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Candida albicans promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium

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Summary *Objective:* The principal aim of this study was to investigate the *in vitro* co-infection of a reconstituted human vaginal epithelium (RHVE) by *Candida albicans* and *Candida glabrata*.
Methods: The ability of both species to invade and colonise the RHVE was examined using species-specific peptide nucleic acid (PNA) probe hybridisation, confocal laser scanning microscopy (CLSM) and a novel qRT-PCR protocol for *Candida* quantification in the tissues. RHVE damage was evaluated by measuring lactate dehydrogenase (LDH) activity. *Candida* virulence gene expression (*HWP1*, *ALS*, *EPA*, *PLB*, *PLD* and *SAP*) was evaluated by quantitative RT-PCR.
Results: The results showed that whilst both species induced damage to the RHVE, this was notably less with *C. glabrata*. Interestingly, there was a significant increase in *C. glabrata* RHVE colonisation and invasiveness when it was added to the tissue with *C. albicans*. The extent of RHVE damage caused by the two species appeared to be primarily dependent on the process of invasion. Of the virulence genes assayed, *HWP1*, *PLD1* and *ALS3* were deemed to be most associated with pathogenicity in the model.
Conclusions: For the first time, we have demonstrated that the RHVE model coupled with specific tools of analysis, allows assessment of *Candida* colonisation and invasion in single and co-

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infection. Using this model we have demonstrated that *C. albicans* enhanced *C. glabrata* colonisation, invasion and tissue damage, which was also evidenced by the expression of virulence genes.

Introduction

Several *Candida* species can colonise human mucosal surfaces as harmless commensals. However, debilitation of the host often results in infection (candidosis), which most frequently manifest as superficial infections of moist mucosal surfaces, such as those of the vagina and oral cavity.[1,2] In the case of vulvovaginal candidosis (VC), *Candida* colonisation rates also vary in type 1 and type 2 diabetic patients.[3] Healthy pregnant women and those using oral contraception and/or antibiotics are also reported to have a high incidence of vaginal candidal carriage.[4] VC is diagnosed in up to 40% of women with vaginal complaints in the primary care setting.[5] Approximately 75% of women experience at least one episode of VC during their lives.[4,6]

Candida albicans is the most frequent *Candida* species involved in both colonisation and infection.[2,7] However, there has been a notable increase in the relative proportion of infections caused by non- *C. albicans Candida* (NCAC) species.[7] An important species in this regard is *Candida glabrata*, which is now the second leading cause of both blood stream and mucosal candidosis in the USA.[7,8] Moreover, several studies have reported enhanced *Candida* species epithelium invasion/colonisation and candidosis episodes caused by co-infection. In 2011, Silva et al.[9] showed enhanced *C. albicans* colonisation and invasiveness of *C. glabrata* in co-infection studies, leading to increased damage in a reconstituted human oral epithelium. In 2013, Kali et al.[10] showed synergistic growth involving *Candida* species and *Mycobacterium tuberculosis* in 40% of patients with pulmonary tuberculosis. In 2010, Martins et al.[11] described *Candida* species oral co-infection episodes in patients attending a dental clinic in Braga, Portugal, where the combination of *C. albicans* - *C. glabrata* was the most commonly found, as noted in other studies.[12–16]

Several model systems have been used to study *Candida*-host interactions, including *in vitro* (reconstituted epithelium)[17] and *in vivo* models (pseudoestrous mice).[18–20] However, since *C. albicans* does not naturally colonise mice, murine models do not adequately reflect human candidosis,[21] which makes results difficult to interpret.

The human epithelium is a complex multi-layered, multi-cellular tissue consisting of increasingly differentiated epithelial cells[22,23] and in recent years, multi-layered organotypic three-dimensional *in vitro* culture systems have been developed to mimic human epithelium.[24,25] Reconstituted human vaginal epithelium (RHVE) is a biological product commercialised by SkinEthic Laboratories (Nice, France) and is based upon the A431 vaginal squamous cell carcinoma cell line. The epithelium has successfully been used to study *in vitro* mechanisms of tissue degradation and virulence gene expression following infection with *Candida* species.[26]

Putative *Candida* virulence factors include the ability to exhibit morphological transition, adhere to host tissues and develop as biofilms, and secrete hydrolytic enzymes. In the case of the latter, secreted aspartyl proteinases (*SAP*s) are considered highly important enzymes in candidosis, which are able to degrade many human proteins at lesional sites, facilitating invasion of *Candida* into the epithelium.[27] Other tissue degrading enzymes produced by *Candida* include phospholipases, which hydrolyse one or more ester linkages of glycerophospholipids.[28] Host cell recognition and colonisation by *Candida* is also facilitated by adhesins, such as Agglutinin-Like Sequence (*ALS*) proteins and Hyphal Wall Protein (*HWP*).[29]

The aim of this study was to characterise *C. glabrata* and *C. albicans* co-infection of a vaginal epithelium by using an RHVE model and to compare with previous work involving oral epithelium.[9] This study was coupled with confocal laser scanning microscopy (CLSM) and quantitative real-time polymerase chain reaction (qRT-PCR). Analysis of *HWP1*, *ALS*, phospholipase B and D (*PLB* and *PLD*), *SAP* and epithelial adhesion (EPA) virulence gene expression was also undertaken.

Material and methods

Microorganisms

Three clinical isolates of *Candida* species were used in this study and included those isolated from the oral cavity (*C. albicans* 324LA/94 and *C. glabrata* D1) and vagina (*C. glabrata* 585626). The oral isolate of *C. albicans* was obtained from the culture collection of the School of Dentistry, Cardiff, UK and the oral strain of *C. glabrata* was acquired from the biofilm group of the Centre of Biological Engineering, Minho University (Braga, Portugal). The strain isolated from vaginal infection was gifted from the culture collection of the Hospital de Braga (Portugal). In addition to these clinical isolates, two reference strains, namely, *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 (obtained from the American Type Culture Collection) were also included in this study. The *Candida* strains were selected based on their previously determined ability to invade a Reconstituted Human Oral Epithelium.[9] The identity of all strains was confirmed by PCR-based sequencing using specific primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[30]

Culture of *Candida*

For each experiment, isolates were cultured on Sabouraud's Dextrose Agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37 °C. The isolates were then sub-cultured overnight in Yeast Nitrogen Base (YNB; BD Diagnostics, Cowley, UK)

medium supplemented with 0.5% (w/v) glucose at 37 °C under agitation at 120 rev/min. After incubation, cells were harvested by centrifugation at 3000 $\times g$ for 10 min at 4 °C and washed twice in 10 ml of Phosphate Buffered Saline (PBS; pH 7). The cellular density of each strain was adjusted to 2×10^6 cells/ml in SkinEthic maintenance medium (SkinEthic Laboratories; Nice, France) using an improved Neubauer haemocytometer (Marienfild, Land-Könicshofen, Germany).

