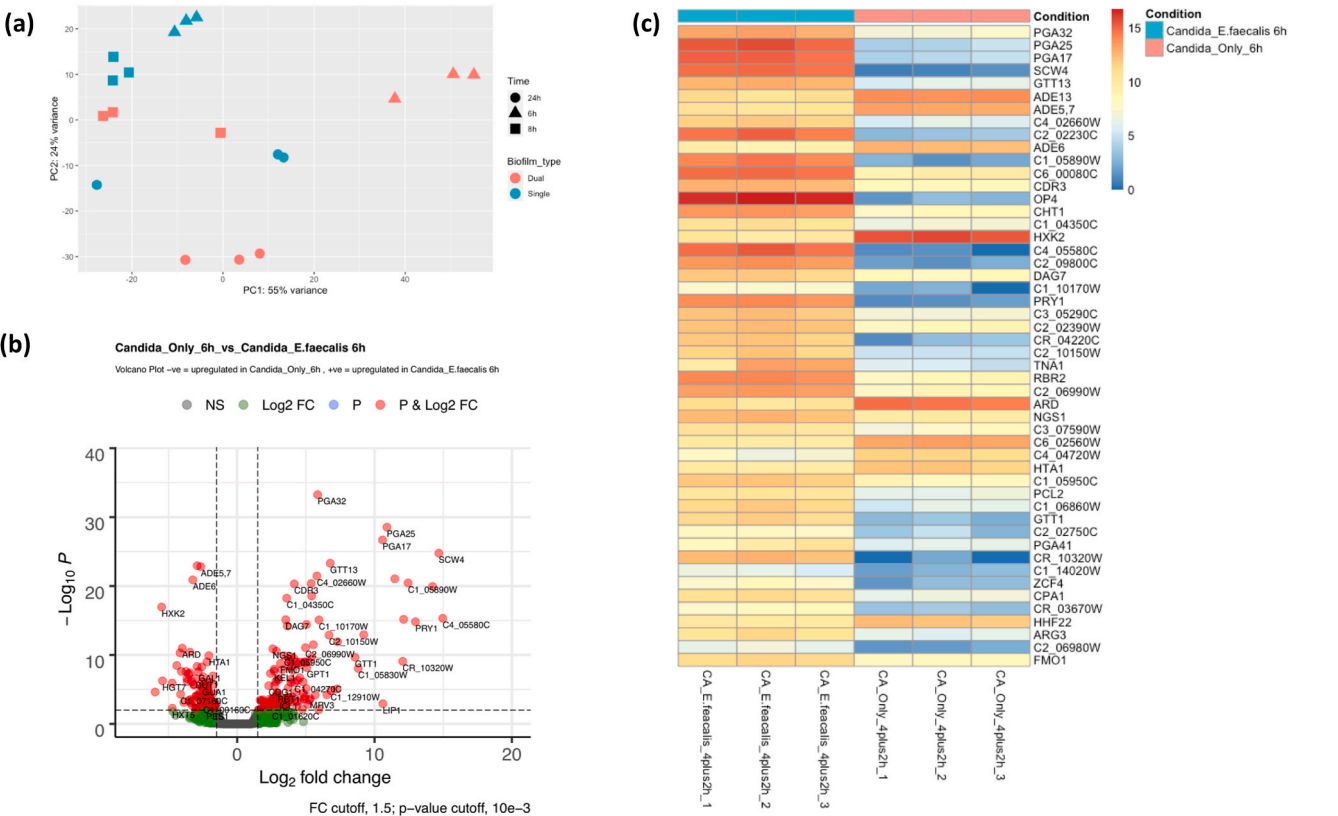

Fig. 3. Overview of the transcriptional analysis of *C. albicans* SC5314 with *E. faecalis* ER5/1 at 4h, 6h and 24h. (a) The principal component analysis of data at all time points. **(b)** Volcano plot of the significantly differentially expressed genes of mono- and dual-species at 6 h. Genes located on the negative x-axis are the up regulated genes in mono-species biofilms. Genes on the positive x-axis are the up regulated genes in dual-species biofilms. **(c)** Heatmap of the top 50 significantly differentially expressed genes of mono- and dual-species biofilms at 6 h.

were mainly responsible for amino acids biosynthesis/metabolism and transmembrane transport activity (Fig. 4a). For the downregulated genes, the main biological functions enriched were purine nucleotide inosine monophosphate (IMP)/nucleotides/purine related biosynthetic processes (Fig. 4b). A simple presentation of the GO term analysis is illustrated in Fig. S1. The increased amino acid uptake, biosynthesis and metabolism in response to *E. faecalis* can be an indication of amino acids or glucose starvation or a stress response. It was also evident that *C. albicans* down-regulated the purine nucleotide biosynthesis pathway, an essential precursor for the nucleic acids DNA and RNA [60]. This may indicate an inhibition in *C. albicans* growth when co-cultured with *E. faecalis*, as shown previously in (Fig. 2a and b).

