



Inactivation of the Complement Lectin Pathway by *Candida tropicalis* Secreted Aspartyl Protease-1

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ABSTRACT

Candida tropicalis is an opportunistic fungal pathogen and is one of the most frequently isolated non-*albicans* species. It can cause localised as well as invasive systemic infections particularly in immunocompromised patients. Increased resistance to common anti-fungal drugs is an emerging problem. In order to establish disseminated infections, *Candida* has evolved several strategies to escape the host immune system. A detailed understanding of how *C. tropicalis* escapes the host immune attack is needed as it can help develop novel anti-fungal therapies. Secreted aspartyl proteinases (Saps) of *C. albicans* have been shown to be determinants of virulence and immune evasion. However, the immune evasion properties of *C. tropicalis* Saps have been poorly characterised. This study investigated the immune evasion properties of *C. tropicalis* secreted aspartic protease 1 (Sapt1). Sapt1 was recombinantly produced using a *Kluyveromyces lactis* yeast expression system. A range of complement proteins and immunoglobulins were screened to test if Sapt1 had any proteolytic activity. Sapt1 efficiently cleaved human mannose-binding lectin (MBL) and collectin-11, which are the initiating molecules of the lectin pathway of the complement system, but not *L*-ficolin. In addition, Sapt1 cleaved DC-SIGN, the receptor on antigen presenting dendritic cells. Proteolysis was prominent in acidic condition (pH 5.2), a characteristic of aspartyl protease. No proteolytic activity was detected against complement proteins C1q, C3, C3b, IgG and IgA. In view of the ability of Sapt1 to cleave MBL and collectin-11, we found that Sapt1 could prevent activation of the complement lectin pathway. RT-qPCR analysis using three different *C. tropicalis* clinical isolates (oral, blood and peritoneal dialysis fluid) revealed relatively higher levels of mRNA expression of Sapt1 gene when compared to a reference strain; Sapt1 protein was found to be secreted by all the tested strains. Lectin pathway and its initiating components are crucial to provide front line defence against *Candida* infections. For the first time, we have shown that a *Candida* protease can proteolytically degrade the key initiating components of lectin pathway and inhibit complement activation. Findings from this study highlight the importance of exploring Sapt1 as a potential therapeutic target. We conclude that *C. tropicalis* secretes Sapt1 to target the complement lectin pathway, a key pattern recognition and clearance mechanism, for its survival and pathogenesis.

1. Introduction

Candida species are opportunistic human pathogenic yeasts. Infections can either be localised that often affects the oral cavities, vagina, skin, or life-threatening invasive candidiasis that can affect multiple organs. There are an estimated 700,000 cases of invasive

candidiasis worldwide annually (Bongomin et al., 2017; Di Mambro et al., 2021). Susceptibility factors include young or old age, immunocompromised status, HIV/AIDS, use of broad-spectrum antibiotics, gastrointestinal surgery, diabetes mellitus, long term hospitalization and catheters or indwelling medical devices (Patil et al., 2015). Around 20 different *Candida* species are known to cause human disease. *C. albicans*

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and *C. tropicalis* are reported to be more virulent followed by *C. glabrata*, *C. parapsilosis* and *C. krusei*, as determined by mortality rates in murine models (Arendrup et al., 2002). The increasing incidence of *C. tropicalis* infections and recent development of anti-fungal resistance merit a closer inspection of the mechanisms through which this species establishes infection and evades the immune system.

The host immune system is activated upon *Candida* colonisation to combat infection. Complement system, a network of ~35 proteins, is often activated early in the course of a *Candida* infection as part of the innate immune response. *Candida* activates all three pathways of complement system: classical, lectin and alternative. Mannan and α -D-glucose on the *Candida albicans* surface are detected by mannose-binding lectin (MBL) and collectin-11 (CL-11) to activate the complement lectin pathway. MBL or CL-11 gene knock-out mice have higher levels of colonisations by *Candida* strains (Choteau et al., 2016; Hansen et al., 2010). However, no association has been established between *Candida* and ficolin, which is another initiating sub-component of the lectin pathway (Endo et al., 2007; Kilpatrick and Chalmers, 2012; Ren et al., 2014). The majority of adult populations experience colonization by *Candida* and develop mannan-specific IgG antibodies (Sendid et al., 2021). These antibodies trigger activation of the classical pathway via C1q in the event of *Candida* infection. During *C. albicans* infection or by treatment with caspofungin, the inner β -glucan components become exposed (Wheeler et al., 2008) that initiate the complement alternative pathway (Boxx et al., 2010). DC-SIGN, a C-type lectin expressed on dendritic cells, binds to mannan residues on the surface of *Candida* and helps in clearance (van Kooyk and Geijtenbeek, 2003; Cambi et al., 2005). DC-SIGN binds to mannose containing glycoproteins such as ICAM-2 and ICAM-3 (CD50) in a calcium dependant manner and recognises pathogens by binding to mannose or Lewis-x carbohydrate structures. DC-SIGN has been shown to be an antigen uptake receptor for *C. albicans* by binding to the N-linked mannan specifically on the surface of *Candida* (van Kooyk and Geijtenbeek, 2003; Cambi et al., 2005).

One of the key factors that makes *Candida* a successful pathogen is its ability to degrade host proteins. This is achieved by the production of extracellular or surface bound proteases which aid the pathogen in penetrating into host tissue and countering the effects of the hosts defence system. Secreted aspartyl proteases (Saps) have been studied in several *Candida* species, in particular, *C. albicans*. Typically, Saps display highest activity at low pH and show an affinity to hydrophobic amino acids, such as phenylalanine, though their substrate affinity varies greatly (Koelsch et al., 2000). There have been several reports of *C. albicans* Saps binding to and cleaving proteins of the host immune system. In particular, the complement system is targeted by Saps1–3 that bind and cleave crucial proteins C3b, C4b and C5, thus, inhibiting complement alternative as well as classical pathways (Gropp et al., 2009). *C. albicans* Saps have also been shown to target antimicrobial peptides, lactoferrin of the saliva, and IgA, thus they manipulate the mucosal and systemic immune response (Naglik et al., 2004). Saps also degrade several other host proteins including collagen, fibronectin, IgG heavy chains, lactoperoxidase, α 2-macroglobulin and β -lactoglobulin (Pichová et al., 2001).