Infection of a reconstituted human vaginal epithelium (RHVE)

To study *in vitro* single and co-infection of vaginal epithelium by *C. albicans* and *C. glabrata*, a commercially available reconstituted human vaginal epithelium (RHVE) (SkinEthic Laboratories) was used as an *in vitro* model of vaginal candidosis. RHVE tissue inserts (0.5 cm^2) were placed in 12-well tissue culture plates. As a negative control, 1 ml of maintenance medium was added to an RHVE tissue preparation. To study single species infection, 5 RHVE tissues were inoculated with 1 ml of standardised suspensions of each *Candida* isolate, prepared as previously described.

In dual species studies, *C. albicans* ATCC 90028 (500 μl; 2×10^6 cells ml^{-1}) or *C. albicans* 324LA/94 was combined with one of *C. glabrata* D1, ATCC 2001, or 585626 (500 μl; 2×10^6 cells ml^{-1}). Each dual species experiment was repeated in 3 independent experiments.

All infected tissues were incubated at 37 °C in a 5% CO_2 environment in saturated humidity for 24 h. After incubation, the tissue was rinsed twice in 1 ml of PBS to remove non-adherent *Candida* cells, and the tissue was then bisected, with one half being used for confocal laser scanning microscopy (CLSM) analysis and the other for qRT-PCR.

Histological techniques

The RHVE tissue for CLSM analysis was fixed in 2% (v/v) formalin and stored at 4 °C until histological processing. Tissues were then dehydrated, cleared, and infiltrated with paraffin wax embedding material. The formalin-fixed, paraffin-embedded (FFPE) tissues were stored at room temperature. The tissues were cut (20 μm sections) and placed on Histobond + coated microscope slides (Raymond A Lamb, East Sussex, UK), de-waxed, and processed through xylene, ethanol, and water before peptide nucleic acid probe hybridization.

Peptide nucleic acid fluorescent *in situ* hybridization

Peptide nucleic acid probe fluorescence *in situ* hybridisation (PNA FISH) was applied to tissue sections using the Yeast Traffic Light (YTL) PNA FISH™ kit (AdvanDx Inc., Woburn MA, USA). These species-specific probes were utilised to study the invasion of the RHVE by *C. albicans* and *C. glabrata*. The YTL PNA FISH™ kit had previously been developed and evaluated using multicolour labelled fluorescent PNA probes targeting specific 26S rRNA sequences of *C. albicans* and *C. glabrata*. The assay was performed in accordance with the guidelines of the manufacturer, with some minor modifications. Tissue sections on microscope slides were overlaid with 1 drop of the respective PNA probes. After 90 min of incubation, in the dark and in a humidified chamber at 55 °C, unbound probe was removed by washing the slides using a previously warmed wash solution at 55 °C for 30 min. The preparation was then mounted in a medium suitable for fluorescence microscopy, which also contained 4',6-diamidino-2-phenylindole (DAPI) for detection of epithelial cell nuclei (*Vectashield, Vector laboratories, California, USA*). Samples were examined by CLSM, where *C. albicans* and *C. glabrata* were represented by bright green and red fluorescence, respectively.

Confocal laser scanning microscopy (CLSM)

Tissue sections (20 μm) hybridised with PNA probes were observed by CLSM, using a Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) to analyse the level of colonisation and morphological characteristics of *C. albicans* and *C. glabrata* in single and co-infection of the surface of RHVE tissues. Sections were scanned through the full depth using appropriate settings for single, double or triple channel fluorescence recordings. Fluorescein-labelled PNA was used for detection of *C. albicans* (laser excitation line 492_{nm} and emissions detected 520_{nm}); Tamara-labelled PNA was used for detection of *C. glabrata* (laser excitation line 565_{nm} and emissions detected 580_{nm}) and DAPI (laser excitation line 485_{nm} and emissions detected $410-485_{nm}$) for nuclear context of keratinocytes. For multi-channel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap between probes. Selected images were presented either as single confocal optical sections or maximum intensity type constructions. A specific classification scale was used to express the level of *Candida* RHVE colonisation. Colonisation categories were as follows: extensive (fungal elements totally covered the surface); moderate (fungal elements covered a large proportion of surface) and sparse (fungal elements covered only limited areas of surface). Similarly, the following categories defined the level of *Candida* RHVE invasion: high (fungal elements totally invaded all RHVE keratinocytes layers); moderate (fungal elements moderately invaded the first RHVE top keratinocytes layers) and low (fungal elements only formed isolated clusters in the first RHVE keratinocytes layer).

Lactase dehydrogenase (LDH) activity

The extracellular activity of LDH released from RHVE cells was monitored as an indicator of tissue damage. LDH released in the maintenance medium of the control RHVE which was devoid of *Candida*, as well as infected RHVE was measured at 24 h using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega). LDH activity from *Candida* cells in a planktonic control (prepared as described for the infection model but devoid of RHVE) was subtracted from the LDH activity of the tissues infected with the different single and co-infection *Candida* species. The LDH released during single or co-infection with the different *C. albicans* and *C. glabrata* strains was then

expressed as relative LDH activity to the untreated control tissue. LDH activity was analysed spectrophotometrically (*FLUOstar OPTIMA*; BMG Labtech, Ortenberg/Germany) by measuring the NADH disappearance rate at 544 nm excitation and 590 nm emission during the LDH-catalyzed conversion of pyruvate to lactate. All experiments were performed in triplicate.

Quantification of *Candida* species in RHVE tissue sections

DNA extraction

After histological processing, four tissue sections of 20 μm were cut from each FFPE tissue and placed in sterile 1.5 ml microcentrifuge tubes (Eppendorf AG, Hamburg, Germany). Paraffin wax was removed using 1 ml of xylene and the tubes centrifuged vigorously for 10 s. After centrifugation (13,000 × *g* for 2 min), the supernatant was discarded and the residual xylene removed from the tissue pellet by adding 1 ml of absolute ethanol. After repeat centrifugation, the supernatant was carefully removed. The tubes were left open for 10 min at room temperature and DNA for PCR analysis was extracted from the tissue pellet using a commercial DNA extraction kit (QIAamp® DNA FFPE Tissue; Qiagen, Crawley, UK).