Next, we aimed to investigate whether the up-regulated amino acid biosynthesis and metabolism was a result of *E. faecalis* induced amino acids or glucose starvation. To achieve this, *C. albicans* SC5314 was grown in THB:RPMI media for 4 h. Afterwards, *E. faecalis* was stand-ardised to the desired cellular density in either THB:RPMI only, THB:RPMI supplemented with amino acids or with glucose and added to *C. albicans* biofilms. *C. albicans* mono-species biofilms with the three different media were also included. After additional incubation of 2 h, mono- and dual-species biofilms were analysed for the expression of amino acids biosynthesis and metabolism genes; *ARG1*, *ARG3*, *CPA1*, *CPA2* and transmembrane transport related genes *GPT1* and *AAP1*. Despite supplementation with amino acids and glucose, all the investigated genes were still upregulated in all the conditions in the dual-species biofilms (Fig. 4c). However, the level of expression of all genes was the highest in THB:RPMI media and lower in glucose supplemented THB:RPMI. Furthermore, the *CaGcn4* gene, *C. albicans* functional homologue of *S. cerevisiae* Gcn4, which mediates *C. albicans* response to

amino acids starvation [61], was not upregulated at any time point, as indicated in RNASeq analysis. These findings suggest that increased amino acid uptake, biosynthesis and metabolism is not caused by amino acids starvation, but instead, it is a direct stress response to *E. faecalis*, its released products, or the environment that it generates.

As recently published by our group [31], amino acid biosynthesis and breakdown related genes were also upregulated in dual-species biofilms of *C. albicans* and *L. crispatus* at 24 h. Indeed, there was 21 shared genes that were upregulated by both *E. faecalis* and *L. crispatus* at 24 h (Fig. 4d). Of note, the transcriptional response of *C. albicans* to *L. crispatus* was delayed (at 24 h) compared to its early response to *E. faecalis* (at 6 h).

Moreover, 10 out of 27 of the enriched GO biological processes in our dual-species biofilms, and 20 out of 22 of the enriched GO biological processes in our mono-species biofilms are also enriched in response to the weak organic acids [29]. In addition, many genes involved in arginine and lysin biosynthesis were induced in *C. albicans* under mild oxidative stress [7,30,62]. Considering that *E. faecalis* is a lactic acid bacteria [63], the similarities observed in *C. albicans* transcriptional response to *E. faecalis*, *L. crispatus*, and weak organic acids, and the fact that acidic environment favours yeast growth [64–66], then we hypothesized that *C. albicans* biofilm and hyphal morphogenesis inhibition observed in dual-species biofilms is pH dependant phenomenon.

3.4. Enterococcus faecalis induced Candida albicans biofilm formation inhibition is not dependent on bacteriocin EntV expression

E. faecalis inhibition of *C. albicans* hyphal morphogenesis, biofilm formations and virulence are reported in various studies [33,34,36,38,
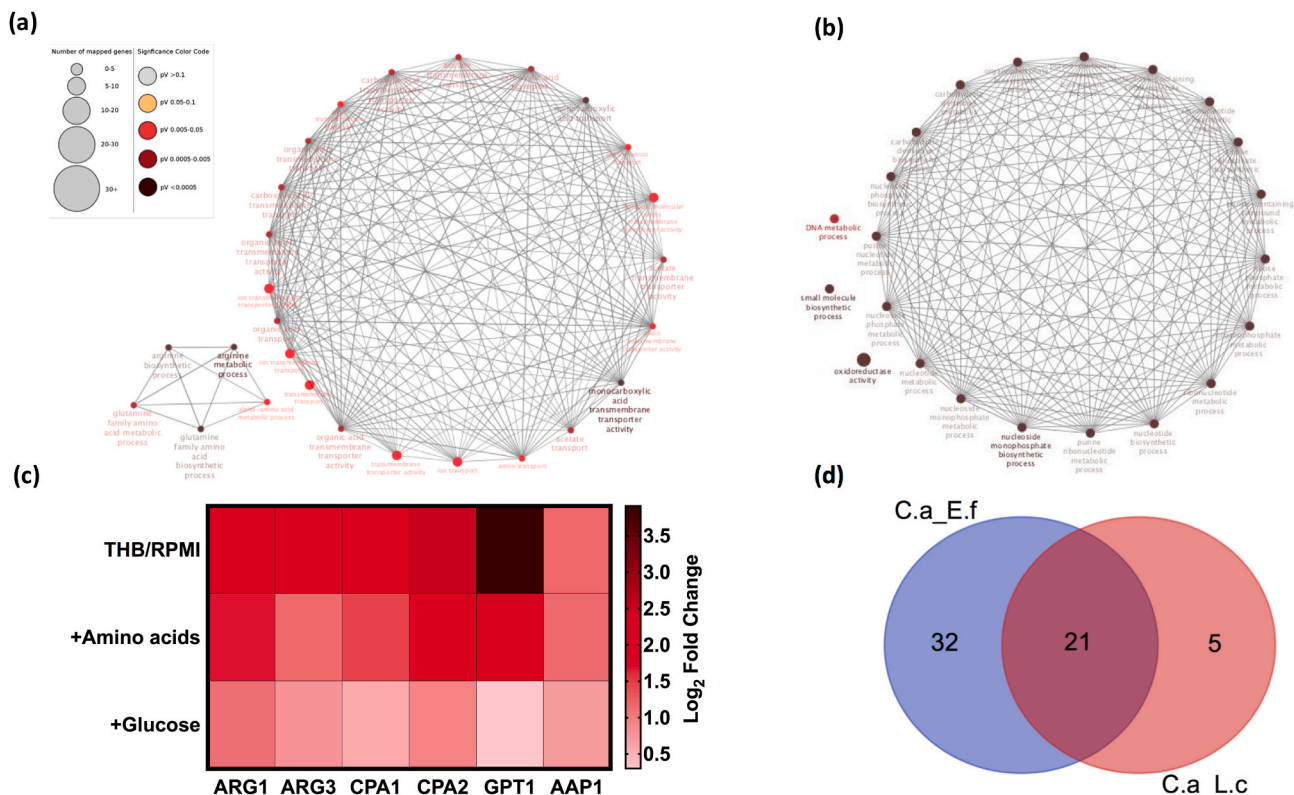
Fig. 4. Gene ontology (GO) enrichment analysis of significant biological, cellular, and molecular functions of mono- and dual-species biofilms at 6h. Amino acids biosynthesis/metabolism and purine related biosynthesis *C. albicans* genes expression in mono- and dual-species biofilms at 6 h. **(a)** Enriched biological processes within the upregulated genes in dual-species biofilms. **(b)** Enriched biological processes within the upregulated genes in mono-species biofilms with ≥10 associated genes. Only enriched pathways of a significant ≤0.05 are displayed. **(c)** Heatmap of key Amino acids biosynthesis/metabolism and transmembrane transport *C. albicans* genes in THB:RPMI media (media), THB:RPMI supplemented with glucose (+Glucose) or THB:RPMI supplemented with amino acids (+Amino acids) at 6 h. Data shown is the mean log fold change relative to mono-species biofilms. **(d)** Number of the unique and overlapped genes that are upregulated at 24 h in *C. albicans* co-cultured with *E. faecalis* or with *L. crispatus*.