Similar to *C. albicans*, *C. tropicalis* also secretes aspartic proteases, with Sapt1 being the most dominantly secreted Sap when grown in medium containing bovine serum albumin (BSA) as the sole nitrogen source (Zaugg et al., 2001; Hube et al., 1994). Three other Sap genes have been detected in *C. tropicalis* strains; thus, the Sap gene family of *C. tropicalis* is likely to be much smaller than that of *C. albicans* which have 10 different Saps.

Sapt1 is the predominantly expressed Sap in *C. tropicalis*, but its proteolytic activity on human immune system proteins remains unknown. Therefore, this study was aimed at characterizing complement evasion properties of *C. tropicalis* mediated by the proteolytic activity of Sapt1. We found that recombinantly produced Sapt1 displayed proteolytic activity against C-type lectins: human MBL, CL-11 and DC-SIGN. Further analysis revealed that Sapt1 targeted the neck and

carbohydrate recognition domain (neck-CRD) of CL-11. Purified recombinant Sapt1 inhibited the activation of complement lectin pathway. Clinical *C. tropicalis* isolates from blood, oral cavity and peritoneal dialysis fluid displayed relatively higher level of Sapt1 expression indicating that Sapt1 could be used as an immune evasion molecule during invasive infections..

2. Materials & Methods

2.1. Strains and proteins

C. tropicalis clinical strains were procured from the National Collection of Pathogenic Fungi (NCPF). Strain NCPF 3111 is a historically MRL antigen strain isolated reference strain in 1928, NCPF 8113 Fluconazole resistant strain was isolated from the oral cavity, NCPF 3463 was isolated from the PD fluid from peritonitis patient, and NCPF is an Azole resistant strain isolated from the blood of a patient with renal failure. Human C1q (Sigma-Aldrich #C1740), C3 (Comptech #A113), C3b (Comptech #A114), IgA (Sigma #12636) and IgG (Sigma #14506) were procured commercially. Human α -D-ficolin and MBL were produced as described (Girija et al., 2007; Wallis and Drickamer, 1997). Human CL-11 was produced as published previously (Venkatraman Girija et al., 2015). Human DC-SIGN was produced as published earlier (Dodagatta-Marri et al., 2017).

2.2. Expression and purification of recombinant SAPT1

Sapt1 gene coding the propeptide and mature peptide sequences (amino acid co-ordinates from 24 to 294; UniProtKB – Q00663) with 10x Histidine tag at the C-terminal end was synthesised by Integrated DNA Technologies. The gene was cloned in-frame downstream to the alpha-mating factor (α -MF) signal sequence in to the Xho I and Eco RI restriction sites of pKLAC2 yeast expression vector. Following sequence confirmation, pKLAC2-Sapt1 was digested with Sac II to produce a linearised expression cassette, and transformed in to *Kluveryomyces lactis* GG799 cells using *K. lactis* protein expression kit (New England Biolabs E1000S). Transformants were selected on Yeast Carbon Base (YCB) with 5 mM acetamide by growing at 30 °C for 2 days.

Identification of clones with the integrated cassette was made by PCR screening using specific integration primers, and by screening clones for Sapt1 expression by SDS-PAGE. Briefly, cells were harvested from YCB agar plates and resuspended into 25 μ l of 1 M sorbitol containing 2 mg/ml lyticase. The mixture was vortexed and incubated for 1 h at 30 °C. Cells were lysed in a thermocycler at 98 °C for 10 min. PCR reaction was set up with primers, integration Primer-1 (5' ACA-CACGTAACGCGCTCGGT 3') and integration primer-2 (5' ATCATCCTTGTACGCGAAAGC 3'), 2 mM dNTPs, ThermoPol buffer, Taq DNA polymerase and made up to 100 μ l with deionised water. PCR cycles consisted of 30 cycles (94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min), followed by 72 °C for 10 min. The PCR products were analysed on 1 % agarose gel for ~2.4 kb product to confirm integration at the LAC4 locus (data not shown). Media supernatant from PCR positive clones was subjected to 12 % SDS-PAGE under reducing conditions to confirm Sapt1 expression.

K. lactis cells expressing recombinant Sapt1 as N-terminal His₁₀ tagged fusion protein were marinated on YCB agar medium with acetamide. A single colony from YCB agar plate was inoculated into 10 ml of YPGal medium (10 g yeast extract, 20 g peptone, and 40 g galactose per liter) and incubated at 30 °C for 8-hours (225 rpm) to produce the primary culture. For large scale expression, 10 ml of this primary culture was inoculated into 1 L of YPGal medium which was incubated under the same conditions overnight. Once the culture had reached an OD₆₀₀ ~25, the cells were harvested, and supernatant containing secreted recombinant Sapt1 was mixed with equal amount of phosphate buffered saline (PBS) and subjected to Nickel-affinity chromatography using 2 ml of Nickel-NTA resin (GE Healthcare 17526801) that was equilibrated

with PBS buffer. The protein was eluted with various concentrations of imidazole made using PBS. Step-gradient process was used to carry out elution starting from 20 mM imidazole, followed by 50 mM, 100 mM, 200 mM and 500 mM imidazole. Eluted fractions were analysed by 12 % SDS-PAGE under reducing conditions and stained with Instant Blue (Expedeon #ISB1L). Fractions containing the purified Sapt1 were pooled together and dialysed in TBS buffer (25 mM Tris pH 7.4, 150 mM NaCl buffer) or citrate buffer pH 5.2 (Sodium citrate dihydrate #10787024 ThermoFisher and Citric acid #10020760 ThermoFisher). Protein band was characterised using MALDI-TOF.

2.3. Proteolytic assays using crude *K. lactis* supernatant expressing Sapt1

K. lactis cells containing pKLAC2-Sapt1 construct were grown in YPgal broth and incubated at 30 °C for 48 h (225 rpm). As a preliminary screening method to test the proteolytic ligands of Sapt1, 20 µl of crude culture supernatant secreting Sapt1 was incubated with 1 µg of C1q, MBL, CL-11 and ι -ficolin, C3 and C3b, DC-SIGN, IgA or IgG. The assay mixture was incubated for 3 h at 37 °C. *K. lactis* cells supernatant secreting male (Maltose Binding Protein) was used as a control for each of the ligand. To monitor proteolysis, the assay mixtures were run on 12 % or 15 % SDS-PAGE under reducing conditions and stained with instant blue (Expedeon #ISB1L).