Quantification of DNA by real-time PCR

Candida were quantified using real-time PCR in an ABI Prism 7000 (Applied Biosystems, Life Technologies Ltd., Paisley, UK). For each sample, 5 μl of extracted DNA was added to a PCR solution containing 12.5 μl of 2 × qPCR Master Mix and 0.25 μl of reference dye (SYBR® Green JumpStart™ *Taq* ReadyMix™, Sigma–Aldrich, Poole, UK), 0.2 μl of 3 μM of each primer (Sigma–Aldrich) (Table 1) and 3.25 μl of dH_2O (Promega, Madison, WI, USA). Negative controls were reaction mixtures with dH_2O (Promega, Southampton, UK) substituting for the template DNA. Template DNA was obtained from FFPE tissues using the DNA extraction protocol described above. PCR cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 s, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s. In each cycle, a dissociation stage at 60 °C was run to generate a melting curve for verification of amplification product specificity.

Calibration curves (Ct vs. Log cells) for each *Candida* isolate were constructed using the same PCR protocol as described above. For these, serial dilutions of known cell concentrations of *Candida* were formalin fixed and the DNA extracted from these using a DNA extraction kit (QIAamp® DNA FFPE Tissue; Qiagen) with some modifications.

Analysis of candidal virulence gene expression

RNA extraction

For gene expression analysis, each *Candida* was cultured as previously described. For RNA extraction, a fresh RHVE tissue was removed with a scalpel directly from the plastic scaffold and placed in a 1.5 ml microcentrifuge tube (QIAshredder®; Qiagen) with 600 μl of RLT buffer containing 500 μl of glass beads (0.5 mm diameter) and 1/100 (v/v) of β-mercaptoethanol. This final mix was homogenised twice for 30 s using a Mini-Bead- Beater-8 (Stratech Scientific, Soham, UK) to lyse cells. The RNeasy Mini Kit (Qiagen) was then used to complete total RNA extraction from the tissue according to the manufacturer's recommended protocol.

Synthesis of cDNA

From each sample, 0.5 μg of RNA was used for complementary DNA (cDNA) synthesis. RNA (14 μl) was incubated with 1 μl (50 μg ml^{-1}) of random primer mix (Promega) in RNase free dH_2O at 70 °C for 5 min. To this solution, 10 μl of a master mix containing 5 μl of M-MLV Reaction buffer (5 ×), 1 μl of each deoxynucleoside triphosphate (dNTP, 10 μM), 1 μl of RNasin Plus RNase inhibitor, 1 μl of M-MLV enzyme and 2 μl RNase free dH_2O was added. All reagents used in these experiments were from Promega. Synthesis of cDNA was performed at 37 °C for 1 h.

Primer design

Primers for 'housekeeping genes' (ACT1_alb and ACT1_gla), and putative virulence genes (*ALS*, *HWP*, *EPA*, *PLB*, *PLD* and *SAP*) were designed using Primer3 web software (http://fokker.wi.mit.edu). Full-length gene sequences were obtained from the *C. albicans* and *C. glabrata* database http://www.candidagenome.org.[31] The specificity of each primer was confirmed by comparing its respective sequences to the *C. albicans* and *C. glabrata* database using BLAST.[32] To verify the specificity of each primer pair for its corresponding target gene, PCR using the various primer pairs was applied to genomic DNA extracted from each of the *Candida* strains.

The sequences of the primers developed in the present study are provided in Table 2.

Reverse transcription quantitative PCR

Real-time PCR was performed in 96-well plates (Bright-White real-time PCR; Primer Design, Southampton, UK)

Table 1 Primers used for real time-PCR screening for *C. albicans* and *C. glabrata*.

Sequence (5′ → 3′)	Primer	Target
GAGCGTCGTTTCTCCCTCAAACCGCTGG	Forward	*C. albicans*
GGTGGACGTTACCGCCGCAAGCAATGTT	Reverse	
ATTTGCATGCGCTTGCCCACGAATCC	Forward	*C. glabrata*
ACGTCTGATCCAATCAATGGCTGGTGA	Reverse	

Table 2 Forward (FW) and reverse (RV) primers used for real-time PCR.

	Sequence (5′ → 3′)	Orientation	Target
Housekeeping Gene (*C. albicans*)	TGCTGAACGTATGCAAAAGG	FW	ACT1_alb
	TGAACAATGGATGGACCAGA	RV	
Housekeeping Gene	TTGCCACACGCTATTTTGAG	FW	ACT1_gla
(*C. glabrata*)	ACCATCTGGCAATTCGTAGG	RV	
HWP1 gene	TCTACTGCTCCAGCCACTGA	FW	HWP1
	CCAGCAGGAATTGTTTCCAT	RV	
ALS genes	CCCAACTTGGAATGCTGTTT	FW	ALS1
	TTTCAAAGCGTCGTTCACAG	RV	
	GCACTTCATTGACTGGAGCA	FW	ALS2
	TCATTGTTGCCACCTTGTGT	RV	
	CTGGACCACCAGGAAACACT	FW	ALS3
	GGTGGAGCGGTGACAGTAGT	RV	
	TCCACAGTTTCTCGTCCACA	FW	ALS4
	ATTGCCACGCTTGTTTTACC	RV	
	GTTCAGACATGCCATCATCG	FW	ALS5
	CCAAGTGATCAGGGTGGACT	RV	
	ATCGGAAGCTCCAAATTCCT	FW	ALS6
	AGGATGTTTAGTGGCGGATG	RV	
	GACCTTTTGTGGATGCGATT	FW	ALS7
	TTTTCTGGAGTCGGGAAATG	RV	
	GTGCCACAATGTGAGAATGG	FW	ALS9
	GTGCCACAATGTGAGAATGG	RV	
EPA genes	ATGTGGCTCTGGGTTTTACG	FW	EPA1
	TGGTCCGTATGGGCTAGGTA	RV	
	TTATGCCGTATGGGGTTCTC	FW	EPA6
	GAGTCAACTGAGGCACACGA	RV	
	AGGATGCACACCCGAAATAG	FW	EPA7
	TTACCAGCCCCAAAATTCAC	RV	
Phospholipase B genes	GCTCTTTTCAACGAAGCGGTGT	FW	PLB1
	GCCATCTTCTCCACCGTCAACT	RV	
	CAATACTAGCCGCGTTGGGAAG	FW	PLB2
	GCCCATGAAAAACCCTGCATTA	RV	
	TCCCAATTGTTGTTGCTGATGG	FW	PLB3
	CCGCATTATCAAACCCACCAAT	RV	
	TCGTCCGGGTCTTCAAGTTCTC	FW	PLB5
	ATCTCCCGAATCCCCGTCTAAA	RV	
Phospholipase D gene	GCCAAGAGAGCAAGGGTTAGCA	FW	PLD1
	CGGATTCGTCATCCATTTCTCC	RV	
Secreted Aspartyl Protease genes	CAATGCTGCCACTGGACAAATC	FW	SAP1
	CAATTCAGCTTGGAAGGCATCA	RV	
	ACCGTTGGATTTGGTGGTGTTT	FW	SAP2
	ATTATTTGTCCCGTGGCAGCAT	RV	
	TGGTCCCCAAGGTGAAATCAAT	FW	SAP3
	TGGATCTTGTCCTTGACCAGCTT	RV	
	GTCAATGTCAACGCTGGTGTCC	FW	SAP4
	ATTCCGAAGCAGGAACGGAAAT	RV	
	ATCTTCCCGCACTTCCCAAAAT	FW	SAP5
	TCGCCGCTTTGAAAACCAATAC	RV	
	AAAATGGCGTGGTGACAGAGGT	FW	SAP6
	CGTTGGCTTGGAAACCAATACC	RV	
	ACGGGTGTTGTTTTGGATACCG	FW	SAP9
	GTCGACTGTTCTGCTGGAGTCG	RV	
	CCCGTTGATTCCAAAAGTCAGC	FW	SAP10
	TCGCCTATCGAAAACCCAAAGA	RV	

