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The role of secreted aspartyl proteinases in *Candida tropicalis* invasion and damage of oral mucosa

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Abstract

Candida virulence attributes include the ability to colonize and invade host tissues, and the secretion of hydrolytic enzymes. Although *Candida albicans* is regarded as the principal fungi causing infections in humans, other species, particularly *Candida tropicalis*, are increasingly being recognized as human pathogens. Relatively little is known, however, about the virulence attributes associated with *C. tropicalis*. The present study aimed to investigate epithelial infection by *C. tropicalis* using a reconstituted human oral epithelium (RHOE) together with confocal laser scanning microscopy and real-time PCR. A comparison of clinical strains was made in terms of tissue colonization, invasion and *C. tropicalis* secreted aspartyl proteinase (*SAPT*) gene expression. All *C. tropicalis* strains were able to colonize RHOE in a strain-dependent manner. After 12 h of infection, *C. tropicalis* was found to be highly invasive, with extensive tissue damage occurring after 24 h. Real-time PCR of *C. tropicalis SAPT1-4* genes showed that expression was strain-dependent, with *SAPT2-4* transcripts being frequently detected and *SAPT1* rarely detected. Tissue invasion and damage was not inhibited by the presence of pepstatin A. Accordingly, and given that an increase in infection time was not accompanied with an increase in *SAPT* gene expression, it can be suggested that the proteinases are not involved in invasion and damage of RHOE by *C. tropicalis*. In summary, *C. tropicalis* can be considered as highly invasive with the ability to induce significant tissue damage. These features, however, do not appear to be related to specific *SAPT* gene expression.

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Introduction

Many *Candida* species inhabit human mucosal surfaces as harmless commensals. However, these organisms can cause opportunistic infections (candidosis), especially in compromised patients such as those individuals with HIV-infection and diabetes mellitus, and well as in those receiving steroid, broad-spectrum antibiotic and cytotoxic drug therapy [1]. *Candida albicans* is regarded as the most prevalent species involved in both human colonization and infection [2,3]. Nevertheless, other *Candida* species are increasingly recognized as important agents of human infection [3–7]. This may reflect the higher level of resistance often exhibited by these species to certain antifungal agents [8] or an association with modern drug therapies, including the more widespread use of immunosuppressive agents and broad-spectrum antibiotics.

Candida tropicalis is a species that is now recognized as an important pathogen of debilitated individuals [9–11]. However, when compared with the more extensively studied *C. albicans* [12–16], relatively less is known about *C. tropicalis* and its ability to cause human disease [17–22]. Thus, a greater understanding of the virulence determinants of this species is important because not only would this provide an insight into its pathogenic mechanisms, but also it might promote the development of more effective treatment strategies.

Virulence attributes of *Candida* species include the ability to adhere to host tissue, exhibit morphological alteration and secrete hydrolytic enzymes [e.g. phospholipases and

secreted aspartyl proteinases (SAPs)] [23]. The change from a commensal to a pathogen has been related to altered expression of these virulence factors together with a debilitation in host defence mechanisms. Moreover, SAPs are considered to be key enzymes that contribute to candidal infection by promoting damage to the host mucosa, thereby facilitating the invasion of the organism into the epithelium [20,24,25]. Furthermore, it is known that *C. tropicalis* possesses at least four genes encoding SAPs, and these are designated *SAPT1* to *SAPT4* [20,26].

The pathogenesis of mucosal candidosis has been investigated in several studies using animal models, primarily with *C. albicans* [13,27]. In recent years, a commercially available reconstituted human oral epithelium (RHOE) has successfully been used to study the *in vitro* mechanisms of tissue invasion by *C. albicans* [12,14,15,25,28–30]. Thus, the present study aimed to investigate the pattern of colonization and invasion of RHOE by *C. tropicalis* and to relate it to SAPs expression. Specifically, a comparison of clinical strains obtained from different body sites was made in terms of tissue colonization, invasion and *SAP* gene expression using the RHOE model, together with confocal laser scanning microscopy (CLSM) and real-time PCR.

Materials and Methods

C. tropicalis strains

A total of six clinical isolates of *C. tropicalis*, originally recovered from the oral cavity (strains AG1 and T2.2), vagina (strains 12 and 75) and urinary tract (strains 519468 and 544123), were used in the present study. A reference strain of *C. tropicalis* from the American Type Culture Collection (ATCC 750^T) was also used. Oral isolates were originally obtained from patients attending the 'Clínica dos Congregados' (Braga, Portugal) and the strains isolated from urinary tract infections were obtained from the 'Hospital de São Marcos' fungal collection (Braga, Portugal). *C. tropicalis* strains 12 and 75 (recovered from the vaginal tract) were provided by the University of Maringá (Maringá, Brazil). The identification of all isolates was confirmed by PCR-based sequencing using specific primers (ITS1 and ITS4) for the 5.8S subunit gene [31].