2.4. Proteolytic assays using purified recombinant Sapt1

Purified Sapt1 was incubated with various substrates (target immune proteins) and the reaction mixture was visualised for the proteolytic activity via 12 % or 15 % SDS-PAGE under reducing conditions and stained with Instant Blue. The optimal environmental conditions for aspartic proteases are generally acidic, and hence, the assays were carried out using pH 5.2 citrate buffer, as well as physiological pH 7.4 using TBS buffer. Reactions were set up with a molar ratio of 1.2:1 of Sapt1: ligand. In addition to the ligands that were used for the crude supernatant assay screen, neck-Carbohydrate Recognition Domain (neck-CRD) of CL-11 was also used. The proteolytic activity was monitored at various time points: zero-h, 0.5 h, 1-h and 3-h. Aspartic protease inhibitor pepstatin A (Merck Millipore #P5318) was used to inhibit Sapt1 proteolytic activity at a final concentration of 10 µM. All the reactions were stopped with Laemmli buffer supplemented with 10 mM DTT upon reaching the incubation time points. The assay mixture containing the proteins were denatured at 95 °C for 2 min prior to being subjected to SDS-PAGE. The gels were stained with Instant Blue and band intensities were analysed using software ImageJ.

2.5. Complement activation assays

To test if Sapt1 had an effect on complement activation, initial assays were carried out using the Wieslab complement system screening kit (Euro Diagnostica, COMPL 300 RUO). Briefly, 1 µM recombinant Sapt1 was mixed with the diluted human serum (1:101 for classical and lectin pathways, 1:32 for alternative pathway) and incubated for 30 min at 37°C. Positive control (serum and the diluent buffer), negative control (heat inactivated serum and the diluent buffer) and buffer blank (diluent buffer) were also set up as provided in the kit. 100 µl of each reaction was added to the wells and incubated for 1 h at 37 °C. Following washes using the wash buffer, 100 µl of alkaline phosphatase labelled antibody specific to the neoantigen in the C5b-9 complex was added to each well, and the plate was then incubated at room temperature for 30 min. After washing steps, 100 µl of substrate solution was added to each well, and incubated for 30 min at room temperature. The absorbance, correlating to the complement activity, was measured at 405 nm using a SpectraMax M5 microplate reader. The blank (diluent) absorbance was subtracted from samples, negative and positive control absorbance. Mean percentage complement activation was calculated using the following formula: [(sample - negative control)/(positive control-negative control)]

x100. To test the effect of Sapt1 on lectin pathway, serial dilutions of Sapt1 were used. Briefly, Sapt1 was diluted with MP diluent (provided with the kit) to a range of concentrations between 0 and 6 µM and then incubated with positive control serum (diluted 1:101 with MP diluent as directed in kit) at room temperature for 30 min. The effect on lectin pathway alone was monitored following the protocol as described above.

2.6. Native Sapt1 protein expression from clinical isolates

All four *C. tropicalis* clinical isolates (reference strain NCPF 3111, PD fluid strain NCPF 3463, oral stain NCPF 8113, and blood strain NCPF 8694) were used to verify if they secreted native Sapt1. The cells were grown overnight with shaking at 30C with 200 rpm in YPD media and cultures were harvested. Sapt1 protein level was tested in both the culture supernatant and cell lysate. *C. tropicalis* culture was centrifuged for 5 min at 7000 rpm; the supernatant was collected. Laemmli buffer containing DTT was added to samples and analysed by western blotting using mouse polyclonal anti-Sapt1 antisera (1:200) (a kind gift by Dr Hasan Yesilkaya, University of Leicester), followed by secondary anti-mouse antibody conjugate (1:2000) (R & D systems, #HAF007).

2.7. qPCR for Sapt1 mRNA expression

C. tropicalis isolates were cultured overnight. Cells (~1 x 10⁸) grown in YPD and YPD supplemented with BSA were lysed using 100U lyticase in 1 M sorbitol/100 mM EDTA. Total RNA was extracted using Isolate II RNA mini kit (Bioline, BIO-52072), as per manufacturers instructions. The samples were treated with DNase I (Invitrogen) to remove DNA contaminants. Concentration and purity of the eluted RNA was determined with Thermo Scientific NanoDrop Lite Spectrophotometer and confirmed by agarose gel electrophoresis.

Complementary DNA (cDNA) was synthesised from 1 µg of RNA using SensiFAST cDNA Synthesis Kit (Bioline #BIO-65053). Reactions without reverse transcriptase served as negative controls. The primers were designed using software Primer3 (<https://frodo.wi.mit.edu/primer3/>) to produce a ~200 bp amplicon and the recommended Tm was used. Primer efficiency was tested and confirmed (between 90 and 110 %), for both primers sets. Specificity was tested by checking amplicon length on an agarose gel, purifying and sequencing the products as well and carrying out melt curve analysis.

qPCR reactions were prepared using cDNA template (20 ng), 10 µl of 2x SensiFAST SYBR Hi-ROX Mix, 400 nM of each primer for the appropriate reaction (Sapt1 forward 5' CAACGCTGATGTGTTTGG 3' and Sapt1 reverse 5' ATCAATTCGAAAGGGGAAC 3'; Act1 forward 5' GACCGAAGCTCCAATGAATC 3' and Act1 reverse 5' AATTGGGA-CAACGTGGGTAA 3') in a total volume of 20 µl. Amplifications were performed with Applied Biosystems QuantStudio 5 Real-Time PCR System, with settings: 2 min at 95 °C, followed by 30 cycles of 5 s at 95 °C, 10 s at 60 °C and 20 s at 72 °C. The gene expression of Sapt1 was normalised against endogenous reference gene, *ACT1*; cycle threshold (Ct) mean value was used to calculate the relative gene expression using 2- $\Delta\Delta$ Ct method. Standard deviation was calculated from the triplicate values (see the results section).

The products were also visualised by agarose gel electrophoresis and compared against gene ruler. These were excised from the gel and extracted with a DNA extraction kit (NucleoSpin® Gel and PCR Clean-up Kit, Macherey-Nagel, Germany). The purified PCR bands were sequence verified using Eurofins Genomics/GATC biotech (Luxemburg) as Light-Run Tube Sanger sequencing reactions.