39]. However, few investigated the mechanism of this interaction. Shekh and Roy (2012) first characterized and purified an anti-candida protein produced by *E. faecalis*, which was heat stable up to 90°C with molecular weight of approximately 43 KDa [40]. Graham and colleagues (2017) also identified the bacteriocin EntV produced by *E. faecalis* as a potent inhibitor of *C. albicans* biofilm formation and virulence [34]. Therefore, we investigated the expression of *EntV* gene in 12 different *E. faecalis* strains and correlated the level of *EntV* expression with the degree of *C. albicans* biofilm inhibition, as quantified by the crystal violet assay. There was a wide variation in the expression of EntV among the screened *E. faecalis* strains at 4 h (Fig. 5a). *E. faecalis* V583, showed the highest level of expression, followed by *E. faecalis* E1 and ER5/1. All other *E. faecalis* strains showed comparable expression levels. The inhibition of biofilm from *C. albicans* SC5314 (Fig. 5b), HBF (Fig. 5c) and LBF (Fig. 5d) was then assessed using the cell free supernatant from the 12 *E. faecalis* strains. Here the biomass inhibition was not correlated with the quantified *EntV* expression, and there was not a statistically significant difference in *C. albicans* biomass inhibition induced by the different *E. faecalis* strains. Moreover, the linear regression analysis showed that there is no correlation between the level of *EntV* expression of different *E. faecalis* strains and the biofilm biomass of C. albicans grown in the supernatant of the corresponding *E. faecalis* strains (Fig. S4).

In order to further investigate the effect of the EntV peptide on

C. albicans, we employed ultrafiltration membranes with 3kD cut-off (EntV was identified as a 7.2-kDa peptide) [34] and heat inactivation of *E. faecalis* supernatant. We aimed to separate any proteins larger than 3kD and heat inactivate the supernatant. *E. faecalis* E2, ER5/1 and V583 were selected for the subsequent analysis based on their variation in *EntV* expression. The supernatant was then used in combination with 10x RPMI to grow *C. albicans*. Despite the ultrafiltration and the heat inactivation of *E. faecalis* supernatant, a significant inhibition in *C. albicans* biofilm biomass still evident in SC5314 (Fig. 6a) and HBF (Fig. 6b). Furthermore, ultrafiltration resulted in increased inhibition compared with normal supernatant in *C. albicans* SC5314 and HBF. Of note, the pH of the normal, ultrafiltered and heat inactivated supernatant was similar around 5.45 before incubation with *C. albicans* (Fig. S2). *C. albicans* is known for its ability to raise extracellular pH [67], and since the RPMI used in this work was unbuffered, the pH of all the conditions increased to around 6–6.3 after 24 h of incubation. Despite this rise in pH, the conditions remained in the acidic range and the biofilm biomass of *C. albicans* SC5314 and HBF was significantly lower in all supernatant containing conditions compared with the *C. albicans* only controls.