using the ABI PRISM 7000 (Applied Biosystems). A total of 5 μl of two-fold diluted cDNA samples and 20 μl of PCR solution (described above) were added to the plates with the respective virulence gene primers. Real-time PCR was performed with an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 s, primer annealing at 54 °C for 30 s and extension at 72 °C for 30 s. In each cycle, a dissociation stage at 60 °C was run to generate a melting curve for verification of amplification product specificity. Control samples were included on each plate to ensure that multiple plates could be compared. The *ACT1* gene of *C. albicans* and *C. glabrata* had previously been used as a reference candidal housekeeping gene[33] and were also used in this study.

All samples were run in triplicate. The Ct value of each sample was determined, and the relative gene expression levels calculated using the ΔCt method, which was normalized to the housekeeping genes described above, as a control.

Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying Tukey multiple-comparisons test, using Graphpad Prism program for Windows, version 6. All tests were performed with a confidence level of 95%.

Results

In vitro single and co-infection of RHVE

The surface colonisation and invasion of fresh RHVE by *Candida* was examined after 24 h incubation with *C. albicans* and *C. glabrata* strains in single and co-infection studies (Figs. 1 and 2 and Table 3). The results showed that all strains were able to colonise the RHVE surface in single and co-infection. However, the level of colonisation was species, strain and combination dependent.

Candida albicans ATCC 90028 and *C. albicans* 324LA/94 (oral strain) exhibited the highest level of colonisation (extensive colonisation) of the vaginal epithelial surface in single and co-infection (Fig. 1A and Fig. 2A—C, Fig. 1B and Fig. 2D—F, respectively).

Although all *C. glabrata* strains colonised the tissue in single infection, this was only sparse when compared with *C. albicans*. In general, after 24 h of single infection, only limited clusters of *C. glabrata* were detected on the surface of the keratinocyte layers (Fig. 1C—E). Additionally, in the presence of *C. albicans*, all *C. glabrata* (Fig. 2) strains demonstrated increased RHVE colonisation. The co-infection of *C. glabrata* 585626 (vaginal strain) with *C. albicans* 324LA/94 (Fig. 2F) actually resulted in the *C. glabrata* having the same level of colonisation as encountered with *C. albicans* strains (*i.e.* extensive colonisation).

Furthermore, when tissue invasion was analysed, similar associations were evident. *Candida albicans* again had a high ability to invade the RHVE model, and it was possible to detect hyphal and yeast elements totally penetrating the RHVE layers (Fig. 1A—B). In single species infection involving *C. glabrata*, yeast invasion of the epithelium was not apparent (Fig. 1C—E) and superficial detachment of keratinocyte layers was not evident. However, as with RHVE colonisation, when *C. glabrata* were combined with *C. albicans*, the level of invasion of *C. glabrata* was increased (moderate invasion in all combinations) (Fig. 2).

Using real-time PCR, it was possible to quantify the number of *Candida* present in the RHVE sections (Table 3). *Candida albicans*, was present in higher numbers (4-fold more) in the RHVE sections than *C. glabrata*, corroborating the CLSM observations. In both single and mixed species infections, *C. albicans* ATCC 90028 (4.76 ± 0.21 $\log_{10}$ number of cells per mL) was present in significantly ($P < 0.01$) higher numbers than *C. albicans* 324LA/94 (4.01 ± 0.24 Log_{10} number of cells per ml). No statistical differences were evident between the levels of *C. glabrata* in single species RHVE infection.

Compared with single species infection, it is important to note that the inoculum used for each individual species was halved in the co-infection studies (Table 3) and statistical comparison between single and co-infection assays was not possible. Despite this, it was evident that in certain cases *e.g. C. glabrata* ATCC 2001, the number of cells increased between the single (0.88 ± 0.41 $\log_{10}$ number of cells per ml) and co-infections with *C. albicans* ATCC 90028 (2.05 ± 0.23 $\log_{10}$ number of cells per ml).

LDH activity as an indicator of tissue damage

In order to determine the extent of RHVE damage induced by *Candida*, the levels of LDH activity were measured after 24 h of single and co-infection (Fig. 3). In single infection, *C. albicans* caused the highest levels of tissue damage ($P < 0.001$), with *C. albicans* 324LA/94 being significantly more damaging than the other strains ($P < 0.001$). In the case of single species infection with *C. glabrata*, only *C. glabrata* 585626 (vaginal strain) resulted in a significant ($P < 0.01$) increase in LDH activity compared to controls.