3. Results

3.1. Expression of recombinant Sapt1 from *K. lactis* cells and screening for proteolytic activity

The cloning strategy was designed to produce a secreted and active Sapt1. Secretion of the recombinant Sapt1 was achieved by replacing the native signal sequence of the SAPT1 gene with the α -MF signal sequence. Recombinant Sapt1 was successfully expressed as a His₁₀ tagged protein using *K. lactis* cells. Reducing SDS-PAGE and Western blot analysis revealed that Sapt1 migrated at a position just above 40 kDa (Fig. 1). This is slightly higher than the calculated molecular weight of ~37 kDa and is likely due to post-translational modification. The ~40 kDa band was subjected to mass spectrometry (MALDI-TOF-MS) analysis which confirmed that the recombinantly expressed protein was Sapt1 (data not shown), and that the transformed *K. lactis* cells secreted only mature Sapt1 protein.

Crude *K. lactis* supernatant expressing Sapt1 was used to test proteolytic activity against a range of complement proteins and immunoglobulins IgA and IgG. Human MBL, CL-11 and DC-SIGN were cleaved by *K. lactis* crude supernatant containing the Sapt1 (Fig. 2). This was observed as either disappearance or significant decrease (>50 %) in the intensity of the intact ligand bands on reducing SDS-PAGE following incubation with the crude *K. lactis* supernatant with Sapt1. None of the other ligands, C1q, C3, C3b, λ -ficolin, IgA and IgG were cleaved or degraded. Equivalent amount of crude *K. lactis* supernatant expressing human maltose binding protein (MalEp), used as a control, did not display any proteolysis on the tested ligands.

3.2. Proteolysis of MBL, CL-11 and DC-SIGN by purified Sapt1

Purified Sapt1, migrating at about 40 kDa, was used to test its proteolytic properties on the target proteins under acidic and physiological conditions, pH 5.2 and 7.4, respectively. Image J was used to scan the band intensities and the data was plotted. Purified Sapt1 cleaved human CL-11, MBL and DC-SIGN at pH 5.2 (Fig. 3 A, B, C), but not the rest of the tested ligands (data not shown). At pH 5.2, about 50 % of both MBL and CL-11 and over 95 % of DC-SIGN were cleaved by Sapt1 following 3 h of co-incubation. This was observed as both decreased in the band intensity

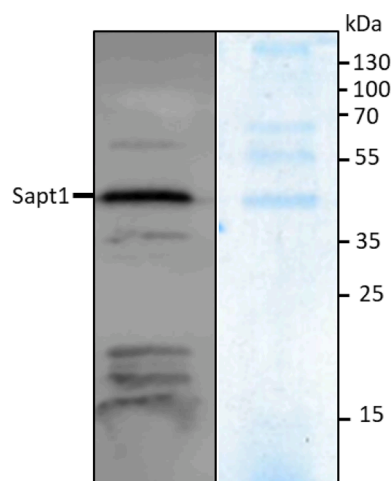


Fig. 1. Expression of recombinant Sapt1 by *K. lactis* cells: pKLAC2-Sapt1-His₁₀ was transformed into *K. lactis* cells and the selected transformants were grown in YPGal medium for 48 h. 20 μ l of supernatant was subjected to 12 % SDS-PAGE under reducing conditions, followed by western blotting using anti-Histidine antibody (left), or instant blue staining (right). Sapt1 was found to be secreted as a ~40 kDa band and identified via MALDI-TOF MS. Image shown for one representative clone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the test ligand as well as appearance of degradation bands (~10–25 kDa) below the corresponding intact ligand bands that were susceptible to Sapt1 proteolysis. It was interesting to note that Sapt1 also underwent auto-degradation over time while cleaving the target ligand.

This can be observed by the ~40 kDa Sapt1 band decreasing in intensity over 3-h when tested at pH 5.2. Pepstatin (10 μ M), an aspartyl protease inhibitor, was used to test if it inhibited the proteolytic activity exhibited by Sapt1 against the target ligands. Pepstatin partially blocked Sapt1 activity as there was still about 20 % MBL cleavage in its presence at pH 5.2 when tested for 3 h (the maximum time point for the assay). It is not uncommon for pepstatin not to exhibit complete inhibitory activity against all aspartyl proteases (Roberts and Taylor, 2003; Aoki et al., 2012).

Under physiological pH 7.4, Sapt1 activity against MBL and CL-11 was much less compared to that at pH 5.2 (Fig. 3D). Only 10 % of both these ligands were found to be cleaved at pH 7.4. However, 40 % of DC-SIGN was cleaved at pH 7.4. Overall, Sapt1, being an acidic protease, exhibited prominent proteolytic activity against the target ligands at pH 5.2 than pH 7.4.

3.3. Cleavage of CL-11-neck-CRD by Sapt1

From our experimental observation, the ligands that were susceptible to Sapt1 proteolysis were found to be C-type lectins MBL and CL-11 and the C-type lectin receptor DC-SIGN: all have neck-Carbohydrate Recognition Domain (neck-CRD) in common. Thus, we tested if Sapt1 targeted neck-CRD. Recombinantly produced CL-11-neck-CRD was incubated with Sapt1 under both pH 5.2 and 7.4 for 3 h as for other ligands. Interestingly, Sapt1 cleaved CL-11-neck-CRD; nearly 50 % and 20 % proteolysis was observed under pH 5.2 and 7.4 respectively (Fig. 4). Pepstatin (10 μ M) almost abolished the Sapt1 effect as there was only ~15 % and ~10 % of proteolysis in the acidic and physiological pH conditions, respectively.

3.4. Effect of Sapt1 on complement activation

To determine the effects of Sapt1 on complement activation, an ELISA kit was used. Sapt1 (0.75 μ M) was pre-incubated with normal human serum to test its influence on all three complement pathways. Lectin pathway was inhibited by ~50 %, while the classical pathway was inhibited by ~20 %. The inhibitory effect on alternative pathway was negligible (<5%) (Fig. 5A). These results correlated with the Sapt1 proteolytic effects on MBL and CL-11 as both are initiating components of complement lectin pathway. Though Sapt1 did not cleave the C1q or C3b under the tested conditions, it is possible that Sapt1 has additional ligands in the complement cascade and that may account for some of the inhibition of classical pathway. Further, a dose-dependent effect of Sapt1 on the lectin pathway was carried out by using serial dilutions of Sapt1 protein ranging from 0 to 6 μ M. Consistent with the initial screening of complement inhibition, ~50 % inhibition of the lectin pathway was achieved at about 0.75 μ M (Fig. 5B).

