PHD THESIS

CHARACTERIZATION OF SECRETED ASPARTYL PROTEASES IN *CANDIDA PARAPSILOSIS*

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LIST OF ABBREVIATIONS

ALS	Agglutinin-like sequence
AP	Alternative pathway
BSA	Bovine serum albumin
CFU	Colony forming unit
CLR	C type lectin receptor
CP	Classical pathway
CR	Complement receptor
CW	Calcofluor white
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FH	Factor H
FHL	Factor H like
FHR	Factor H related
FITC	Fluorescein isothiocyanate
HiS	Heat inactivated serum
IL	Interleukin
LDH	Lactate dehydrogenase
LiAc	Lithium acetate
LP	Lectin pathway
mt	Mitochondria
NAT	Nourseothricin N-acetyltransferase
NCAC	Non *Candida albicans Candida*

NHS	Normal human serum
NLRP	Nucleotide-binding oligomerization domain, Leucine rich repeat and Pyrin domain containing
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
PBMC-DM	Peripheral blood mono-nuclear cells derived macrophages
PS	Penicillin-Streptomycin
PTX	Pentraxin
qRT-PCR	Quantitative real time polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SAP	Secreted aspartyl protease
SDS	Sodium dodecyl sulphate
SSC	Saline-sodium citrate
TAE	Tris-acetate-EDTA
TDH3	Glyceraldehyde-3-phosphate dehydrogenase 3
TF	Transcription factor
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRIS	Tris- (hydroxymethyl) aminomethane
UV	Ultra Violet
WGA-TRITC	Wheat germ agglutinin conjugated to tetramethylrhodamine isothiocyanate-dextran
XTT	2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H tetrazolium
YCB	Yeast carbon base
YNB	Yeast nitrogen base

1. INTRODUCTION

1.1 Aspartyl proteases

Aspartyl proteases[1] are also named as aspartic-, aspartate-, or acid proteases (enzyme class: 3.4.23). They are a family of proteolytic enzymes that usually functional at acidic pH (pH 1.9–4.0). Aspartyl proteases belong to endopeptidase family, share common catalytic apparatus and cleave dipeptide bonds between two hydrophobic amino acid residues. Aspartyl proteases have a conserved sequence Asp-Gly-Thr (DTG) and/or Asp-Ser-Gly (DSG) at the active site and highly susceptible to pepstatin (pentapeptides produced by various species of *Actinomyces*)[1,2]. These proteases are present in diverse range of organisms including protozoa, viruses, bacteria, plants, and higher vertebrates such as humans[2–6]. Some of these important proteases are described in table 1. In higher organisms, aspartyl proteases play range of important roles including protein processing (renin, cathepsin D, gastric enzymes) and degradation, whereas, viral, bacterial and fungal secreted aspartyl proteases are crucial for their nutrient acquisition and virulence[7,8]. Mostly, aspartyl proteases are synthesized as single chain proenzymes or zymogens (inactive precursor of enzymes) having molecular weight (MW) approximately 40,000 Da. These proenzymes are further processed to form active enzyme (approx. MW 35,000 Da) by removing 45 residues from the N-terminal peptide residues. One of the oldest studied aspartyl proteases, pepsin has substrate-binding cleft with two catalytically competent aspartic (Asp) residues (Fig. 1). These aspartic residues are present in the center of the cleft formed by two domains and large enough to accommodate a substrate or inhibitor with seven amino acid residues[9]. Hypothesis of sharing common catalytic site by all aspartyl proteases also supported by the fact that they are universally inhibited by pepstatin (exception includes secreted aspartic protease from *Candida albicans*: Sap7)[10,11]. Catalytic mechanism of aspartyl proteases which generally involves the hydrolysis of amide bond through an active site water molecule named as “push-pull” mechanism is now generally accepted and described elsewhere in detail[3,12].

[1] In this thesis term aspartyl protease is used uniformly for aspartic and aspartate proteases.

1.1.1 Human aspartyl proteases

There are several well studied human aspartyl proteases including pepsin, gastricsin, cathepsins D and E. Pepsin is one of the predominant aspartyl protease produced by fundus (upper part of the human stomach). Pepsin is secreted in its inactive form called pepsinogen which further processed by hydrochloric acid released by stomach parietal cells to form active form of pepsin. In the acidic environment of the stomach (pH 1.5-2.5) pepsin hydrolyzes the peptide bonds of most of the proteins, breaking them down into smaller polypeptide fragments[13]. Role of pepsin in inflammation has also been investigated in some clinical studies[14,15]. One such study highlights the inflammatory role of pepsin in otitis media, a group of inflammatory diseases of the middle ear. This study clearly showed that the presence of higher concentration of pepsin A in nasopharynx correlates with higher level of inflammatory cytokines, such as tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and interleukin-8 (IL-8). Increased level of pro-inflammatory cytokines aggravates preexisting disease condition of otitis media[15]. Being a soluble lysosomal aspartyl endopeptidase, cathepsin D (CD) is synthesized as preprocathepsin D and transported to intracellular compartments such as to lysosomes, endosomes, phagosomes after removal of the signal peptide. Removal of 44 amino acids (aa) N-terminal from propeptide in acidic endosomal and lysosomal compartment results in a 48 kDa single-chain intermediate active enzyme[16]. CD plays many crucial roles in degradation and activation of polypeptide hormones and growth factors, activation and processing of