3.5. Candida albicans biofilm inhibition is pH dependent

As suggested above, we aimed to investigate the effect of *E. faecalis*
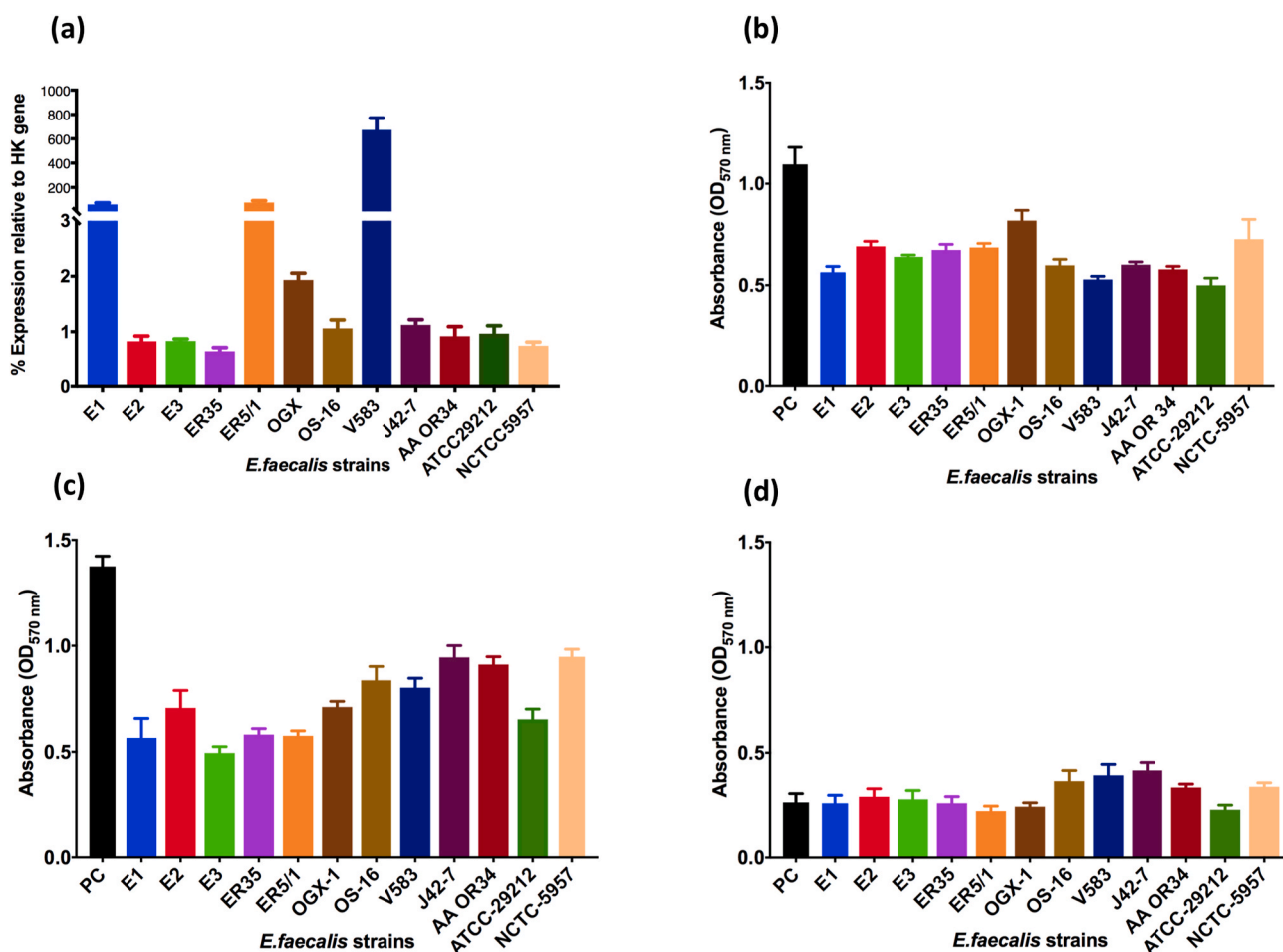
Fig. 5. Inhibition of *C. albicans* hyphal morphogenesis when cocultured with *E. faecalis* is not dependent on bacteriocin *EntV* expression. (a) Percentage of *EntV* expression in 12 *E. faecalis* strains relative to ddl housekeeping gene at 4 h with real time qPCR. The expression of ddl housekeeping gene was stable cross all the 12 *E. faecalis* strains with a variation within 2 cycle threshold (Ct) values. **(b–d)** Cell free *E. faecalis* supernatant obtained from 24 h incubation of E2, ER5/1 or V583 in THB:RPMI was supplemented with 10x RPMI. *C. albicans* biofilm biomass of the mono-culture (control) and *C. albicans* grown in 12 *E. faecalis* strains supernatant was assessed by crystal violet at 24 h **(a)** SC5314 **(b)** HBF (BC146) **(c)** LBF (BC023). Data obtained from triplicates of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
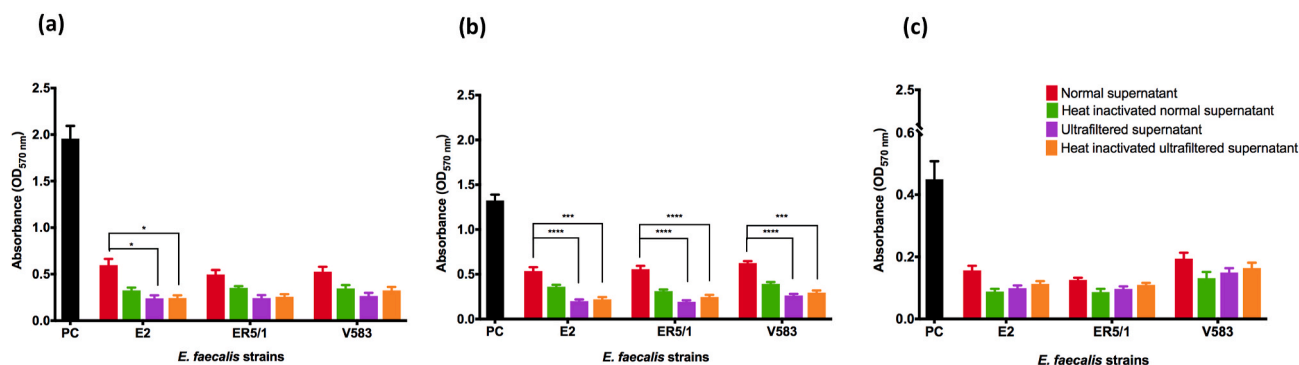
Fig. 6. Inhibition of *C. albicans* biofilm formation by *E. faecalis* normal, ultrafiltered and boiled supernatant. *C. albicans* biofilm biomass of the mono-culture (control) and *C. albicans* grown in *E. faecalis* E2, ER5/1 and V583 supernatant supplemented with 10x RPMI as assessed by crystal violet at 24 h. Four types of each strain supernatant were used; normal, boiled, ultrafiltered and boiled ultrafiltered supernatant **(a)** SC5314 **(b)** HBF **(c)** LBF. Statistical significance was presented as * p < 0.05, ***p < 0.001, ****p < 0.0001. Results represent data from three independent experiments. PC = *C. albicans* only control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