3.5. Expression of Sapt1 by *C. tropicalis* clinical isolates

Lectin pathway is one of the key innate immune mechanisms against *Candida* infections. Since recombinant Sapt1 exhibited proteolytic activities against both MBL and CL-11 and inhibited lectin pathway activation, it underscored the importance of Sapt1 in *C. tropicalis* infections. Thus, we tested if Sapt1 was expressed by clinical *C. tropicalis* isolates. Three clinical isolates, one each from blood, oral cavity and peritoneal dialysis fluid, were used to test Sapt1 expression. Both mRNA and native Sapt1 protein expression were examined and compared against a reference strain. Quantitative PCR (qPCR) analysis of Sapt1 transcripts for all the four *C. tropicalis* strains were carried out after growing in two different medium conditions: YPD broth with and without BSA (1 %) as few previous studies have indicated that secreted aspartic proteases

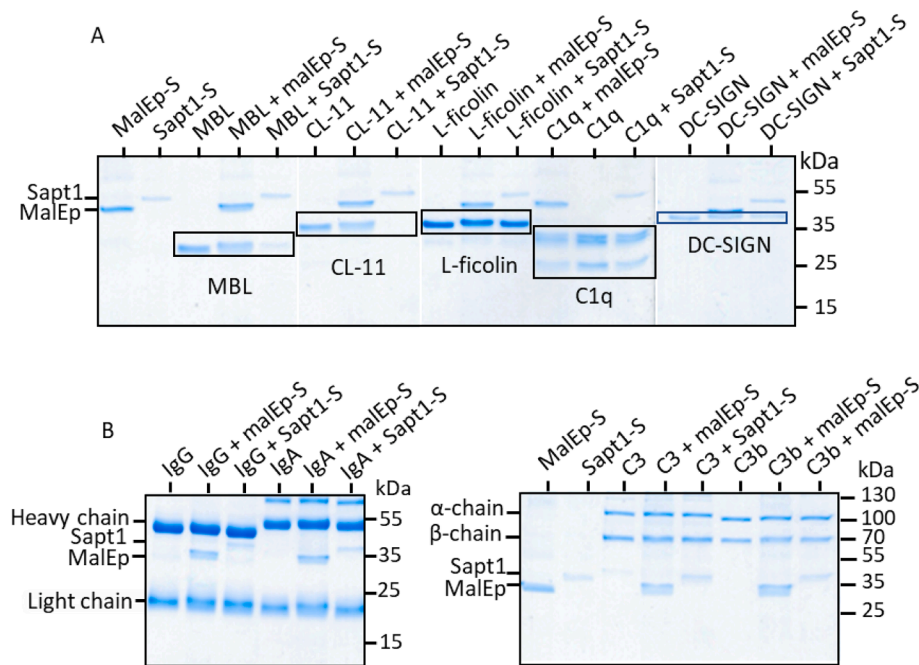


Fig. 2. Proteolytic screening of *K. lactis* crude supernatant containing Sapt1: Incubation of medium supernatant of *K. lactis* cells secreting Sapt1 with the target ligands for 3 h at 37 °C showed that MBL, CL-11 and DC-SIGN either disappeared or showed significant reduction (>50 %) in the band intensity. L-ficolin and C1q were not proteolytically degraded (A); C3, C3b, IgG and IgA were also not degraded (B).

expression is induced in media supplemented with BSA (Singh et al., 2019; White and Agabian, 1995). The Sapt1 expression data was normalised against the housekeeping gene, *ACT1* (Table 1).

When grown in YPD medium, both NCPF 8113 (oral) and NCPF 8694 (blood) isolates showed significantly higher expression levels of Sapt1 transcripts, with ~10–14-fold upregulation when compared to the reference strain. NCPF 3463 PD fluid isolate showed ~2-fold higher expression of Sapt1 transcripts. Our qPCR results from strains, grown in the presence of BSA, did not show a significant difference in the mRNA levels except that for the reference strain and PD fluid isolate. It is possible that mRNA expression reached saturation in the clinical isolates (oral and blood) with the exception of PD fluid isolate and reference strain where BSA containing medium showed ~2–3-fold higher levels. Following qPCR, the products were also visualised on 1.5 % agarose gel stained with SYBR safe. A single band was present migrating at the expected 200 bp mark for all the strains when Sapt1 primers and housekeeping gene actin primers were used. The bands were gel purified and sequence verified (data not shown).

All four *C. tropicalis* strains were grown in YPD medium overnight (18 h) and equal volume of media supernatant from each was subjected to western blot using anti-Sapt1 antibody. Native secreted Sapt1 was detected in all four strains (Fig. 6). Image J analysis indicated that NCPF 8113 (oral) and NCPF 8694 (blood) isolates secreted ~1.7–2-fold more native Sapt1 than the reference strain. No significant difference was found in the secretion level of Sapt1 from the NCPF 3463 (PD fluid) isolate when compared to the reference strain.

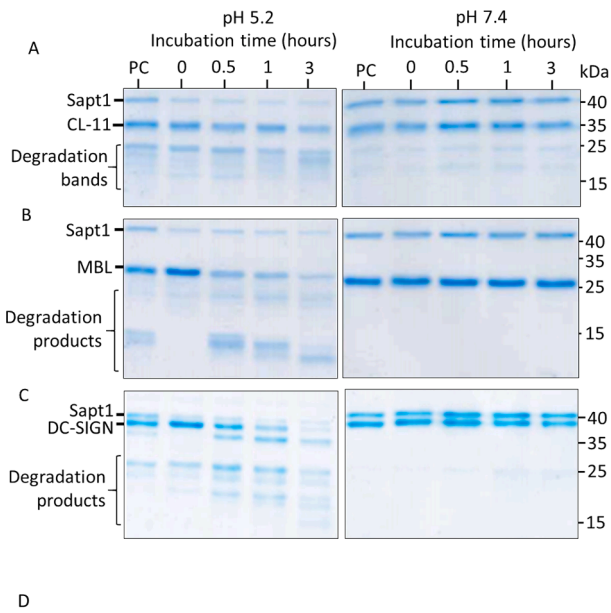
4. Discussion

Here, we show that a recombinant form of *C. tropicalis* Secreted aspartyl protease-1 (Sapt1) proteolytically degraded key innate immune molecules such as MBL, CL-11 and DC-SIGN. Sapt1 also inhibited the activation of the complement lectin pathway. Interestingly, clinical isolates of *C. tropicalis* displayed higher levels of Sapt1 expression both at mRNA level and native protein.

