

Candida glabrata and *Candida albicans* co-infection of an *in vitro* oral epithelium

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BACKGROUND: *Candida albicans* is regarded as the leading of candidosis. However, *Candida glabrata* has emerged as an important pathogen of oral mucosa, occurring both singly or in mixed species infections, often with *C. albicans*. Compared with *C. albicans*, little is known about the role of *C. glabrata* in oral infection. The aim of this study was to examine single and mixed species infection of oral epithelium involving *C. glabrata* and establish its ability to invade and damage tissue.

METHODS: A reconstituted human oral epithelium (RHOE) was infected only with *C. glabrata*, or simultaneously with *C. glabrata* and *C. albicans*. The ability of both species to invade the tissue was examined using species specific peptide nucleic acid (PNA) probe hybridization and confocal laser scanning microscopy. Epithelial damage was assessed by measuring lactate dehydrogenase (LDH) activity.

RESULTS: *Candida glabrata* strains were able to colonize the RHOE, in a strain dependent manner. *Candida glabrata* single infection after 12 h, generally revealed no invasion of the RHOE, which contrasted with extensive tissue invasion demonstrated by *C. albicans*. Mixed infection showed that *C. albicans* enhanced the invasiveness of *C. glabrata*, and led to increased LDH release by the RHOE, which paralleled the observed histological damage.

CONCLUSIONS: The results obtained demonstrating enhanced invasion and increased tissue damage caused by mixed *C. glabrata* and *C. albicans* infections have important clinical significance and highlight the need to identify *Candida* species involved in oral candidosis.

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Introduction

Candidosis is a frequent opportunistic infection in immunocompromised individuals (1). *Candida albicans* remains the main aetiological agent in oral candidosis, accounting for 70–80% of isolates from oral mucosal lesions (2, 3). However, in recent years, *Candida glabrata* has emerged as a notable pathogenic agent of the oral mucosa, either co-infecting with *C. albicans* (4–7), or occurring in single species infections (2, 4–6). Furthermore, *C. glabrata* is the third most common cause of mortality in hospitals (8). *Candida glabrata* exhibits significant differences from *C. albicans*, including relative physical size, morphological properties and biochemistry. *Candida glabrata* yeasts are smaller (1–4 µm) than *C. albicans* yeast (4–6 µm) and have a much narrower spectrum of carbohydrate utilization (8). Furthermore, in contrast to *C. albicans*, *C. glabrata* is generally not polymorphic, and only occurs in the yeast form within the oral cavity (8). One possible explanation therefore for the relatively frequent occurrence of co-infection of *C. albicans* and *C. glabrata* is that these significant differences limit the extent of inter-species competition allowing the organisms to occupy similar oral niches. Importantly, *C. glabrata*-associated oropharyngeal candidosis in HIV and cancer patients tends to be more severe and more difficult to treat than infections caused solely by *C. albicans* (5, 6, 9).

An understanding of the interactions between these *Candida* species has significance for the development of strategies for oral candidosis management. This is important since, although oral *Candida* infections are not associated with mortality, they are a major cause of morbidity, and trigger chronic pain or discomfort upon mastication, which may limit nutritional intake in immunocompromised or elderly individuals (5, 10). In addition, oral carriage of *Candida* has also been proposed to be a pre-disposing factor for systemic infection in severely immunocompromised patients (11).

Fluorescent *in situ* hybridization (FISH) is rapidly becoming established as a valuable research and

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diagnostic tool in molecular pathology laboratories, having previously been used to detect and differentiate microorganisms in histological sections (12–14). Peptide nucleic acid (PNA) probes are synthetic equivalents of nucleic acids where the phosphate–sugar polynucleotide backbone has been substituted with a peptide polymer which provides the basis for linking nucleobases. Unlike DNA, the peptide backbone of PNA probes is not charged and this offers greater stability in nucleic acid–probe hybridization. Furthermore, PNA probes are more resistant to degradation from nuclease and protease activity. Given such advantages, it is not surprising that PNA probes have emerged as valuable tools in the study of infectious diseases and represent a new generation of therapy-directed diagnostics (14–16). To exemplify this, the Yeast Traffic Light PNA FISH™ (YTL; AdvanDx, Inc., Woburn, MA, USA) system is a newly developed and commercialized PNA probe kit for the rapid detection and distinction of the five most common and clinically relevant *Candida* species (17, 18).