supernatant pH on *C. albicans* hyphal morphogenesis and biofilm formation. Firstly, we measured the pH of the *E. faecalis* cell free supernatant obtained from 24 h *E. faecalis* biofilms grown in THB:RPMI media. The pH of the supernatant was approximately 5.0. The pH of this supernatant and THB:RPMI media was then modified to different pH levels. Following the growth of *C. albicans* in various pH conditions, the biofilm biomass was assessed. Interestingly, there was a positive correlation between raising the pH of the *E. faecalis* supernatant and increased *C. albicans* biofilm biomass of SC5314 and HBF (Fig. 7a and b, respectively). *C. albicans* biofilm biomass was also increased with a pH rise of in pH modified media. In all conditions tested, the biomass was

significantly higher at pH 7.0 compared to that of pH 5.0. More importantly, *E. faecalis* supernatant showed a similar effect trend on *C. albicans* biofilm formation to that of the pH modified media at the same pH value. This similarity is further phenotypically confirmed using calcofluor white images of the same conditions (Fig. 7d). More abundant and longer *C. albicans* hyphae can be observed with the rise in pH of both the ER5/1 supernatant and the media. As shown, with pH 5.0, yeast is the predominant phenotype of *C. albicans*. With pH increase to 6.0, more hyphae are evident, which becomes the dominant phenotype at pH 7.0. The biofilm biomass of LBF was not affected by pH modification of the supernatant and media except for lactic acid modified media, in which
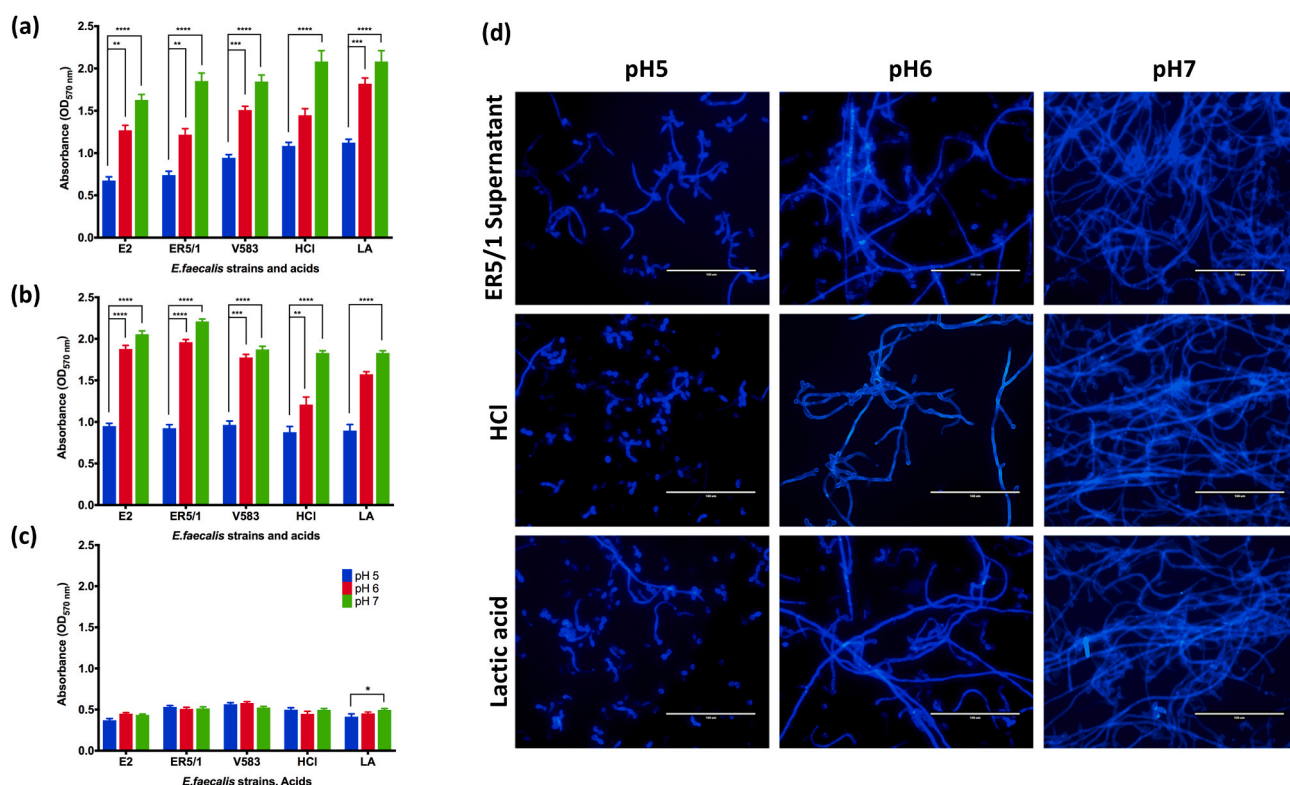
Fig. 7. The effect of pH modified *E. faecalis* supernatant and acid modified media on *C. albicans* biofilm biomass. Cell free *E. faecalis* supernatant of E2, ER5/1 or V583 supplemented with 10x RPMI used with its original pH 5 or adjusted to pH 6 and pH 7 using NaOH. THB:RPMI media also used at its normal pH 7 or adjusted to pH5 and pH6 using hydrochloric acid (HCl) or lactic acid (LA). pH modified supernatant and media was used to grow *C. albicans* SC5314 **(a)** HBF **(b)** and LBF **(c)** for 24 h and biofilm biomass assessed using crystal violet. **(d)** *C. albicans* SC5314 biofilms stained with Calcofluor white following incubation in the above-mentioned conditions for 24 h. Scale bars are 100 μm. Statistical significance was presented as * p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Results represent data from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the biofilm biomass was significantly higher in pH 7.0 compared with pH 5.0 (Fig. 7c). These observations indicate the potential role of *E. faecalis* supernatant pH on *C. albicans* hyphal morphogenesis and biofilm formation.