Isolates were initially cultured on Sabouraud Dextrose Agar (SDA; Merck, Darmstadt, Germany) for 48 h at 37°C, followed by culture in yeast nitrogen base medium (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 yeast cells were then counted using an Improved Neubauer haemocytometer (Marienfield, Lauda-Königshofen, Germany) and adjusted to a concentration of 2×10^6 cells/mL.

In vitro RHOE infection model

Tissue inserts (0.5 cm^2) of commercially available (SkinEthic Laboratories, Nice, France) human oral epithelium, derived from a squamous carcinoma of the buccal mucosa (human keratinocyte cell line TR 146) and formed on a support membrane, were placed in a new 24-well tissue culture plate. Standardized suspensions of *C. tropicalis* (1 mL containing 2×10^6 cells in MCDB-153 defined medium (Clonetics, San Diego, CA, USA) containing 5 mg/L insulin, 1.5 mM $CaCl_2$, 25 μL/mL gentamicin and 0.4 mg/L hydrocortisone) were then added to each RHOE tissue insert. Control samples were inoculated with 1 mL of medium devoid of *Candida* cells. Tissues were subsequently incubated at 37°C in a 5% CO_2 environment with saturated humidity for 12 and 24 h. In addition, planktonic cultures of *C. tropicalis*, used as controls, were prepared using the same tissue culture medium and incubation conditions, but in the absence of RHOE. These planktonic cultures were subsequently used for standardizing lactate dehydrogenase (LDH) activity and also as controls for *SAP* gene expression studies (24 h).

After incubation, the tissues were rinsed twice with PBS to remove non-adherent *Candida* cells and then bisected, with one-half used for CLSM analysis and the other for RNA extraction and real-time PCR studies. The culture medium was also analyzed for *Candida* induced damage using a LDH assay.

In separate experiments, the SAP inhibitor, pepstatin A (Sigma, Poole, UK) was added, simultaneously with the *C. tropicalis* inoculum, to the tissue inserts to assess the effect of SAPs on the infection process. Briefly, pepstatin A was dissolved in 100% methanol and added directly to the prepared *Candida* inoculum to achieve a final concentration of pepstatin A of 15 mg/L. These tissue inserts were examined after 24 h for invasion of *Candida* and LDH production.

CLSM

Colonization and morphological characteristics of *C. tropicalis* on the surface of fresh tissues were assessed using CLSM, following direct labelling of *Candida* cells with 100 μL of concanavalin A lectin conjugated with Alexa 594 (Molecular Probes-Invitrogen, Paisley, UK: 25 mg/L in PBS) for 20 min at room temperature. Briefly, infected tissue was fixed in 2% (v/v) paraformaldehyde (in ultrapure water) for 24 h at 4°C and embedded in paraffin wax using standard histological techniques. Tissue sections (20 μm) were cut and placed on Histobond+ coated microscope slides (Raymond A Lamb, Eastbourne, UK), de-waxed by processing through xylene,

followed by immersion in ethanol and then water. The prepared sections were overlaid with 50 μL of Alexa 594-conjugated concanavalin A lectin (Molecular Probes-Invitrogen; 25 mg/L in PBS) for 20 min at room temperature. For keratinocyte nuclear staining within the RHOE sections, Hoechst 33258 dye (Sigma; 1 mg/L) was applied for 30 min at room temperature and then washed with PBS. Tissues were mounted in Vectashield (Vector Laboratories, Peterborough, UK) fade-retarding mountant and then observed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany). The sections were scanned through full depth using appropriate settings for single- and double-fluorescence recordings of Hoechst 33258 (laser excitation line 485 nm and emissions detected 410–485 nm) and Alexa 594-conjugated concanavalin A (laser excitation line 546 and emissions detected 600–660).

LDH assay

The release of LDH from the RHOE into the culture medium was used as a measure of epithelial cell damage. The LDH concentration in the medium from both control and infected tissues was measured at 12 and 24 h (in both presence and absence of pepstatin A) using the CytoTox-ONE™ kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. A control for LDH activity was also prepared using a replicate preparation of *Candida*, cultured in medium devoid of RHOE. The LDH activity of this control was subtracted from the LDH activity of the tissue infected with yeast. The LDH released during infection with the different *C. tropicalis* strains was then expressed as a relative activity determined against the untreated control tissue. All experiments were performed in duplicate.