As MBL and CL-11 are involved in sensing and clearing *Candida*, cleavage of these proteins by *C. tropicalis* Sapt1 inhibits activation of the

complement lectin pathway. These proteins, MBL in particular, are involved in opsonisation, modulation of inflammation, and apoptotic cell clearance (Hickling et al., 2003). Sapt1 cleaves neck-CRD of CL-11. Thus, the CRD region seems the likely target of Sapt1 as other proteolytic targets, MBL and DC-SIGN, also have neck-CRD. Interestingly L-ficolin, another initiator of lectin pathway, and C1q, the initiator of classical pathway, were both resistant to Sapt1 cleavage. Collagen-like domain is present in C1q, MBL, CL-11 and ficolin. If collagen-like domains were to be the target, L-ficolin and C1q would have been susceptible to Sapt1 proteolysis. To our knowledge, no association between ficolin and *Candida* have been found yet. It is interesting that Sapt1 seems to target the components of the human innate immune system which normally are involved in the recognition and elimination of *Candida*. Soluble complement proteins are present mainly in the blood, but also found in serous exudates on mucosal surfaces (Reichhardt and Meri, 2016). This is particularly noticeable under pathological conditions when there is mechanical damage or infection such as periodontitis (Cekici et al., 2013). MBL expression is induced in the gut in response to *C. albicans* sensing and is required for intestinal homeostasis and host defence against *C. albicans* (Choteau et al., 2016). Patients with Crohn's disease (CD) have much higher levels of *C. tropicalis* compared to those without CD (Hoarau et al., 2016). This highlights the importance of fungal biome in the inflammatory bowel disease. It is possible that Sapt1 plays an important role in minimising the level of intestinal MBL, thus, favouring the overgrowth of *C. tropicalis*.

The mucosal linings of the oral cavity, gastrointestinal tract and the vagina harbour dendritic cells, which express the C-type lectin receptor, DC-SIGN. DC-SIGN recognises mannose-rich structures on *C. albicans*. It is possible that Sapt1 can affect the number of DC-SIGN receptors on the surface of dendritic cells. This in turn can negatively influence the binding to intracellular adhesion molecule-3, an endogenous DC-SIGN ligand expressed on naive T-lymphocytes which is thought to be involved in T-helper type 1 cytokine signalling (Huang et al., 2011). Thus, Sapt1 can negatively affect recognition of *Candida* cells by dendritic cells to avoid clearance via potentiation of the adaptive immune response. Escaping the host immunity would lead to overgrowth of microorganisms leading to oral candidiasis, which is often caused by a



Proteolytic activity of Sapt1 against MBL, CL-11 and DC-SIGN

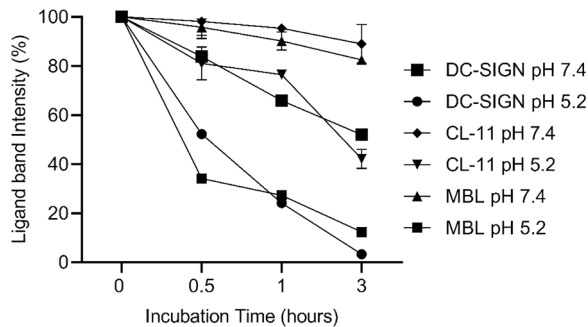


Fig. 3. Proteolytic activity of purified recombinant Sapt1 against C-type lectins. Purified Sapt1 and the ligands were incubated for up to 3 h at 37 °C in a molar ratio of 1.2: 1, either in citrate buffer pH 5.2 or Tris buffered saline pH 7.4. Sapt1 and the ligands were also co-incubated for the maximum time point of 3 h in the presence of 10 μM pepstatin (pepstatin control –PC). At various time points, aliquots of the assay mixture were analysed by SDS-PAGE under reducing conditions. (A, B & C). CL-11, MBL and DC-SIGN were susceptible to Sapt1 proteolysis, which was more prominent at pH 5.2. Multiple degradation bands ranging from ~25–15 kDa were observed below the uncleaved ligands. There was minimal proteolysis (~10–35 %) observed at 3 h in the presence of pepstatin, indicating that Sapt1 activity is not completely abolished by the protease inhibitor. Proteolytic activity was quantified from the amount of remaining uncleaved ligands over various time points by scanning gels, using Image J. The data plotted show that Sapt1 was more active at acid pH 5.2 than pH 7.4 (D).

change in the local environment by factors such as impaired salivary gland functions, immunosuppression and use of dentures that favour the growth of opportunistic *Candida* (Van Boven et al., 2013). Sapt1 ability to be highly active under acidic conditions to cleave crucial immune molecules suggests its importance as a virulence factor in niches of acidic environments, such as the gastrointestinal tract, stomach or vaginal mucosal surfaces. It is not surprising that Sapt1, being an acidic aspartyl protease, was highly active at pH 5.2 than at pH 7.4. Studies carried out on *C. albicans* suggest that under acidic conditions, the cell wall undergoes remodelling, which enhances recognition by the host innate immune system. During the remodelling of the fungal cell wall, increased exposure of β-glucan and chitin components, activates the complement system, facilitating their recognition by macrophages,