3.6. Enterococcus faecalis induced pH drop correlates with inhibition of Candida albicans hyphal morphogenesis

To gain insight into the chronology of *C. albicans* hyphal and biofilm formation in mono- and dual-species cultures with *E. faecalis*, the biofilm of both conditions is stained and imaged at 2, 4, 6, 8 and 24 h. The pH of the supernatant of each condition was also measured at the corresponding time points. It was shown that *C. albicans* starts to form germ tubes at the first hour of incubation in both conditions when the pH was 7.0 (Fig. 8). No differences were observed between the two conditions at 2 h, where true hyphae start to form and elongate whilst the pH of the mono-species remains neutral, whereas dropping to 6.5 in the co-culture. From 4 h onwards, the difference between the two conditions becomes visibly evident. Here the mono-species *C. albicans* biofilms showed denser and longer hyphae that continued to increase over time, whilst the pH remained neutral across all time points. Conversely, the abundance and length of the hyphae appeared to be unchanged from 4 to 24 h in the co-cultures. Interestingly, the 4 h time point also represents the point in which the media becomes more acidic and drops to 5.5 in the co-cultures. The pH of the co-culture remained acidic (pH 5.5) at 4, 6, and 8 h, and starts to rise slightly at 24 h to reach 6.0, which indicates some buffering attempts exerted by *C. albicans*. The pH values of *C. albicans* mono-species, *E. faecalis* mono-species and their co-culture at the different time points is available in Fig. S3. Collectively, these observations further confirm that *E. faecalis* induced inhibition of *C. albicans* hyphal morphogenesis and biofilm formation is based on the ability of *E. faecalis* to acidify the environment which in turns drives a predominantly yeast phenotype [68].

4. Discussion

There is a growing fascination of how *C. albicans* interacts with bacterial species within complex interkingdom communities [31,56,69, 70]. These studies have been instrumental in furthering our understanding of how this opportunistic yeast co-operates, or otherwise, with key bacterial species. Our previous work has shown how interactions with different species of *Lactobacillus* leads to differential health outcomes in patients with recurrent vulvovaginal candidiasis, and that denture wearing patients have resilient populations of *C. albicans* that support bacterial biofilm populations [31,71]. In this new study we describe changes in *C. albicans* growth, phenotype, virulence and transcriptome when co-cultured with *E. faecalis in vitro*. In agreement with previous *in vitro* and *in vivo* studies, our preliminary investigation showed an inhibition of *C. albicans* growth, hyphal morphogenesis and biofilm formation in dual-species biofilms [18,33–40]. Subsequent RNASeq showed that *E. faecalis* rapidly and significantly alters *C. albicans* gene expression. Our enrichment analysis findings and the degree of overlapping with responses observed to lactobacilli and weak organic acids directed our interest toward the role of pH and stress response in this inter-kingdom interaction.