Analysis of *SAP* gene expression

RNA extraction. Tissues for RNA extraction were stored at 4°C in 2 mL microtubes containing RNAlater® solution (Ambion, Huntington, UK). Prior to RNA extraction, lysis buffer was prepared by adding 10 μL of β-mercaptoethanol per mL of RLT buffer (Qiagen, Crawley, UK). Then, 600 μL of lysis buffer and glass beads (0.5 mm diameter, approximately 500 μL) were added to each tissue and these were homogenized twice for 30 s, using a Mini-BeadBeater-8 (Stratech Scientific, Soham, UK). After tissue disruption, the RNeasy Mini kit (Qiagen) was used for total RNA extraction according to the manufacturer's recommended protocol. Potential DNA contamination was removed by RNase-Free DNase I (Qiagen) treatment. Additionally, RNA was also extracted, following the same approach, from *C. tropicalis* planktonic cells (absence of RHOE) prepared and cultured in the same manner as the RHOE-infecting strains.

Primer design. Primers for real-time PCR were designed using the PRIMER3 web-based software (http://frodo.wi.mit.edu/primer3) and these primer sequences are listed in Table 1. To verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from ATCC 750^T genomic DNA. Control S14 primers were also used [32] to detect human recombinant DNA.

Synthesis of cDNA. From each sample, 10 μL of extracted RNA was used for cDNA synthesis. First, the RNA was incubated with 5 μL (50 mg/L) of oligo-dT primer (Promega, Southampton, UK) at 70°C for 5 min. Subsequently, RNA was added to a reaction mixture containing RT Buffer [50 mM Tris (pH 8.3), 3 mM $MgCl_2$, 10 U of RNasin Plus RNase inhibitor (Promega)], 20 μM of each deoxynucleoside triphosphate and 200 U of Moloney murine leukaemia virus-reverse transcriptase (Promega), in a final reaction volume of 50 μL. cDNA synthesis was then performed at 42°C for 1 h. The reaction was stopped by heating for 5 min at 95°C.

Real-time PCR. Real-time PCR was used to determine the relative levels of *SAPT1-4* mRNA transcripts in the RNA samples, with *ACT1* used as a reference housekeeping gene. A 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) was used in accordance with the manufacturer's instructions. Each reaction mixture consisted of the working concentration of Power SYBR® Green PCR master mix (Applied Biosystems), 300 nM forward and reverse primer and 1 μL of cDNA, in a final reaction volume of 20 μL. Negative (water) controls were included in each run. The relative quantification of *SAPT1-4* gene expression was performed by the ΔC_T method, using the control gene (*ACT1*) to normalize the data. Each reaction was performed in tripli-

TABLE 1. Primers used for real time-PCR analysis of *SAP* and control gene expression

Sequence (5′ to 3′)	Primer	Target	PCR product size (bp)
GGAAGATCTGATGTGCCAACTACATTGA	Forward	*SAPT1*	1005
CGTGCGGCCGCTCTACAAAGCCGAGATGT CT	Reverse		
TTCTTCTAGTGGTACCTGGGTCAAAG	Forward	*SAPT2*	762
CATAGATCTCTAAACAATAGTGACATTAGA	Reverse		
ACTTGGATTTCCAGCGAAGA	Forward	*SAPT3*	165
AGCCCTTCCAATGCCTAAAT	Reverse		
GTACTCGAGCTCCTACAACTTCACCTCCT	Forward	*SAPT4*	1130
CATGGATCCCTATGTAAGTGGAAGTATGTT	Reverse		
GACCGAAGCTCCAATGAATC	Forward	*ACT1*	181
AATTGGGACAACGTGGGTAA	Reverse		
CAGGTCCAGGGGTCTTGGTCC	Forward	Human	
GGCAGACCGAGATGAATCCTCA	Reverse	*S14*	143

cate and mean values of relative expression were analyzed for each *SAP* gene.

Statistical analysis

Results were compared using one-way analysis of variance by applying Levene's test of homogeneity of variance and Tukey's multiple-comparisons test, using SPSS (SPSS Inc., Chicago, IL, USA). All tests were performed with a 95% CI.

Results

In vitro RHOE infection

Fresh RHOE tissues were used to study surface colonization and candidal morphology after 12 h of incubation (Fig. 1 and Table 2). The results showed that all *C. tropicalis* strains were able to colonize the RHOE. However, the extent of colonization was strain-dependent. By contrast to the other *C. tropicalis* strains, strain 544123 (urinary tract isolate) exhibited a low colonization level. Interestingly, the different *C. tropicalis* strains exhibited diverse and strain-dependent differences with respect to cell morphologies on the RHOE surface (Fig. 1 and Table 2). *C. tropicalis* T2.2 and AG1 (oral strains) and *C. tropicalis* 12 (vaginal strain) formed a network of both yeast and filamentous morphology. This contrasted with *C. tropicalis* 519468 (urinary isolate) and *C. tropicalis* ATCC 750^T, with both exhibiting predominantly filamentous growth on the RHOE surface. *C. tropicalis* 75 (vaginal isolate) and *C. tropicalis* 544123 (urinary tract) strains colonized and grew on the RHOE primarily in yeast form.