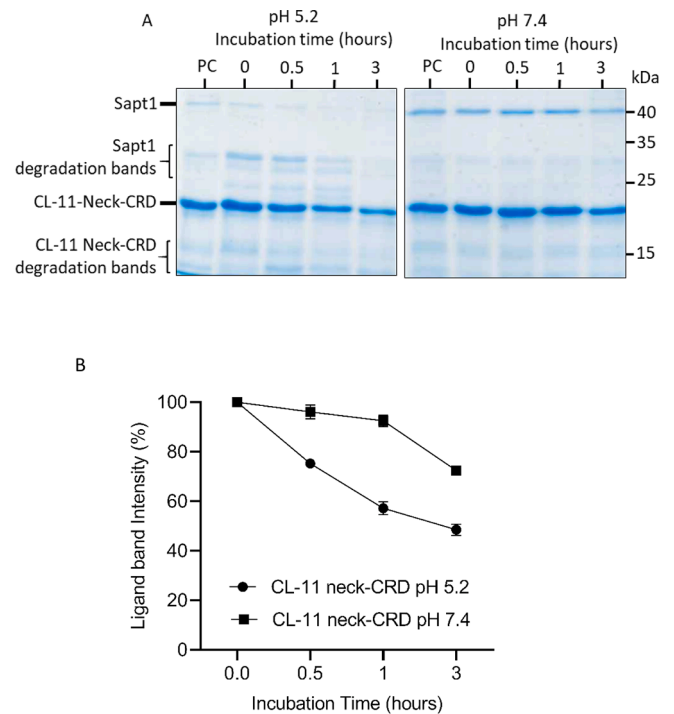


Fig. 4. Sapt1-mediated proteolysis of CL-11 neck-CRD region: Purified Sapt1 and recombinant neck-CRD of CL-11 were co-incubated for up to 3 h at 37 °C in a molar ratio of 1.2:1, either in citrate buffer pH 5.2 or Tris buffered saline pH 7.4. Sapt1 and CL-11-neck-CRD proteins were also incubated for the maximum time point of 3 h in the presence of 10 μM pepstatin (pepstatin control–PC). At various time points, aliquots of the assay mixture were separated by SDS-PAGE under reducing conditions (A). Proteolytic activity was quantified from the amount of remaining uncleaved CL-11 neck-CRD by scanning gels, using Image J. At 3 h, ~50 % of neck-CRD of CL-11 was cleaved at pH 5.2 while > 25 % cleavage was observed at pH 7.4 (B). There was minimal proteolysis (~10 %) observed at 3 h in the presence of pepstatin, indicating that Sapt1 activity is not completely abolished by the protease inhibitor.

neutrophils and stimulating the production of pro-inflammatory cytokines (Sherrington et al., 2017). The different pH conditions in the host could give *C. tropicalis* the advantage to prevent pathogen clearance by secreting proteases such as Sapt1 to evade immune mechanisms and colonise on the host mucosal surface. Interestingly, gene expression of Sapt1 was found to be higher in the oral clinical isolate than the NCPF 3111 reference strain.

The pro-peptide domain of proteases such as Sapt1 functions to maintain its latency, allow correct folding of the proteins, and ligand binding (Boon et al., 2020). This could be one reason for Sapt1 auto-catalytic properties as it loses its pro-enzyme region before being secreted as an active protease. As noted in this study, pepstatin, a common aspartyl protease inhibitor, does not completely inhibit Sapt1. The variation in protonation state in the acidic pH and lack of pro-enzyme region can lead to an active state of Sapt1 and not being able to retain its bonding to pepstatin. Further work on Sapt1 enzyme kinetics and substrate specificity would reveal the characteristics of Sapt1 activity including its optimal pH range, temperature and other environmental conditions.

To our knowledge, this is the first study that reports microbial degradation of collectin proteins, MBL and CL-11. Two major house dust mite allergens, *Dermatophagoides pteronyssinus* 1 (Der p1) and *Dermatophagoides farinae* 1 (Der f1), have been shown to proteolytically degrade pulmonary surfactant protein-A and -D (SP-A and SP-D), which also belong to the collectin family (Deb et al., 2007). SP-A and SP-D are structurally similar to MBL and CL-11 containing collagen-like domain and CRD. Interestingly, SP-D has been shown to bind to *C. albicans* and

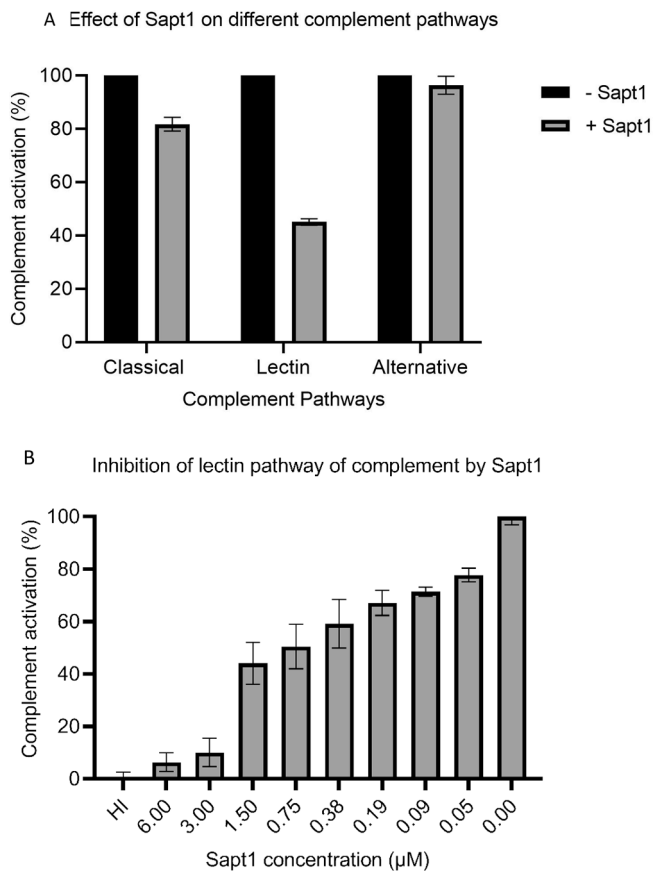


Fig. 5. Effect of Sapt1 on complement activation. (A) Normal Human Serum with or without Sapt1 was tested for activation of all three complement pathways. Alkaline phosphatase labelled anti-C5-9 antibody was used to measure the complement activation. Complement activation in the absence of Sapt1 was calculated as 100 % for reference. Sapt1 treated samples showed ~55 % of inhibition of lectin pathway and ~20 % inhibition of classical pathway. The inhibitory effect on alternative pathway was negligible (<5%). (B) Dilutions of Sapt1 (0–6 μM) were incubated with normal human serum and lectin pathway activation was monitored. Sapt1 inhibited lectin pathway in a dose-dependent manner; approximately 50 % inhibition was achieved at ~0.75 μM concentration of Sapt1. Heat Inactivated (HI) serum was used as a control.

Table 1
qPCR analysis of Sapt1 gene expression by *C. tropicalis* strains. Mean Sapt1 mRNA levels for each of the tested strain. Data obtained by quantitative real-time PCR and presented as relative values to *ACT1* (Actin) transcript level in each strain.