Previously, a reconstituted human oral epithelium (RHOE) was successfully used to investigate colonization and infection of tissue by *Candida* (19–23). Coupled with species specific PNA probes and confocal laser scanning microscopy (CLSM), co-infection of RHOE using *C. glabrata* and *C. albicans* can therefore be readily examined. The aim of this study was to generate single and mixed species infections of RHOE with *C. albicans* and *C. glabrata* and ascertain the role of *C. glabrata* in the infection using species specific PNA probes, CLSM and lactose dehydrogenase (LDH) activity (as a measure of tissue damage) in infected tissue.

Materials and methods

Organisms

A total of six clinical isolates of *C. glabrata*, originally recovered from the oral cavity (strains D1 and AE2), vagina (strains 534784 and 585626) and urinary tract (strains 562123 and 513100) were used in this study. A reference strain of *C. glabrata* from the American Type Culture Collection (ATCC 2001) was also included. Oral isolates were obtained from the biofilm group of the Centre of Biological Engineering, Minho University (Braga, Portugal). Strains isolated from urinary and vaginal infections were kindly provided from the culture collection of the Hospital of São Marcos (Braga, Portugal). An oral isolate of *C. albicans* (strain 324LA/94) from the culture collection of Cardiff Dental School (Cardiff, UK) was selected based on its ability to invade RHOE (21). The identity of all isolates was initially confirmed by PCR-based sequencing using specific primers (ITS1 and ITS4) for the 5.8S subunit gene (24).

Growth conditions

For each experiment, isolates were cultured on Sabouraud Dextrose Agar (SDA) (Merck, Darmstadt, Germany) for 48 h at 37°C. Isolates were then subcultured in Yeast Nitrogen Base medium (YNB; BD

Diagnostics, Cowley, UK) supplemented with 0.5% glucose (w/v) for 12 h at 37°C. After incubation, cells were harvested by centrifugation and washed three times with phosphate buffered saline (PBS; pH 7). The yeasts were then enumerated using an Improved Neubauer haemocytometer and adjusted to a concentration of 2×10^6 cells/ml in MCDB-153 defined medium (Clonetics, San Diego, CA, USA).

Reconstituted human oral epithelium infection

To investigate *in vitro* single- and mixed species infection of oral mucosa by *C. glabrata* and *C. albicans* strains, a reconstituted human oral epithelium (RHOE) (human keratinocytes derived from a squamous carcinoma of the buccal mucosa, cell line TR 146, SkinEthic Laboratories, Nice, France) model was used. RHOE tissue inserts (0.5 cm²) were placed in 24-well tissue culture plates with MCDB 153-defined medium containing 5 µg/ml insulin, 1.5 mM CaCl₂, 25 µl/ml gentamicin, and 0.4 µg/ml hydrocortisone, according to the manufacturer's instructions. For single species infection, 1 ml of standardized suspensions (2×10^6 cells/ml) of *C. glabrata* or *C. albicans* was placed directly onto the RHOE tissue inserts. For mixed species infection, 500 µl of *C. glabrata* (2×10^6 cells/ml) was combined with 500 µl of *C. albicans* (2×10^6 cells/ml) suspension and this preparation placed onto the RHOE tissue inserts. Control samples were inoculated with 1 ml of medium devoid of *Candida*. Infected tissues were incubated at 37°C in a 5% CO₂ environment with saturated humidity for 12 h. After 12 h incubation, the maintenance medium was removed and the RHOE tissues rinsed twice with PBS to remove non-adherent *Candida* cells. The RHOE specimens were then fixed in 2% (v/v, in ultrapure water) paraformaldehyde for 24 h, at 4°C and embedded in paraffin wax using standard histological techniques. Tissue sections (20 µm) were cut and placed onto Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, UK), de-waxed and processed through xylene, ethanol and water.