To assess the invasive capability of *C. tropicalis*, the RHOE tissues were fixed, sectioned, stained and observed by CLSM at 12 and 24 h after infection (Figs 2 and 3 and Table 2). Fig. 2, illustrates the ability of *C. tropicalis* to invade the RHOE after 12 h of incubation. All *C. tropicalis* strains extensively invaded the RHOE, with the exception of *C. tropicalis* 544123 (urinary isolate) that only presented clusters of yeast attached to the tissue surface. Invasion was more extensive after 24 h of infection (Fig. 3) and disruption of the epithelial structure was also more evident. Infection with *C. tropicalis* AG1 and T2.2 (both oral isolates) and *C. tropicalis* 12 (vaginal isolate) induced vacuolization between keratinocytes, promoting a high level of disorganization of the epithelium structure. Tissue alteration caused by *C. tropicalis* 75 (vaginal isolate) and *C. tropicalis* 519468 (urinary isolate) resulted in the detachment of the superficial keratinocytes layer. Furthermore, the high number of filamentous forms of both strains appeared to be responsible for the detachment of the RHOE from the support membrane. By contrast, changes caused by the non-invasive *C. tropicalis* 544123 (urinary isolate) and by *C. tropicalis* ATCC 750^T were limited, despite the latter being able to invade the RHOE.

To quantify tissue damage, the levels of released LDH were determined after 12 and 24 h of infection (Fig. 4). Co-culture of tissue with each of the isolates caused a moderate increase in the LDH levels after 12 h of incubation compared to uninfected controls. Furthermore, the relative LDH activity was similar for all *C. tropicalis* strains analyzed ($p > 0.05$). The levels of LDH markedly increased (approximately five-fold) with longer infection time ($p > 0.05$). The results obtained for LDH activity (Fig. 4) positively correlated with the extent of tissue damage observed microscopically (Fig. 3). *C. tropicalis* 544123 caused the least histologically observed damage (not invasive) and also produced the lowest LDH levels, although this was still significantly higher than the uninfected control tissue (Fig. 4 and Table 2).

To provide insight into the mechanism of *C. tropicalis* RHOE invasion and induction of tissue damage, the effect of the specific aspartyl proteinase inhibitor, pepstatin A on the infection process was evaluated (Figs 4 and 5). Importantly, invasion of *C. tropicalis* was not inhibited by the presence of pepstatin A (Fig. 5). Furthermore, the inclusion of pepstatin A did not reduce the level of tissue damage as assessed his-

TABLE 2. *Candida tropicalis* infection profile of reconstituted human oral epithelium (at 12 and 24 h) as assessed by confocal laser scanning microscopy

Candida tropicalis strains	Origin	Surface colonization (12 h)	Strain morphology in presence of tissue (12 h)	Invasion/integrity of RHOE	
				12 h	24 h
AG1	Oral	+++	Yeast and filament forms	High/undamaged	High/damage
T2.2		+++	Yeast and filament forms	High/undamaged	High/damage
519468	Urinary	+++	Predominantly filament forms	High/undamaged	High/damage
544123		+	Predominantly yeast	Low/undamaged	Low/undamaged
12	Vaginal	++	Yeast and filament forms	High/undamaged	High/damage
75		++	Predominantly yeast	High/undamaged	High/damaged
ATCC 750^T	Reference	+++	Predominantly filament forms	High/undamaged	High/undamaged