In co-infection studies (Fig. 3 ii) it is again important to highlight that half of the number of *Candida* cells was present in the starting inoculum. Interestingly, the higher tissue damage previously seen with *C. albicans* 324LA/94 compared with *C. albicans* ATCC 90028 in single infection was not apparent in co-infection assays. *Candida albicans* ATCC 90028 and 324LA/94 combinations with *C. glabrata* 585626 resulted in significantly higher tissue damage ($P < 0.01$; $P < 0.001$, respectively), compared with other co-infection combinations. In all co-infection assays, the tissue damage demonstrated a similar profile with respect to that induced by the respective C. *glabrata* strains. The LDH values corroborated with the degree of tissue damage observed by CLSM (Figs. 1 and 2 and Table 3).

Gene expression

qRT-PCR revealed a wide range of expression of Hyphal Wall Protein (*HWP1*), Agglutinin-Like Sequence (*ALS*), Phospholipase B and D, Secreted Aspartyl Proteinase (*SAP)* (Table 4) and Epithelial Adhesin (*EPA*) genes (Table 5) for RHVE single and co-infection combinations. The results were expressed as the mean percentage expression, relative to the expression of *ACT1* during RHVE infection. *ACT1* gene expression levels were constant in all assays.

HWP1 expression by *C. albicans* was up regulated during co-infection with all *C. glabrata* strains (Table 4). Expression of *ALS* genes was generally detected, however *ALS9* expression was not encountered in any of the analyses.

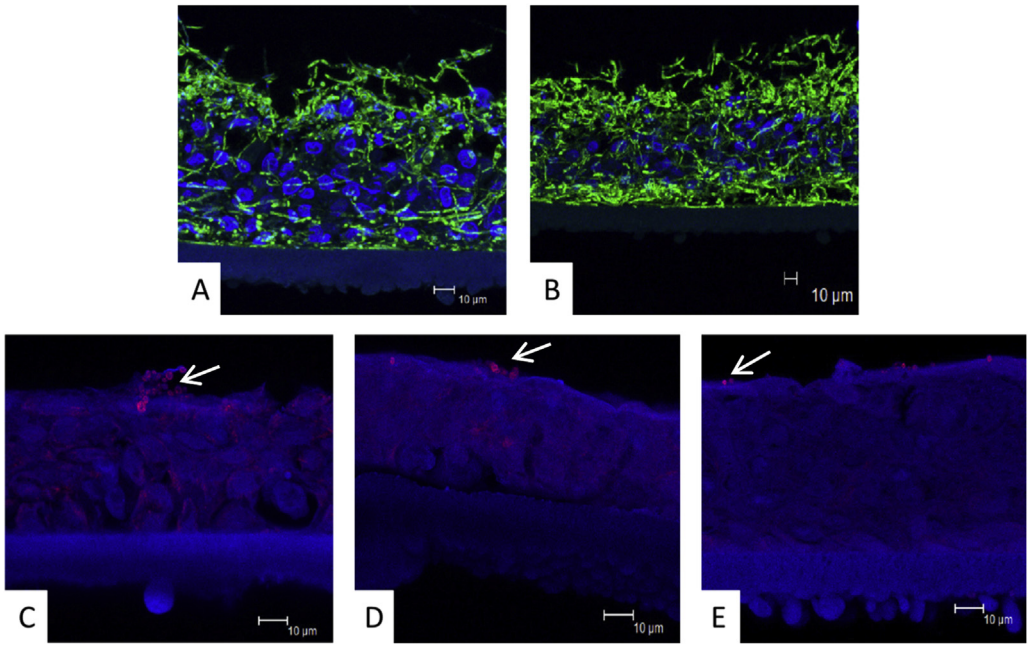

Figure 1 *C. albicans* and *C. glabrata* single infection of RHVE after 24 h, assessed by CLSM and YTL PNA FISH™. (**A**) *C. albicans* ATCC 90028, (**B**) *C. albicans* 324LA/94 (**C**) *C. glabrata* ATCC 2001, (**D**) *C. glabrata* D1 and (**E**) *C. glabrata* 585626. Arrows indicate the localisation of *C. glabrata* cells in the RHVE.

Compared with single species infection, *C. albicans ALS1* expression was up-regulated for *C. albicans* ATCC 90028 in association with *C. glabrata* ATCC 2001. *ALS3* expression demonstrated similar results when both *C. albicans* strains were combined with *C. glabrata* 585626 (vaginal strain). Furthermore, this gene was up-regulated for *C. albicans* 324LA/94 in the presence of all the other *C. glabrata*, and contrasted with the other ALS genes that were down-regulated when this strain was combined in infection with *C. glabrata*. Expression of *Phospholipase B* and *D* gene families by *C. albicans* was always detected in the samples (Table 4). All these genes were up-regulated during co-infection of *C. albicans* 324LA/94 and *C. glabrata* 585626. Expression of PLD1 was also up-regulated when *C. albicans*