Peptide nucleic acid fluorescent in situ hybridization (PNA FISH)

The Yeast Traffic Light PNA FISH™ (YTL-PNA FISH) kit was used to examine invasion of the RHOE by *C. glabrata* and *C. albicans*. The *Candida* PNA FISH assay was performed according to the manufacturer's instructions with some modifications (17, 18). Sections of RHOE were prepared as described above and then washed briefly in pre-warmed 1 × wash solution. Hybridization with PNA probes was performed using 1 drop of *C. albicans*/*C. glabrata* PNA probe mixture and the overlay incubated in the dark in a humidified chamber for 90 min at 55°C. After probe hybridization, excess PNA probe was removed by immersion of the slides into pre-warmed 1 × wash solution (55°C) for 30 min. For nuclear staining, keratinocytes within the RHOE sections were counterstained with Hoechst 33258 for 30 min at room temperature, before washing in 1 × wash solution. The preparation was then mounted using

Vectashield® fade-retarding mountant (Vector Laboratories, Peterborough, UK).

Confocal laser scanner microscopy

Sections hybridized with PNA probes were observed by CLSM using a Leica TCS SP2 AOBs spectral confocal microscope (Leica, Wetzlar, Germany). The 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 Hoechst 33258 (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. *Candida glabrata* and *C. albicans* were identified by red and green colouration respectively, whilst keratinocytes were depicted blue. All experiments were performed in duplicate.

Lactate dehydrogenase activity

The release of LDH from RHOE into the culture medium was used as a measure of epithelial cell damage. The LDH concentration in the medium from control and infected tissues was determined after 12 h using the CytoTox-ONE™ kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The LDH activity of *Candida* cells in cell culture medium devoid of tissue was subtracted from the LDH activity of the tissue infected with yeasts. The LDH released during single or co-infection with the different *C. glabrata* strains and *C. albicans* was then expressed in relation to the untreated control tissue. All experiments were performed in duplicate.

Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variance and the Tukey multiple-comparisons test, using SPSS software [SPSS 17 (Statistical Package for the Social Sciences), Inc., Chicago, IL, USA]. All tests were performed with a confidence level of 95%.

Results

Candida glabrata and Candida albicans single species RHOE infection

The ability of *C. glabrata* and *C. albicans* strains to colonize and invade human oral epithelium was examined after 12 h of infection using the RHOE model. Results showed that all strains colonized the RHOE surface, although the extent of colonization was highly strain dependent (Table 1 and Fig. 1). *Candida glabrata* 534784 and 585626 (both vaginal strains) and *C. albicans* exhibited the highest level of RHOE colonization, whilst *C. glabrata* D1 and AE2 (both oral strains)

Table 1 *Candida glabrata* and *C. albicans* single and co-infection profile of reconstituted human oral epithelium (at 12 h), as assessed using confocal laser scanning microscopy and peptide nucleic acid probe hybridization

<i>Candida</i>	Origin	Single infection		Co-infection
		Colonization	Invasion	Invasion
<i>C. glabrata</i> (D1)	Oral	+	None	Moderate
<i>C. glabrata</i> (AE2)		+	None	Moderate
<i>C. glabrata</i> (562123)	Urinary	+	None	Moderate
<i>C. glabrata</i> (513100)		++	Moderate	High
<i>C. glabrata</i> (534784)	Vaginal	+++	None	Moderate
<i>C. glabrata</i> (585626)		+++	None	High
<i>C. glabrata</i> (ATCC 2001)	Reference	++	None	High
<i>C. albicans</i> (324LA/94)	Oral	+++	High	-

and 562123 (urinary isolate) colonized the tissue to a lower extent (Fig. 1).

In general, after 12 h of infection, only clusters of *C. glabrata* yeast were detected at the surface of the keratinocyte layers (Fig. 1A,B). Furthermore, strains of *C. glabrata* were generally unable to invade the epithelium, although *C. glabrata* 513100 (urinary isolate), was found within the upper (second or third epithelial cell layer) region of the RHOE (Fig. 1B). In contrast, *C. albicans* showed a greater ability to invade the RHOE with hyphal elements completely penetrating all the epithelial layers (Fig. 1D).