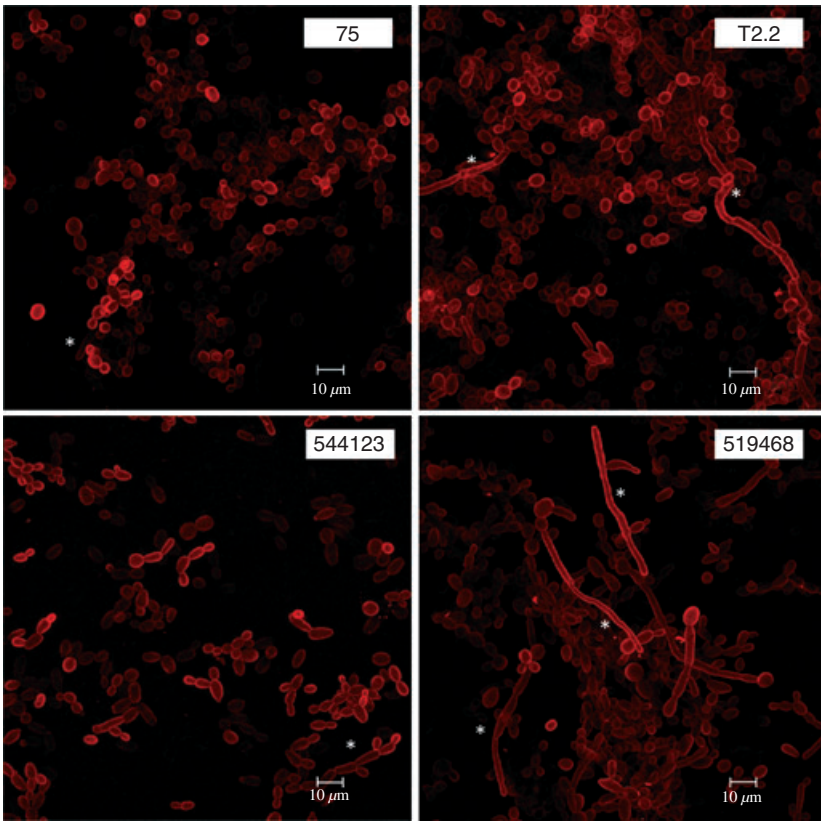

FIG. 1. Colonization profile of *Candida tropicalis* strains infecting human oral epithelium after 12 h of incubation. *Presence of filamentous forms.

tologically (Fig. 5) and by LDH activity (Fig. 4). At the concentrations used, pepstatin A did not affect *C. tropicalis* colonization or the appearance of the uninfected RHOE.

SAP gene expression

Table 3 presents the mean percentage *SAP* gene expression levels relative to *ACT1*, for *C. tropicalis* infecting RHOE for 12 and 24 h, and for planktonic cells (after 24 h of incubation using the same conditions, but in the absence of RHOE).

Real-time PCR analysis revealed a wide range of expression profiles of *SAP* genes for the seven *C. tropicalis* strains in the three conditions examined (Table 3). In general, *SAPT2-4* transcripts were frequently detected, whereas *SAPT1* was only rarely detected. After 12 and 24 h of incubation, *C. tropicalis* strains exhibited different *SAPT2-4* gene expression profiles. An increase in the expression of *SAP* genes with a longer incubation time was only evident with *C. tropicalis* ATCC 750^T and *C. tropicalis* 75 (*SAPT2-4*), and for *C. tropicalis* AG1, *C. tropicalis* T2.2 and *C. tropicalis* 544123 (*SAPT3*). Furthermore, all *C. tropicalis* strains growing for 24 h in the planktonic form expressed all the *SAPT2-4* genes, with the exception of *SAPT2* for *C. tropicalis* 519468 and *C. tropicalis* 12. Additionally, there was an apparent increase in the expression of most *SAPT* genes for planktonic cells (24 h) compared to their 24 h RHOE counterparts. For example, with the oral isolate *C. tropicalis* AG1, real-time PCR analysis revealed an increased expression of *SAPT3* and *SAPT4* genes, by approximately four- and 20-fold, respectively, compared to RHOE-infecting cells.

Discussion

The pathogenesis of mucosal candidosis, particularly those infections caused by *C. albicans*, has been investigated in several studies [12,14–16,28–30] using RHOE as a successful surrogate model to animals. *C. tropicalis* has been recognized as an increasingly important pathogen of debilitated individuals [9–11], with several studies demonstrating that *C. tropicalis* virulence in mice can be similar to, or indeed higher than, *C. albicans* [21,22]. However, compared to *C. albicans*, relatively few investigations have been performed to assess the virulence of *C. tropicalis*, particularly in human tissues [14,33,34]. Thus, the primary aim of the present study was to investigate the pattern of colonization and invasion of RHOE by a variety of *C. tropicalis* strains from different body sites.

The results obtained (Fig. 1 and Table 2) showed that the oral epithelium was successfully colonized by *C. tropicalis*, although the extent of colonization was noticeably strain-dependent. The study also revealed that *C. tropicalis* was able