At the outset of this work we reviewed the published mechanisms of interaction between *C. albicans* and *E. faecalis*, where the secreted bacteriocin EntV was reported as a potent inhibitor of *C. albicans* hyphal morphogenesis, biofilm formation, and virulence, albeit with limited strain selection [34,40]. Despite this carefully characterised phenomenon we were unable to demonstrate a correlation between the expression of *EntV* and the level of inhibition of *C. albicans* biofilm biomass, suggesting that an alternative additional interaction was important. It has been reported that for optimal functionality EntV requires post-translational gelatinase processing [36], though our own assessment of gelatinase activity showed there was no difference observed between the strains with positive activity, and those lacking this property in supressing *C. albicans* biofilm (data not shown). Moreover, despite the heat inactivation and the removal of any *E. faecalis* supernatant proteins of molecular weight of more than 3 kDa, *C. albicans* hyphal and biofilm formation was not recovered, again suggestive of another mechanism. EntV is a heat stable peptide, therefore, recovery of *C. albicans* biofilm activities was not anticipated following heat treatment. However, the increased inhibition associated with ultrafiltered supernatants was surprising. There is no clear explanation of why ultrafiltration enhanced biofilm inhibition, however, it is possible that the proteins and molecules from the supernatant had a subtle buffering capacity, so removal of these exposed *C. albicans* solely to a pH effect. We surmised that differences in growth media and the *E. faecalis* strains used in our work could account for the difference in findings. The ability
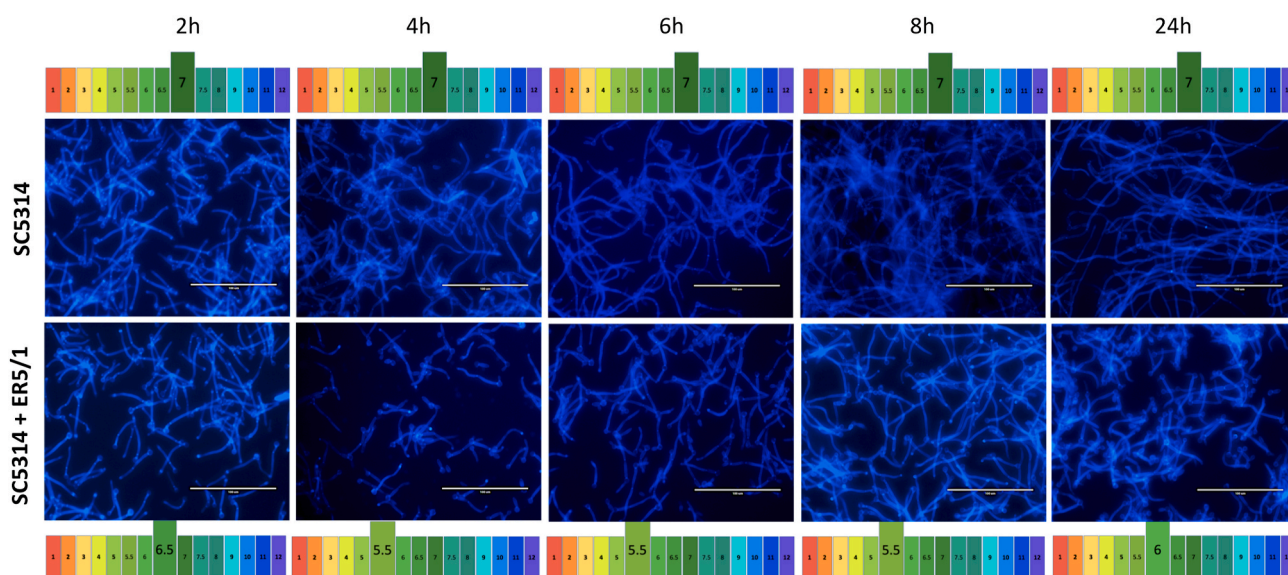

Fig. 8. *C. albicans* hyphal formation and elongation in mono-culture and coculture with *E. faecalis* at 2, 4, 6, 8 and 24h. *C. albicans* SC5314 grown in THB: RPMI in mono- and dual-species biofilms with *E. faecalis* ER5/1. At every time point, supernatant removed, biofilms washed with PBS and stained with calcofluor white and supernatant's pH is measured. Images are for SC5314 and ER5/1 with Scale bars of 100 μm and pH measurements of the supernatant of each condition at the relevant time point.

of *E. faecalis* to metabolise nutrients and thereby produce organic acids may differ according the media used. Metabolising THB:RPMI by *E. faecalis* resulted in a significant drop in pH after 24 h. However, similar drop may be not be achieved with other growth media such as artificial saliva used in EntV based studies. Indeed, post-translational modification following gene expression may explain the absence of correlation between the high EntV gene expression in three of the *E. faecalis* strains and the levels of *C. albicans* biofilm inhibition. It is also possible that the low expression of *EntV* gene in most *E. faecalis* strains was sufficient, or reached an expression threshold, beyond which any extra expression fails to cause more inhibition of *C. albicans* biofilm formation. We cannot rule out the possibility that EntV requires undefined optimal conditions for full functionality, including a specific pH range. Therefore, it is possible that raising the supernatant pH may have impaired the functionality of *EntV* and renders it less effective in inhibiting *C. albicans* biofilm formation. However, the evidence gathered does not support previous reports showing the singular importance of EntV in preventing *C. albicans* hyphal growth and biofilm formation [34].

To investigate for alternative mechanisms, we conducted a large-scale transcriptional analysis, and in doing so revealed the possibility of pH dependent mechanisms. Amino acid starvation was excluded for the reasons explained above. Some studies reported enrichment of *C. albicans* amino acids biosynthesis/metabolism in response to acids [29], lactic acid producing bacteria [31] and to oxidative stress [30,62]. Moreover, the vast majority of enriched pathways in *C. albicans* mono-species biofilms were also enriched in the response to the weak organic acids [29]. Upregulation in arginine biosynthesis and oxidative stress related genes were also shown in *C. albicans* when internalized by macrophages [62]. However, none of the reported oxidative stress genes in their study was upregulated in our dual-species biofilm. This further focussed the subsequent investigation to pH dependant mechanisms.