Histological examination of the RHOE after infection with *C. albicans*, demonstrated noticeable changes (Fig. 1D) compared with tissue infected with *C. glabrata* (Fig. 1A–C). It was evident that *C. albicans* RHOE infection induced superficial detachment of keratinocyte layers (Fig. 1D).

In order to quantify tissue damage, the levels of LDH released by epithelial cells after infection were measured (Fig. 2) and this showed that infection with *C. albicans* produced higher relative levels of LDH activity ($P < 0.05$) compared with *C. glabrata* strains. Furthermore, a moderate but statistically significant increase in relative LDH activity levels was induced by *C. glabrata* strains compared with uninfected controls ($P < 0.05$). Statistical differences with respect to LDH activity released from the RHOE were not evident for *C. glabrata* RHOE infection ($P > 0.05$).

Candida glabrata and Candida albicans RHOE mixed species infection

Tissues simultaneously infected with *C. glabrata* and *C. albicans* were subjected to appropriate histological procedures, and the *Candida* species differentiated by hybridization with specific *C. glabrata*/*C. albicans* PNA probes. The relative pattern of infection and ability to invade the RHOE was then observed by CLSM.

Contrary to *C. glabrata* single species infection (Fig. 1 and Table 1), when co-infected with *C. albicans*, the majority of *C. glabrata* strains were found to invade the RHOE (Fig. 3 and Table 1). Yeast of oral isolates (*C. glabrata* D1 and AE2), the urinary isolate (*C. glabrata*

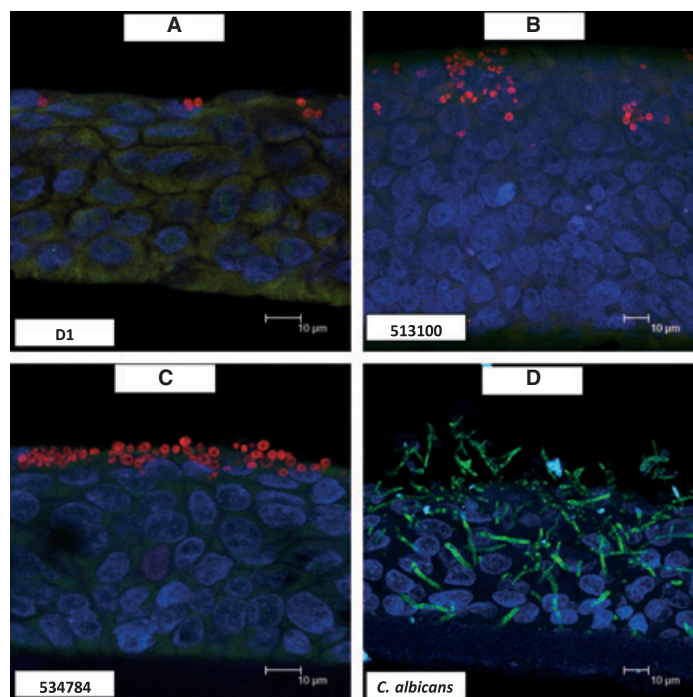


Figure 1 Confocal laser scanner microscopy of *Candida* strains after 12 h of RHOE single infection, using YTL PNA FISHTM. *C. glabrata* cells appear red, *C. albicans* are green, and keratinocytes nuclei are blue. (A) *C. glabrata* D1 showing sparse colonization (+) and no invasion; (B) *C. glabrata* 513100 showing moderate colonization (++) and moderate invasion; (C) *C. glabrata* 534784 showing extensive colonization (+++) and no invasion; (D) *C. albicans* showing extensive colonization (+++) and high invasion.