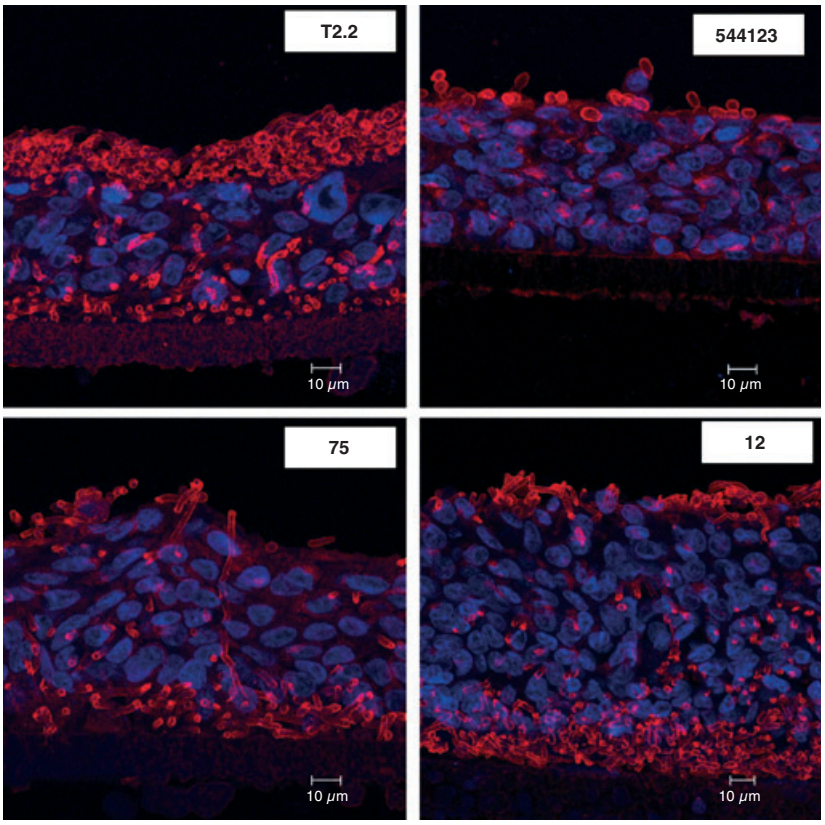

FIG. 2. Confocal laser scanning microscopy images of *Candida tropicalis* strains infecting human oral epithelium after 12 h of incubation.

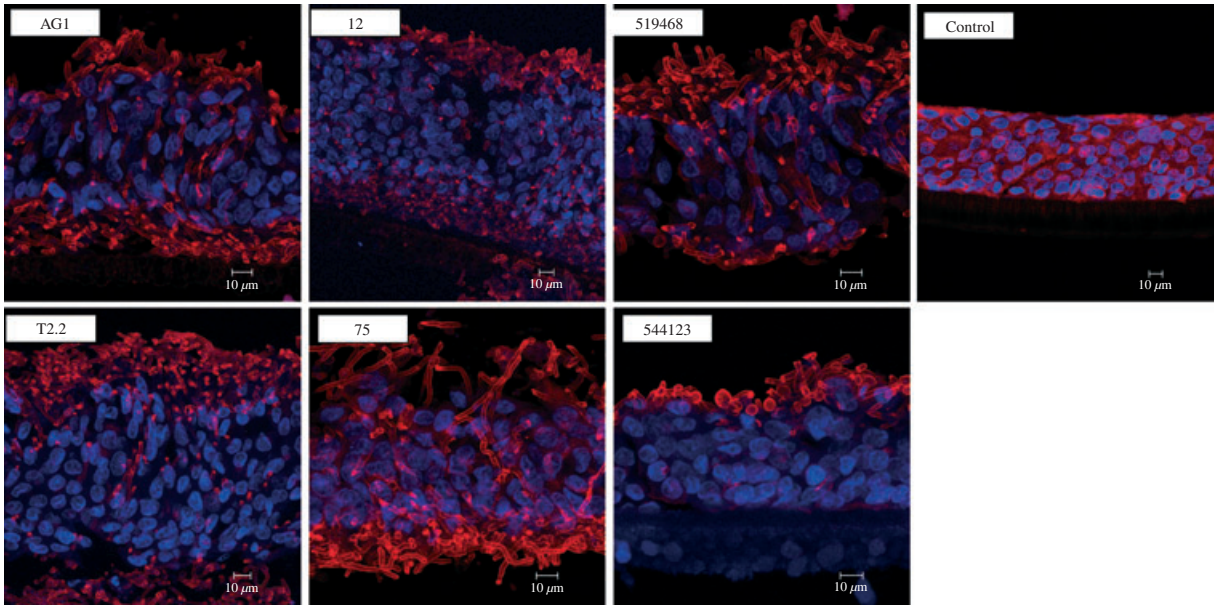

FIG. 3. Confocal laser scanning microscopy images of *Candida tropicalis* strains infecting human oral epithelium after 24 h of incubation.

to develop filamentous forms on the tissue surface that could promote colonization. Furthermore, CLSM images showed that, similar to *C. albicans* [14,15,34], *C. tropicalis* was also able to invade the RHOE (Fig. 2). In this regard, invading fungal elements were found to penetrate to the deepest layers of the RHOE within just 12 h of infection (Fig. 2 and Table 2). These observations are not in total agreement with previous studies [14,33,34], where *C. tropicalis* has been

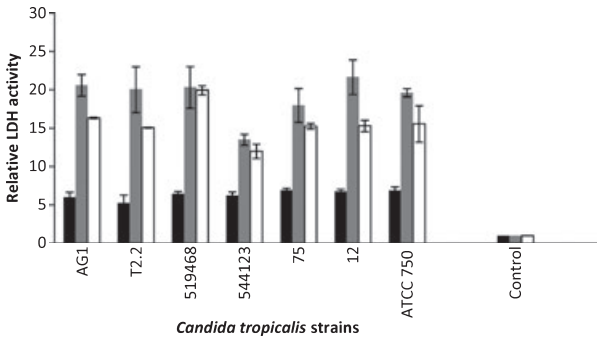

FIG. 4. Relative lactate dehydrogenase activity measured in the human oral epithelium tissue culture supernatant after 12 h (■) and 24 h (■) incubation with different *Candida tropicalis* strains; and in the presence of pepstatin A at 24 h (□). *Strain statistically different from the other strains ($p < 0.05$). Error bars represent the SD.

described as a non-invasive species, despite having an ability to colonize. However, as revealed in the present study, strain differences in the capability to invade were apparent.