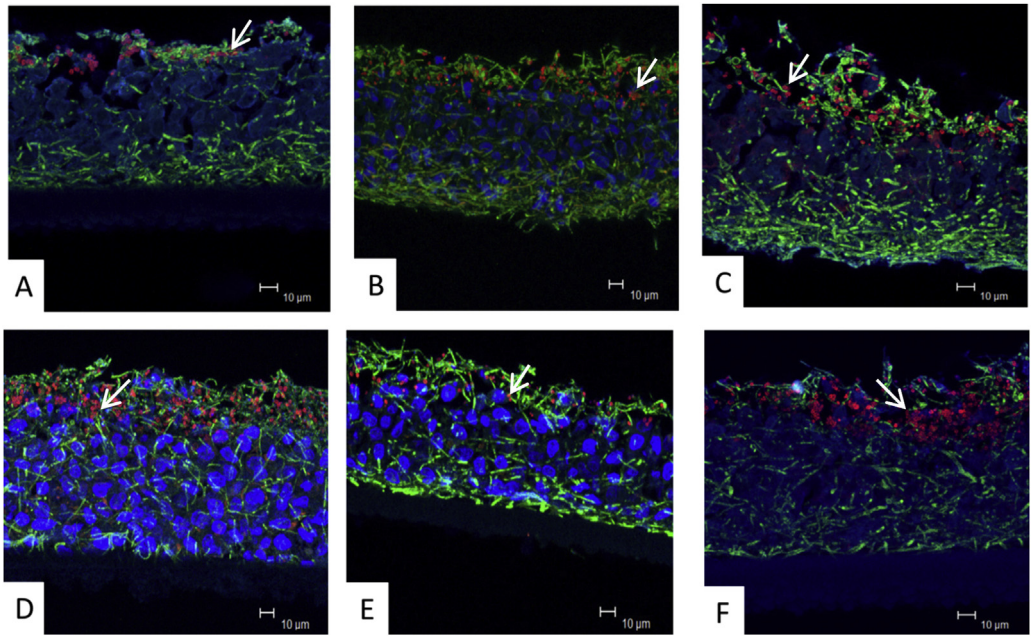

Figure 2 *C. albicans* and *C. glabrata* strains co-infection of RHVE after 24 h, assessed by CLSM and YTL PNA FISH™. (**A**) *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001, (**B**) *C. albicans* ATCC 90028 and *C. glabrata* D1, (**C**) *C. albicans* ATCC 90028 and *C. glabrata* 585626, (**D**) *C. albicans* 324LA/94 and *C. glabrata* ATCC 2001, (**E**) *C. albicans* 324LA/94 and *C. glabrata* D1 and (**F**) *C. albicans* 324LA/94 and *C. glabrata* 585626. Arrows indicate the localisation of *C. glabrata* cells in the RHVE.

Table 3 Summary findings of *C. glabrata* and *C. albicans* single and co-infection of reconstituted human vaginal epithelium at 24 h.

Candida strain	Origin	Single infection			Co-infection *C. albicans* ATCC 90028			Co-infection *C. albicans* 324LA/94		
		Colonisation	Invasion	qRT-PCR (Log number of cells/ml)	Colonisation	Invasion	qRT-PCR (Log number of cells/ml)	Colonisation	Invasion	qRT-PCR (Log number of cells/ml)
							C. albicans ATCC 90028			C. albicans 324LA/94
							C. glabrata strains			*C. glabrata* strains
C. albicans ATCC 90028	Reference	+++	High	4.76 ± 0.21	−	−	−	−	−	−
C. albicans 324LA/94	Oral	+++	High	4.01 ± 0.24**	−	−	−	−	−	−
C. glabrata ATCC 2001	Reference	+	Low	0.88 ± 0.41	++	Moderate	3.87 ± 0.39 2.05 ± 0.23	++	Moderate	2.46 ± 0.35 1.97 ± 0.39
C. glabrata D1	Oral	+	Low	0.89 ± 0.46	++	Moderate	3.99 ± 0.39 1.89 ± 0.29	++	Moderate	3.01 ± 0.23 2.03 ± 0.22
C. glabrata 585626	Vaginal	+	Low	1.30 ± 0.38	++	Moderate	4.66 ± 0.26 2.62 ± 0.21	+++	Moderate	3.38 ± 0.26 2.55 ± 17

(+) sparse colonisation; (++) moderate colonisation; (+++) extensive colonisation.
** Statistically different to reference strain ($P < 0.01$).

ATCC 90028 was co-infected with *C. glabrata* 585626. *SAP* genes were generally detected in all samples. However, *SAP3* was not expressed by *C. albicans* 324LA/94 during infection of RHVE. For this gene family, it is important to note that in co-infection, both *C. albicans* with *C. glabrata* 585626 (vaginal strain) demonstrated a noticeable up-regulation of *SAP4-10* genes.

In contrast, *Epithelial Adhesin* gene expression by *C. glabrata* did not appear to correlate with the level of RHVE colonisation and invasion (Table 5). In all mixed species combinations, expression of this gene family was shown to be principally down regulated, and in some cases absent.

Discussion

The mucosal epithelium is highly important in host defense and immune surveillance, as it is the first cell layer that initially encounters environmental microbes with pathogenic potential.[34] As a result, the characteristic feature of vaginal candidosis is chronic inflammation of the mucosa. In recent years, a number of studies have investigated colonisation and invasion of reconstituted human epithelium by *Candida*.[26,27,35] The work presented revealed that in single species infection (Fig. 1A and B and summarised in Table 3), both *C. albicans* strains tested extensively colonised and highly invaded the RHVE. Furthermore, *C. albicans* induced a significant change in the vaginal keratinocytes structure, disruption of the superficial epithelium and cleft formation between the cells in the upper layers. These features have previously been reported in studies using RHOE.[9,35–37] After 24 h infection, CLSM revealed that *C. glabrata* were generally unable to invade the RHVE (very low invasion, Table 3) with few clusters of cells (Fig. 1C–E) evident in the first layers of the epithelium (sparse colonisation). These findings were in agreement with those of Schaller et al., 2002 and Jayatilake et al., 2006, who also classified *C. glabrata* as being non-invasive of RHOE. The differences between both species could be explained by the ability of *C. albicans* to form hyphae and/or pseudohyphae, and the absence of this property by *C. glabrata*.[8,29] Polymorphism and the movement of hyphae are indeed previously recognised factors enhancing RHOE colonisation and invasion.[35,36,39]

In addition to CLSM observations, a molecular method was applied to enumerate the amount of *Candida* able to colonise and invade RHVE. It was interesting to note that although the inoculum used in co-infection studies for each strain was halved compared with the single infection, the degree of colonisation and invasion of *C. albicans* in the presence of *C. glabrata* was not affected. Additionally, and as had previously been noticed using RHOE infection,[9] colonisation by *C. glabrata* cells was highest (extensive colonisation) when combined with *C. albicans*. Furthermore, *C. glabrata* invasion also increased in co-infections studies. Thus, the presence of *C. albicans* would appear to enhance invasiveness of *C. glabrata* in this model system, although the mechanism of this remains unclear. One possible explanation is that *C. glabrata* invasion is enhanced because of the damage induced to the epithelium by *C. albicans* hyphae (Fig. 2). This would facilitate access of *C. glabrata* to lower epithelial layers. It is also possible that *C. glabrata*