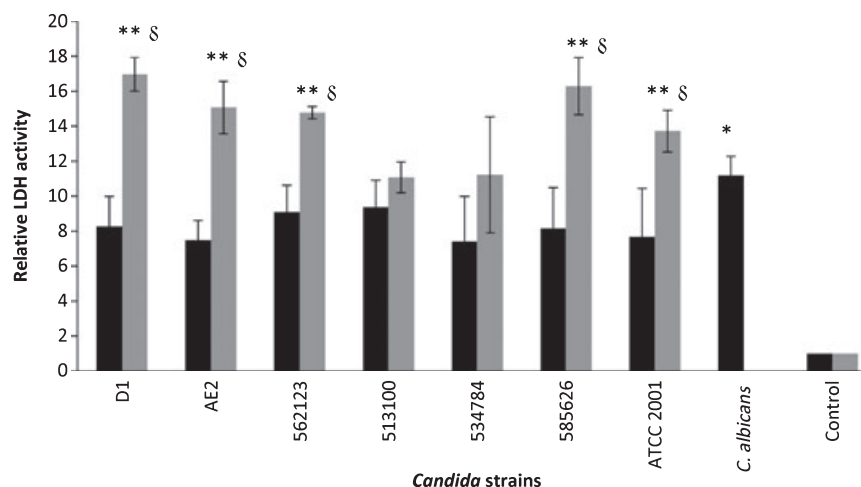


Figure 2 Relative lactate dehydrogenase (LDH) activities measured in the RHOE supernatant after 12 h of single infection (■) and co-infection (▒) with *C. glabrata* and *C. albicans*. Control was performed without *Candida* cells. Error bars represent standard deviation. *LDH activity of *Candida albicans* single infection is statistically different from *C. glabrata* strains single infections ($P < 0.05$). **LDH activity of co-infection is statistically different from the respective single infection by each *C. glabrata* strain ($P < 0.05$). §LDH activity of *C. albicans* single infection is statistically different from co-infection values ($P < 0.05$).

562123) and the vaginal isolate (*C. glabrata* 534784) penetrated to the third epithelial layer of the RHOE (Fig. 3A). In the case of *C. glabrata* 513100 (urinary isolate), *C. glabrata* 585626 (vaginal isolate) and the reference strain (*C. glabrata* ATCC 2001) penetration to the lowest epithelium layers (Fig. 3B) was evident. Within the tissue, *C. glabrata* yeast were localized close to vacuolized tissue portions (Fig. 4A) and observed

in close proximity to *C. albicans* hyphal elements (Fig. 4B,C).

For several strains, the LDH levels released by the RHOE epithelial cells infected by both species increased significantly compared to single species infection (Fig. 2). Specifically, for tissues co-infected with *C. albicans* and the oral isolates (*C. glabrata* D1 and AE2), the vaginal isolate *C. glabrata* 585626 and the

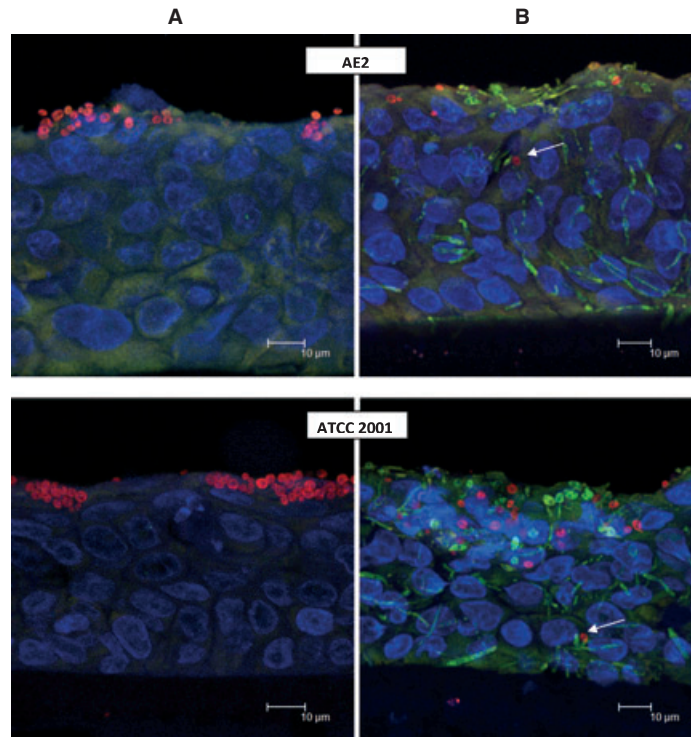


Figure 3 Single (A) and co-infection (B) of *C. glabrata* and *C. albicans* of RHOE after 12 h, assessed by CLSM and YTL PNA FISHTM. *C. glabrata* cells appear red, *C. albicans* green, and keratinocytes nucleus blue. *Candida glabrata* AE2 presents moderate invasion while *C. glabrata* ATCC 2001 presents high invasion in the presence of *C. albicans*.