Subsequent examination of epithelial invasion revealed significant differences between 12- and 24-h infection periods, indicating progressive tissue damage related to the duration of infection. There was induction of significant tissue disruption in the tissue structure (Fig. 3) compared to uninfected tissues after 24 h infection. The degree of tissue damage was further evaluated quantitatively through the measurement of relative LDH activity (Fig. 4), and the results obtained positively correlated with the *C. tropicalis* infection temporal pattern and histologically observed damage. Interestingly, after 12 h of infection, the LDH activities of tissues infected with the different *C. tropicalis* strains were largely similar, implying equivalent degrees of the damage caused. More pronounced differences were found after 24 h of infection, which might reflect a variation in the ability of strains to continue to grow within the epithelium and induce damage after initial invasion. These features were similar to those described in previous studies [14,34], where it was demonstrated that *Candida dubliniensis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* and particularly *C. tropicalis*, were causative agents of dramatic histopathological tissue alteration.

Of note, the six strains classified in the present study as 'high invaders' were derived from three different superficial body sites, possibly indicating that strain origin is not important with respect to invasive capability. However, further studies involving a greater number of strains, including those from systemic infections, would be of benefit in confirming this view. One strain (*C. tropicalis* 544123) did not produce extensive filamentous forms and, notably, this strain was the only one that was unable to invade the oral epithelium (Fig. 2 and Table 2). This result would support the view that it is the filamentous forms that facilitate tissue invasion.

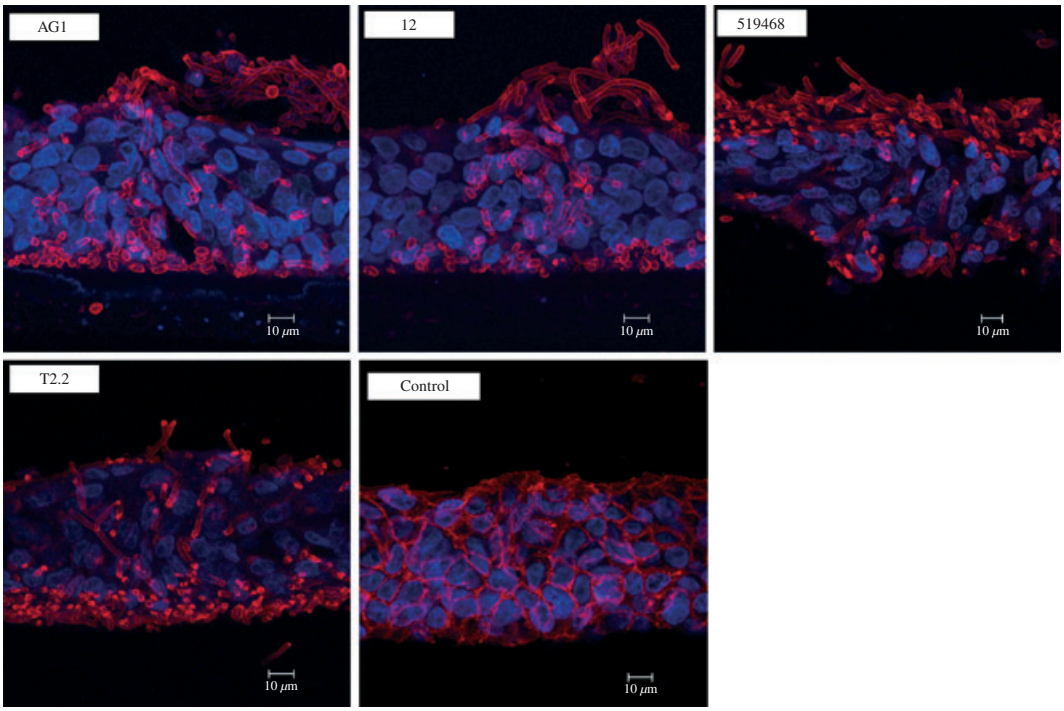

FIG. 5. Confocal laser scanning microscopy images of *Candida tropicalis* strains infecting human oral epithelium after 24 h in presence of 15 μL/mL pepstatin A.