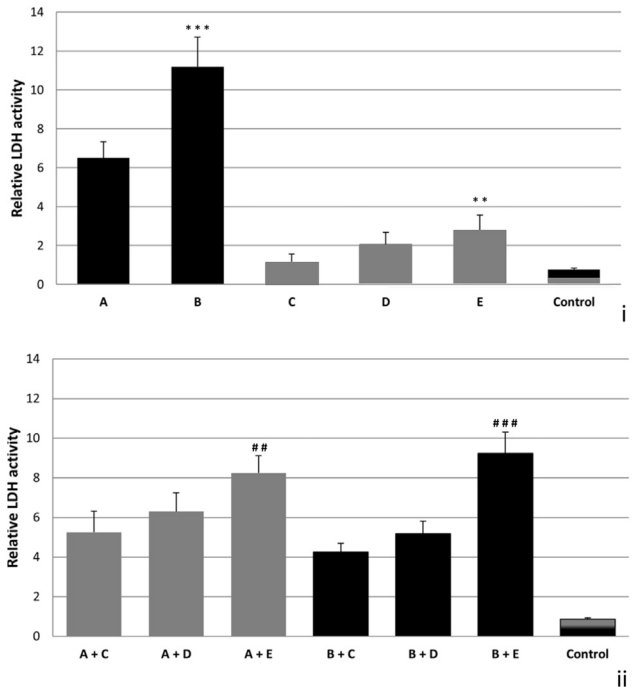

Figure 3 Relative lactate dehydrogenase (LDH) activity measured in the human vaginal epithelium tissue culture supernatant after 24 h incubation with different *C. albicans* and *C. glabrata* strains in single infection (i) and co-infection (ii) (**A** — *C. albicans* ATCC 90028; **B** — *C. albicans* 324LA/94; **C** — *C. glabrata* ATCC 2001; **D** — *C. glabrata* D1; **E** — *C. glabrata* 585626). **,***Candida strain statistically different to respective reference strain ($P < 0.01$; $P < 0.001$); ##, ###Candida glabrata co-infection statistically different to combination of *C. glabrata* ATCC 2001 with *C. albicans* ATCC 90028 and *C. albicans* 324LA/95, respectively ($P < 0.01$; $P < 0.001$).

yeast could adhere to *C. albicans* hyphae and be transported to lower tissue layers.

Adhesion of *C. albicans* to host cells is a key virulence mechanism and is associated with expression of *Hyphal Wall Protein* (*HWP*).[40] It is known that the glutamine residues in the N-terminal domain of *HWP1* are cross-linked by host transglutaminase activity to unidentified host proteins and this results in covalent attachment of the hyphae to host epithelial cells.[41] In this current research, qRT-PCR showed that in co-infection, both *C. albicans* strains exhibited up-regulation of *HWP1* compared with single infection. In 2001, Hornby et al.[42] had shown that the predisposition of the cells to germinate into the hyphal form was improved in cell densities of less than 10^6 cells ml^{-1} and decreased in higher concentrations of culture medium, under conditions which predispose the cells to germinate into the hyphal form. This has been called the inoculum effect and has been associated with regulation by quorum sensing.

Based on the qRT-PCR findings it is tempting to speculate that RHVE invasion and colonisation by *C. glabrata* cells is enhanced by the presence of *C. albicans* hyphae following *HWP1* up-regulation.

Expression of adhesins may also be responsible for the different colonisation profile and therefore expression of genes of the Agglutinin-Like Sequence (*ALS*) and Epithelial Adhesins (*EPA*) family were also evaluated. Most ALS proteins have adhesin function,[43] and the binding domain for most substrates is located in the N terminal region. *ALS3* is a hyphal-specific gene expressed by *C. albicans* hyphae and pseudohyphae, but not yeast.[44,45] The expression pattern of the *ALS* genes (Table 4) in *C. albicans* RHVE single infection assays was generally similar to that observed in the co-infections, with exception of *ALS3*, which was highly increased. Since *ALS3* is *C. albicans* hyphal specific, its up-regulation in co-infection studies, might be reflected by increased hyphal production leading to enhanced invasion of *C. glabrata* yeast.

In vitro adherence of *C. glabrata* to epithelial cells is primarily mediated by *EPA* genes, which encode cell wall proteins of a large family of putative adhesins.[46] The *EPA* gene family consists of 17—23 members depending on the strain, and along with *EPA1*, at least two other genes have been shown to encode functional adhesins, namely *EPA6* and *EPA7*.[47] *EPA1* exhibited highest expression in all the single *C. glabrata* assays (Table 4). However, in co-infection, the majority of the *EPA* genes studied were down-regulated or absent, suggesting that these adhesins were not associated with increased RHVE colonisation or invasion by *C. glabrata* in the co-infection assays. Therefore, other factors might be responsible for this behaviour.

The degree of tissue damage caused by *Candida* during infection, was evaluated in single and co-infection (Fig. 3) by LDH activity and results agreed with the CLSM observations (Figs. 1 and 2). Single species infection revealed that *C. albicans* generated more damage than *C. glabrata*. This result was expected given the fact that *C. albicans* is deemed more pathogenic.[48] LDH activity after co-infection yielded a profile similar to *C. glabrata* in single species infection, but understandably at higher levels because the presence of *C. albicans*. Furthermore, the previously observed differences between both *C. albicans* strains in single colonisation assays were not evident in co-colonisation. The differences between the *C. glabrata* strains were, however, still apparent with highest LDH activity occurring for *C. glabrata* 585626 combined with both *C. albicans*. In the case of *C. glabrata* 585626 and *C. albicans* ATCC 90028 co-infection, tissue damage was higher than with *C. albicans* ATCC 90028 alone, despite the initial inoculum of *C. albicans* ATCC 90028 being half of that used in the single species infection. Based on these findings, it is evident that RHVE damage is species and strain dependent with potential synergism occurring in mixed species infection.

In order to better elucidate pathogenic differences, phospholipase and secreted aspartyl proteinase gene expression was measured. A variety of phospholipases (PL), are produced by *C. albicans* and the function of these enzymes includes the metabolism of phospholipids in biological membranes. Expression of these enzymes can therefore lead to disruption of membrane structure and function.[49] The current study showed that expression of *PLB and PLD* gene families was always detected, with *PLD1* expressed at a higher level in all single and *C. glabrata* 585626 co-infections assays, indicating a potential role of this factor in RHVE damage. However, these findings contrast with those of Malic et al., 2007,[35] where

Table 4 Relative expression of *Hyphal Wall Protein 1* (*HWP1*), *Agglutinin-like Sequence* (*ALS*), *Phospholipase* (*B* and *D*) and *Secreted Aspartyl Protease (SAP)* gene families after 24 h of reconstituted human vaginal epithelium single and co-infection by *Candida albicans* strains using quantitative RT-PCR.