C. albicans tolerates and adapts to a wide pH range, and this dynamic feature plays an important role in changing gene expression and morphology [64,72]. In an acidic environment, *C. albicans* grows as yeast cells, while neutral and alkaline environment induces filamentation [68]. In low pH, *C. albicans* uses amino acids as a carbon source in glucose limiting environment and produce ammonia which in turn rises the extracellular pH and induce hyphal morphogenesis. This ability of *C. albicans* to raise the extracellular pH was also observed in our results. As shown in Figs. 8 and S3, despite the early drop, the pH of dual-species biofilms started to rise at 24 h. Therefore, *E. faecalis* induced inhibition of C. albicans can be time and media dependant where the ability of *E. faecalis* to acidify the media and the time required for C. albicans to raise the extracellular pH and restore hyphal morphogenesis play a major role. C. *albicans* environment alkalization was also associated with upregulation in arginine biosynthesis [67]. This finding further supports the close association between amino acid biosynthesis upregulation and pH response in *C. albicans*. Moreover, *C. albicans* utilization of amino acids for environment alkalization is repressed by glucose. This may explain the reduced expression of amino acid biosynthesis and transport genes in glucose supplemented media noted in our data shown in Fig. 4c.

As *E. faecalis* favours the growth in the yeast form of *C. albicans*, it suppresses the latter's virulence. We showed previously that *C. albicans* is more susceptible to endodontic irrigants when co-cultured with *E. faecalis* compared with its mono-species biofilm [73]. Ishijima and colleagues (2014) also showed that oral administration of heat killed *E. faecalis*, in the form of the commercially available probiotic, protected immunocompromised mice from oral candidiasis [37]. They reasoned this protection to the direct binding of heat killed *E. faecalis* to *C. albicans* yeast, pseudohypha and hyphae, and therefore reduces *C. albicans* adherence to surfaces. This is another proposed mechanism of *C. albicans* and *E. faecalis* interaction. Likewise, it has been shown that the survival of *Caenorhabditis elegans* was significantly higher when co-infected with *C. albicans* and *E. faecalis* compared with the infection with single

species. Similar results were also reported by Cruz and colleagues [33], which indicates that *C. albicans* and *E. faecalis* are inhibiting one another's virulence, and instead enhance commensalism. When considering endodontic infections of the root canals of teeth where these two microorganisms are frequently co-isolated from recurrent infections, this interkingdom interaction may influence their persistence in the root canals. The standard endodontic treatment regimen of root canals involves dressing the canals with calcium hydroxide as antimicrobial medicament between treatment visits. The antimicrobial efficiency of calcium hydroxide is attributed to its high alkalinity (pH 12.5) [74]. Although, most endodontic pathogens are sensitive to calcium hydroxide, *C. albicans* and *E. faecalis* are shown to be tolerant [75]. The ability of *E. faecalis* to produce lactic acid may account for its ability, and *C. albicans* through coexistence, to neutralize and survive the high alkalinity of calcium hydroxide.

Finally, we show the importance of E. *faecalis* induced pH change as inducer of *C. albicans* inhibition of hyphal and biofilm formation. However, the interkingdom interaction between *C. albicans* and *E. faecalis* is dynamic and complicated process that is further influenced by the environment in which this interaction takes place. Therefore, it is expected that this interaction between the two microorganisms can vary in different environments such as in the root canals of teeth and the gut. It is plausible to consider that the biofilm microenvironment within root canals has a positive influence in their ability to coexist and take advantage of the dentinal tubules [76]. The low pH can drive dissolution and enlargement of the tubules, which can then enhance invasive capacity. When *C. albicans* elevates the pH within this microenvironment, then its ability to induce hyphae and invade into the tubules is enhanced [77]. Furthermore, it cannot be claimed that *E. faecalis* effect is based solely on pH and subsequently, the other reported mechanisms namely, EntV bacteriocin [34], anticandidal protein [40] and direct binding [37] cannot be excluded. Notably, we observed that despite a downregulation of hyphal regulated genes *HWP1* and *ECE1*, we did not observe an expected reciprocal downregulation of the key adhesin *ALS3*. This may indicate that the presence of *E. faecalis* stimulates *C. albicans* to maintain its extracellular adhesins to support potential interactions, or at least the organisms within the same microenvironments.

Overall, it is more reasonable to consider that the effect of *E. faecalis* on *C. albicans* is a collective effect of a combination of different mechanisms that ultimately manifested by *C. albicans* inhibition. How the interaction between *C. albicans* and *E. faecalis* fits for potential antimicrobial strategies remains unclear and should be further explored. In addition, to gain a full picture of this interkingdom interaction, the impact of *C. albicans* on *E. faecalis's* biology should be also evaluated.

5. Conclusions

This study highlights the importance that the microenvironment can make in driving dysbiosis or rebiosis within complex interkingdom interactions. We cannot assume antagonism from a simple phenotypic change, but instead we need to carefully assess whether this change benefits the competing species. We have shown that modulation of the pH microenvironment influences these perceived enemies to become friends (frenemies) and drive co-existence to support invasion of dentinal tubules.

CRediT authorship contribution statement

Om Alkhir Alshanta: participated in study design, experimental procedures, data analysis, and wrote the manuscript. **Khawlah Albashaireh:** participated in study design, experimental procedures and data analysis. **Emily McKloud:** participated in study design, experimental procedures and data analysis. **Christopher Delaney:** participated in study design, experimental procedures and bioinformatic data analysis. **Ryan Kean:** conceived the study, participated in study design, and was responsible for producing the final manuscript. **William McLean:**

conceived the study, participated in study design, and was responsible for producing the final manuscript. **Gordon Ramage:** conceived the study, participated in study design, and was responsible for producing the final manuscript. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Data availability

Sequenced transcriptome data has been deposited to the NCBI sequence read archive (SRA) database and can be found under accession number PRJNA731052.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biofilm.2022.100072.

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