TABLE 3. Detection of secreted aspartyl proteinase (*SAPT1-4*) gene expression associated with pathogenesis during *Candida tropicalis* reconstituted human oral epithelium infection (at 12 and 24 h) and planktonic culture using a quantitative real-time PCR

Candida tropicalis strains	Relative expression of SAP genes[a]											
	Cells that infected RHOE 12 h				24 h				Planktonic cells 24 h			
	SAPT1	*SAPT2*	*SAPT3*	*SAPT4*	*SAPT1*	*SAPT2*	*SAPT3*	*SAPT4*	*SAPT1*	*SAPT2*	*SAPT3*	*SAPT4*
AG1	ND	0.01 ± 0.00	0.18 ± 0.02	0.28 ± 0.02	ND	ND	0.32 ± 0.15	0.06 ± 0.02	ND	0.02 ± 0.01	1.29 ± 0.42	1.24 ± 0.93
T2.2	ND	0.04 ± 0.02	0.48 ± 0.15	2.70 ± 0.64	ND	ND	0.69 ± 0.27	0.06 ± 0.02	ND	0.03 ± 0.00	1.59 ± 0.16	0.30 ± 0.08
519468	0.03 ± 0.02	0.22 ± 0.14	0.45 ± 0.13	4.57 ± 0.99	ND	0.22 ± 0.09	ND	0.95 ± 0.54	ND	ND	0.86 ± 0.07	0.16 ± 0.02
544123	ND	0.22 ± 0.03	0.04 ± 0.00	1.58 ± 0.62	ND	0.01 ± 0.00	0.60 ± 0.06	0.02 ± 0.00	ND	0.01 ± 0.01	0.97 ± 0.44	0.10 ± 0.02
12	ND	ND	0.23 ± 0.04	0.01 ± 0.00	ND	0.01 ± 0.01	0.20 ± 0.02	0.05 ± 0.03	ND	ND	0.07 ± 0.03	0.25 ± 0.06
75	ND	ND	0.48 ± 0.06	0.03 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	1.58 ± 0.35	10.07 ± 2.47	0.06 ± 0.01	0.04 ± 0.00	2.27 ± 0.71	9.78 ± 0.41
ATCC 750^T	ND	ND	0.76 ± 0.10	0.01 ± 0.07	ND	0.13 ± 0.07	3.92 ± 1.17	0.21 ± 0.07	ND	0.04 ± 0.02	1.07 ± 0.20	0.16 ± 0.13

ND, no gene expression detected.
[a]Mean arbitrary messenger RNA transcript levels based upon triplicate measurements, presented as a percentage relative to the respective *ACT1* transcript level.

SAPs are considered hydrolytic enzymes contributing to *Candida* infection by promoting damage to the host mucosa, thus facilitating invasion of the epithelium [20,24]. There are at least four known genes encoding for SAPs in *C. tropicalis* [16,20,26] and the expression of these *C. tropicalis* genes and the role of SAPs in the infection process were assessed in this present study. For all *C. tropicalis* strains, *SAPT* gene expression was assessed using real-time PCR, after 12 and 24 h of infection (and after 24 h of incubation for equivalent planktonic preparations). Overall, the expression of *SAP* genes by *C. tropicalis* was strain-dependent, with *SAPT1* not expressed by the majority of strains, suggesting its limited involvement in invasion and tissue damage. Interestingly, *SAP* gene expression was not significantly different between infecting and planktonic strains of *C. tropicalis*. Furthermore, the effect of the aspartyl proteinase inhibitor, pepstatin A, on the infection process was also evaluated to provide a complementary insight into potential involvement of SAPs in *C. tropicalis* invasion and damage to the RHOE. Pepstatin A is a specific inhibitor of acidic proteinases and has been widely used as an inhibitor of *Candida* aspartyl proteinase activity [25,29,30]. Interestingly, treatment with pepstatin A did not prevent tissue invasion or apparent histological damage induced by *C. tropicalis* (Fig. 5). Furthermore, LDH activity was also not reduced by incorporation of pepstatin A, reinforcing the view that the extent of tissue damage was maintained even when SAP activity was inhibited. It would appear therefore that SAPs do not play a significant role in tissue invasion and damage caused by *C. tropicalis*. These results appear to be in agreement with recent studies involving *C. albicans*, which demonstrated that SAPs were not required for invasion [30], and damage to RHOE [29].

In summary, the present study confirms the effectiveness of RHOE as an *in vitro* model to study *Candida* virulence attributes and broadens our knowledge on *C. tropicalis* virulence mechanisms. The CLSM approach has conclusively shown that *C. tropicalis* was not only capable of colonizing, but also could invade RHOE, leading to significant damage in the tissue structure. Furthermore, to our knowledge, this is the first report of *C. tropicalis SAPT* gene expression during tissue invasion, and the results obtained imply a limited role of these enzymes in *C. tropicalis* invasion and tissue damage.

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Transparency Declaration

The authors have no conflict of interest to declare.

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