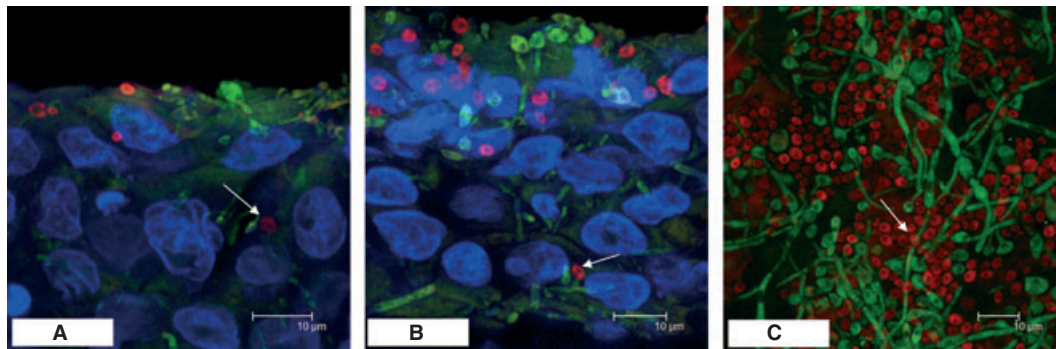


Figure 4 *Candida glabrata/Candida albicans* co-infection of RHOE after 12 h, assessed by CLSM and YTL PNA FISHTM. Panel A shows the presence of *C. glabrata* yeast at a vacuolized tissue portion (see arrow), panel B illustrates shows *C. glabrata* cells near *C. albicans* hyphal elements (see arrow) and panel C shows the adhesion and co-aggregation of *C. glabrata* cells on hyphal forms of *C. albicans* (see arrow).

reference strain (*C. glabrata* ATCC 2001) the levels of LDH increased approximately two-fold, whilst a slightly lower (1.5-fold) increase was evident with *C. glabrata* 562123 (urinary isolate). This increase in LDH activity was found to be significant ($P < 0.05$). However, no statistically differences were found in the LDH activity released by single and co-infection of *C. glabrata* 513100 (urinary isolate) and *C. glabrata* 534784 (vaginal isolate). Furthermore, an increase in the levels of LDH activity was verified between *C. albicans* single infection and co-infection concerning both oral isolates (*C. glabrata* D1 and AE2), one vaginal isolate (*C. glabrata* 585626), one urinary isolate (*C. glabrata* 562123) and the reference strain (*C. glabrata* ATCC 2001)

($P < 0.05$). This augment in activity was not found for *C. albicans* co-infecting with one urinary isolate (*C. glabrata* 513100) and one vaginal isolate (*C. glabrata* 534784) ($P > 0.05$).

Discussion

Although *C. albicans* is the most frequently encountered pathogen (2, 3), several other species, and especially *C. glabrata*, have recently emerged as oral pathogens (5–7). *Candida glabrata* is unable to grow in filamentous forms and is therefore only seen as relatively small yeast cells in humans (8). In contrast, *C. albicans* is polymorphic being able to grow as yeast, pseudohyphae and hyphae

(25). In terms of tissue invasion, the ability of *C. albicans* to generate hyphae is seen as advantageous as such filamentous growth enables migration of the fungal element from the point of adherence. Thigmotropic movement is also a property of *Candida* hyphae (26) which helps to promote invasion of the epithelium (14, 26, 27). The morphological differences and potentially different patterns of growth exhibited by *C. albicans* and *C. glabrata* could allow these species to occupy the same location within the oral cavity with limited competition for space, thereby allowing co-existence in many instances.

In recent years, *C. albicans* pathogenesis in mucosal candidosis has been investigated by several authors (19–21, 28) using RHOE as a surrogate model to animals. Since mixed infection with both *C. albicans* and *C. glabrata* species has often been reported in the mouth (5, 6), we aimed to examine for the first time the effect of mixed infections in the RHOE model. This in turn would indicate the possible pathogenic significance of mixed infection in the oral cavity.