Culture medium	Strains			
	Reference NCPF3111	Oral isolate NCPF3463	PD fluid isolate NCPF8113	Blood isolate NCPF8694
YPD	0.32 ± 0.17	3.01 ± 0.23	0.56 ± 0.06	4.43 ± 0.11
YPD + BSA	0.96 ± 0.15	3.96 ± 0.53	1.07 ± 0.54	3.02 ± 0.03

agglutinate. Der p1 and Der f1 are cysteine proteases and cleave SP-A and SP-D at multiple sites. Der p1 has also been shown to proteolytically cleave DC-SIGN and DC-SIGN receptor (DC-SIGNR) at multiple sites. While our study shows that Sapt1 cleaves DC-SIGN, it will be interesting to test if it has any proteolytic activity on SP-A and SP-D (Furmonaviciene et al., 2007).

We found that the recombinant Sapt1 inhibited the activation of the complement lectin pathway. Though Sapt1 was found to be most active at acidic pH, there was some proteolytic activity detected at pH 7.4. The optimum condition for aspartic proteases is an acidic environment;

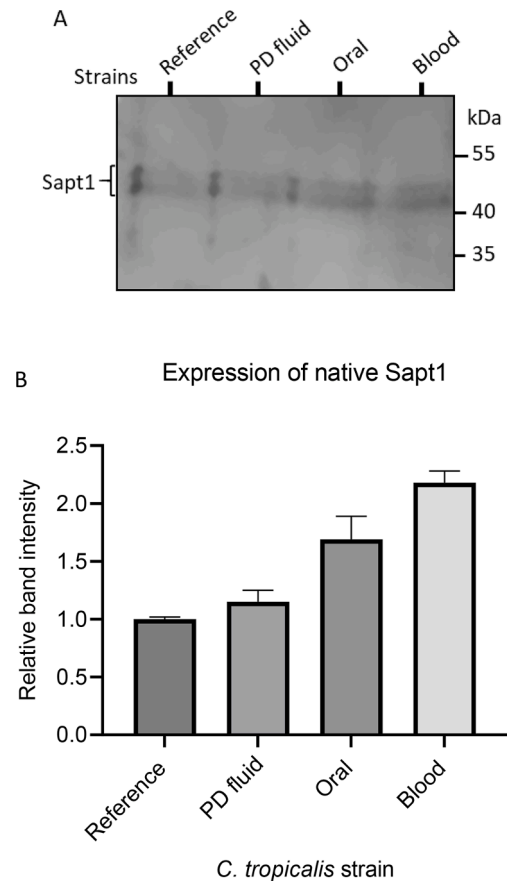


Fig. 6. Expression of native Sapt1 by *C. tropicalis* strains: Cells were grown in YPD medium at 30C for 48 h. 20 μl culture supernatant was loaded on to 12 % reducing SDS-PAGE and then subjected to western blotting using anti-Sapt1 serum. Blot was developed using Chemiluminescence system. (A) Native Sapt1 was secreted by all four strains. Sapt1 band seems to migrate as a doublet just above 40 kDa, probably representing differentially glycosylated Sapt1. (B) Band intensities were determined using Image-J. When compared to reference strains, oral and blood isolates secreted approximately 1.5- and 2-fold more of Sapt1, respectively, while PD fluid strain secreted slightly higher levels.

however, there are multiple factors involved in the proteolysis. Firstly, the enzyme-substrate fit, for example, the interaction between Sapt1 and complement proteins. Secondly, the susceptibility is determined by the cleavage site on the ligand and its availability. It is possible that MBL-MASP and CL-11-MASP complexes in the serum hold a different conformational structure rendering them vulnerable to Sapt1 proteolysis despite the pH being not highly optimal. A change in the protein conformation is very likely upon target recognition. It would be interesting to test complement inhibition in acidic conditions as the emerging evidence shows excessive complement activation can happen in the gastrointestinal system of both inflammatory bowel disease and colorectal cancer patients (Ning et al., 2015).

Among the broad spectrum of invasive *Candida* diseases, *Candida* peritonitis, an inflammation of the peritoneal lining of the abdomen, is one of the most common manifestations of infection and a frequent complication in peritoneal dialysis, which is used by patients with kidney diseases (Salzer, 2018). Interestingly, we detected Sapt1 expression in PD fluid isolate strain NCPF 3463, though the levels were only comparable to the reference strain. Evidence of *Candida* in PD fluid is becoming more common. *Candida* peritonitis can be life threatening if left untreated, as it can lead to systemic *Candida* infection, with an estimated mortality rate of up to 70 %. PD fluids creating a favourable environment for opportunistic pathogens, such as high glucose levels, hyperosmolarity and acidic pH, are considered biologically

unfavourable for the host causing damage to the peritoneal membrane. These factors allow the microorganisms to spread and cause systemic infections (Spampinato and Leonardi, 2013). Opportunistic pathogens such as *Candida* are able to take advantage and use immune evading strategies such as secretions of virulent proteins like Sapt1.

The complement system is activated by *Candida* species, and hence, interaction between complement components and *Candida* is of paramount interest in the pathogenesis caused by *Candida* species (Speth et al., 2004; Pappas et al., 2018). Candidemia is a major issue in hospitals and health care settings. *C. tropicalis*, after *C. albicans*, is the second most frequently isolated species from the blood cultures of patients with various diseases such as liver or kidney failure, neutropenia, neurological disease, diabetes, and cardiac disease in multiple countries (Mathews et al., 2001; Nucci and Colombo, 2007) Sapt1 expression levels were significantly higher (>10-fold) in the NCPF 8694 (blood) isolate from a patient with renal failure. Expression of native Sapt1 from *C. tropicalis* clinical isolate is likely to be mirrored in animal model and clinical studies.

In conclusion, Sapt1 can cleave key molecules of the innate immune system and negatively affect complement activation, especially the lectin pathway. This highlights one of the key evasion/escape mechanisms through which *C. tropicalis* could exploit the host immune system to establish infections. *Candida* secreted aspartyl proteases are strong candidates as therapeutic targets. One particular focus has been to combat recurrent episodes of vaginal candidiasis by targeting secreted aspartic protease 2 (Sap2) by *C. albicans*. Sap2 exhibits enzymatic activity on immune molecules at the mucosal site and allows adherence to host cells for fungal cell evasion (De Bernardis et al., 1995; Naglik et al., 2003). Pevion Biotech have produced a r-Sap2 virosome vaccine, which is currently in clinical trial for recurrent vulvovaginal candidiasis due to *C. albicans* (De Bernardis et al., 2014). Further studies on the interactions between the secreted aspartyl proteases and the host immune system will be important to combat both localised and invasive *Candida* infections.

Declaration of Competing Interest

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

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