Gene	Single infection		Co-infection					
			C. albicans ATCC 90028			*C. albicans* 324LA/94		
	C. albicans ATCC 90028	*C. albicans* 324LA/94	C. *glabrata* ATCC 2001	C. *glabrata* D1	C. *glabrata* 585626	C. *glabrata* ATCC 2001	C. *glabrata* D1	C. *glabrata* 585626
HWP1	42 ± 7	50 ± 13	160 ± 13	220 ± 14	227 ± 27	521 ± 53	678 ± 46	750 ± 41
ALS1	216 ± 53	53 ± 7	859 ± 59	325 ± 22	426 ± 19	12 ± 5	18 ± 3	23 ± 2
ALS2	110 ± 11	128 ± 21	93 ± 5	141 ± 8	135 ± 9	67 ± 6	82 ± 10	75 ± 8
ALS3	345 ± 41	273 ± 29	575 ± 68	588 ± 24	1016 ± 166	308 ± 39	329 ± 16	485 ± 41
ALS4	224 ± 26	304 ± 33	201 ± 25	242 ± 14	187 ± 27	144 ± 13	187 ± 9	243 ± 8
ALS5	206 ± 21	208 ± 22	174 ± 8	129 ± 17	107 ± 9	106 ± 10	94 ± 7	65 ± 8
ALS6	374 ± 31	355 ± 40	484 ± 23	84 ± 5	222 ± 27	38 ± 4	44 ± 4	47 ± 5
ALS7	13 ± 2	32 ± 3	49 ± 7	80 ± 5	88 ± 3	23 ± 2	21 ± 3	ND
PLB1	31 ± 8	19 ± 5	38 ± 9	14 ± 3	34 ± 3	23 ± 2	10 ± 1	71 ± 10
PLB2	135 ± 10	102 ± 8	122 ± 18	99 ± 12	166 ± 18	76 ± 3	36 ± 2	206 ± 21
PLB3	218 ± 11	119 ± 3	179 ± 11	172 ± 9	238 ± 6	74 ± 3	18 ± 3	162 ± 20
PLB5	248 ± 6	159 ± 11	236 ± 8	126 ± 8	210 ± 2	94 ± 5	82 ± 3	366 ± 31
PLD1	278 ± 11	214 ± 4	270 ± 6	211 ± 12	390 ± 21	144 ± 5	94 ± 4	389 ± 30
SAP1	65 ± 9	21 ± 1	62 ± 8	39 ± 4	59 ± 2	26 ± 3	16 ± 1	33 ± 5
SAP2	182 ± 25	103 ± 6	196 ± 11	116 ± 6	134 ± 2	83 ± 4	34 ± 1	67 ± 6
SAP3	112 ± 11	ND	139 ± 15	78 ± 6	72 ± 3	ND	ND	ND
SAP4	118 ± 14	343 ± 10	224 ± 18	153 ± 14	266 ± 9	257 ± 10	134 ± 4	567 ± 24
SAP5	56 ± 6	123 ± 17	224 ± 15	214 ± 12	363 ± 22	187 ± 15	69 ± 7	298 ± 19
SAP6	63 ± 6	243 ± 10	104 ± 6	68 ± 4	117 ± 2	159 ± 12	115 ± 8	366 ± 25
SAP9	387 ± 17	329 ± 13	454 ± 24	351 ± 15	524 ± 4	244 ± 10	150 ± 9	555 ± 31
SAP10	103 ± 10	73 ± 3	114 ± 16	78 ± 6	180 ± 7	65 ± 6	31 ± 2	140 ± 14

ND, no gene expression was detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the respective *Candida* housekeeping gene product (ACT1).

phospholipase gene expression did not correlate with RHOE invasion. Assuming that hyphal forms are more adept at epithelium invasion, expression of PLD1 could be significant in this process as Hube et al., 2001[50] reported that *PLD1* was necessary for the yeast-to-hypha transition in *C. albicans*.

The most widely studied extracellular hydrolytic enzymes of *C. albicans* are *SAPs*.[51–53] This present study revealed that *SAP* gene expression by *C. albicans* was strain dependent in single and co-infection assays. From qRT-PCR analysis, highly invasive *C. albicans* strains, in single infections were consistent producers of *SAP4–10*. Of note is that Naglik et al., 2003[52] have shown that *SAP4–6* are important for yeast-to-hypha transformation. It is also important to highlight that *SAP3* was not expressed by *C. albicans* 324LA/94 which was a 'high invader'. Korting et al., 2003[54] determined that the epithelial cell damage by *C. albicans* correlated with expression of *SAP3*. Based on the

Table 5 Relative expression of *Epithelial Adhesin* (*EPA*) gene family after 24 h of reconstituted human vaginal epithelium single and co-infection by *Candida glabrata* using quantitative RT-PCR.

	Single infection			Co-infection					
				C. *glabrata* ATCC 2001		*C. glabrata* D1		*C. glabrata* 585626	
	C. glabrata ATCC 2001	*C. glabrata* D1	*C. glabrata* 585626	*C. albicans* ATCC 90028	*C. albicans* 324LA/94	*C. albicans* ATCC 90028	*C. albicans* 324LA/94	*C. albicans* ATCC 90028	*C. albicans* 324LA/94
EPA1	72 ± 5	110 ± 12	124 ± 10	60 ± 5	75 ± 3	70 ± 3	63 ± 5	96 ± 5	47 ± 7
EPA6	18 ± 1	25 ± 3	28 ± 5	13 ± 1	22 ± 2	ND	ND	ND	18 ± 3
EPA7	39 ± 1	49 ± 6	ND	18 ± 3	41 ± 8	22 ± 3	32 ± 2	ND	ND

ND, no gene expression was detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the respective *Candida* housekeeping gene product (ACT1).

results of this current study, and as indicated by Naglik et al., 2008, it would appear that SAPs do not play a significant role in *C. albicans* invasion and damage of RHVE.

In summary, the present study confirms the effectiveness of RHVE as an *in vitro* model to study *Candida* virulence attributes and facilitated study of *C. glabrata* pathogenicity, which compared with *C. albicans* has received limited attention. Use of CLSM and LDH approaches and the developed methodology of RT-PCR for *Candida* cells quantification in the tissue, has conclusively shown that *C. albicans* is a much more extensive RHVE coloniser and invader compared to *C. glabrata*. However, it is important to highlight that *C. albicans* co-infection with *C. glabrata* enhanced colonisation and invasiveness of the latter. To our knowledge, this is the first report of *HWP1*, *ALS*, *EPA*, *PLB*, *PLD* and *SAP* gene expression in *C. albicans* and *C. glabrata* co-infecting vaginal epithelium and the results suggest an important role of *HWP1*, *PLD1* and *ALS3* virulence factors in *C. albicans* and *C. glabrata* pathogenicity.

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