Initially, the colonization patterns and invasion of RHOE by several *C. glabrata* strains originating from different body sites were examined. The results obtained (Table 1, Figs 1A–C and 3A) showed that the surface of the RHOE was successfully colonized by all *C. glabrata* strains tested, although this was noticeably strain dependent. Furthermore, CLSM revealed that *C. glabrata* were generally unable to invade the RHOE (Fig. 2). One exception was the urinary isolate *C. glabrata* 513100 which appeared to invade the superficial epithelial layers of the RHOE. These results highlight the diversity of *C. glabrata* strains in colonization and invasiveness capability, and mirror those previously reported for *C. parapsilosis* (22) and *C. albicans* (21). Schaller et al. (27) and Jayatilake et al. (20) have both previously classified *C. glabrata* strains as being non-invasive in RHOE infection.

It is important to note that six of the seven strains analysed in this present study and classified as ‘non-invaders’ were sourced from different body sites, possibly indicating that strain origin is not an important aspect in *C. glabrata* colonization and invasion of oral epithelium. For example, the oral isolates (AE2 and D1) exhibited a lower ability to colonize the RHOE, compared with vaginal isolates (534784 and 585626).

Generally, most *C. albicans* isolates infecting the RHOE model exhibit histological signs of hyphal invasion (14, 19, 25, 27). This current study also confirmed the high invasiveness of *C. albicans* (Fig. 1D) and particularly so when compared with *C. glabrata* (Figs 1A–C and 3A). In fact, after 12 h of infection, invasion of the RHOE by hyphal elements of *C. albicans* was clearly evident, leading to marked disorganization of RHOE and detachment of superficial layers of the tissue from the insert (Fig. 1D); this was in contrast to the lower level of damage caused by *C. glabrata* strains (Figs 1A–C and 3A). The extent of tissue damage was quantitatively assessed by measurement of relative LDH activity (Fig. 2). The results were in accordance with the extent of *Candida* colonization and tissue damage

observed histologically (Fig. 1). The levels of LDH released during single species infection was highest for *C. albicans* which was expected given the previous histological observations and considering that this species is generally the most virulent (19, 27).

Interestingly, mixed species infection revealed (Table 1 and Fig. 3B) that *C. albicans* promoted the invasiveness of *C. glabrata*. In fact, all six *C. glabrata* strains classified as ‘non-invaders’ in single infection studies were found to penetrate the oral epithelium in the presence of *C. albicans*. It is known that *C. albicans* hyphal formation is a critical determinant in invasive pathogenesis especially in comparison with non-*Candida albicans* *Candida* species, and it has been shown that *C. albicans* with mutations in genes involved in promoting hyphal development were less invasive than wild type strains (20).

The exact mechanism of how *C. albicans* might promote *C. glabrata* penetration of the oral epithelium remains unclear. Damage to the integrity of the epithelial surface caused by the growing tips of the *C. albicans* hyphae (Fig. 4A,B) seems to provide access to lower epithelial layers for *C. glabrata* yeast. In fact, *C. glabrata* cells were observed in close proximity to *C. albicans* hyphae and transported in this adhered form to deeper tissue layers (Fig 4C, arrows). In this context, Ez-Azizi et al. (29) showed the efficient adherence of *C. albicans* to a pre-formed *C. glabrata* biofilm in a catheter model, suggesting the co-aggregation of these two species *in vivo*.

The increased LDH activity (Fig. 2) reported after mixed infection with *C. albicans* and *C. glabrata* supports a finding of enhanced pathogenicity (7). Of additional concern is that strains of *C. glabrata* often have higher resistance to azoles antifungal agents compared with many other *Candida* species, including *C. albicans* (5, 6, 30). In such instances, mixed infection would not only exhibit enhanced pathogenicity (7), but could also be more problematic to treat. Many diagnostic laboratories are limited in the identification of mixed *Candida* infection, particularly when non-differential primary isolation agars such as Sabouraud dextrose agar are used. The importance of recognizing the occurrence of such mixed infection is however highlighted by the results of this study and represents an area that should be considered by both diagnostic laboratories and clinicians alike.

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