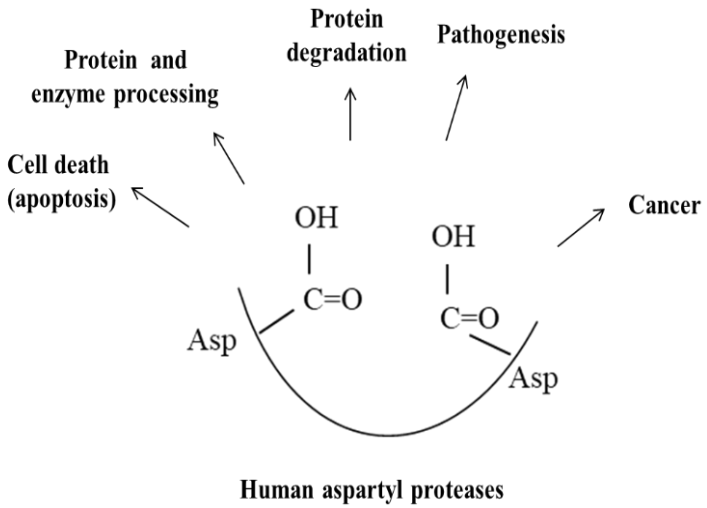


Figure 1: Roles of human aspartyl proteases. Human aspartyl proteases play various roles in cellular processes.

enzymes, antigen processing and regulation of caspase-dependent cell death (Fig.1)[16]. Role of human CD in the metastasis of breast cancer and in Alzheimer's disease is under extensive investigation[16–18]. Renin is also an important factor in body's homeostasis. Secreted mainly by the kidney and some other tissues such as brain and the adrenal gland, renin plays an important role in controlling the blood pressure and maintaining body homeostasis, bone marrow differentiation, and regulating defense mechanism against injuries[19–21]. These studies show the important roles of aspartyl proteases in human physiology and disease which signifies their importance as an alternative target to treat disease.

1.1.2 Aspartyl proteases in fungi

Several fungi from order Mucorales and some species of genus *Aspergillus* are the source of commercially important aspartyl proteases. Members of genera *Mucor, Rhizomucor, Absidia,* and *Cunninghamella* are commonly used in industry as microbial sources of renins and other aspartyl protease[22,23]. *Endothia parastica*, a member of Ascomycota is also reported to produce renin[24].

Aspergillopepsin produced by different *Aspergillus* species; mucorpepsin produced by *Rhizomucor miehei*; penicillopepsin by *Penicillium janthinellum*; rhizopuspepsin by *Rhizopus microsporus*; endothiapepsin from *Cryphonectria parasitica*; trichodermapepsin from *Trichoderma reesei*; saccharopepsin/proteinase A/Yapsins from *Saccharomyces cerevisiae,* candidapepsin (Sap1-10) from *C. albicans*; candidaparapepsin (Sapp1-3) from *C. parapsilosis*; canditropsin (Sapt1-8) from *C. tropicalis* are known[25–27].

1.1.3 Synthesis and processing of fungal aspartyl proteases

Secretory pathway of fungal aspartyl proteases is best studied in *C. albicans*. In *C. albicans* newly synthesized mRNA is transported to the cell cytosol, where it binds to the ribosome and gets translated into the pre-proenzyme and enters to rough endoplasmic reticulum (ER) through 16–18 residues of signal sequence (N-terminal hydrophobic peptide) mediated entry. In ER, N-terminal signal peptide is removed by

specific peptidase. As the journey progresses the proenzyme is transferred to the Golgi apparatus which leads it further processing by a Kexin-like proteinases (Kex2 proteinase). Kex2 recognizes Lys-Arg sequence and removes N-terminal extension. This N-terminal sequence also known as pro-region or pro-part or pro-peptide and serves as a stabilizer of inactive form of protein. It also play role in proper folding of the domains and further keeping zymogens inactive[28]. Once packed into the secretory vesicles, the enzymes are transported to the plasma membrane, where it may later be incorporated into the cell wall or released to the extracellular micro-niche of the host.

1.1.4 Inhibitors of aspartyl proteases

Role of aspartyl proteases received enormous attention because of their significance in human diseases, such as renin in hypertension, cathepsin D in cancer, and the HIV proteases in acquired immune deficiency syndrome (AIDS). Therefore, designing of inhibitor drugs against aspartyl proteases could be an effective and medically relevant therapy. Scientists have successfully designed inhibitors of aspartyl proteases by mimicking the transition state formed during amide hydrolysis of a protein substrate by an aspartyl protease[29].

Aspartyl proteases are universally inhibited by pepstatin, a peptide produced by various species of *Streptomyces*[30,31]. Pepstatin also inhibits the activities of cathepsin D, cathepsin E, renin, pseudorenin and aspartyl proteases produced by microorganisms. Recently, one study showed that *C. albicans* aspartyl protease 7 (Sap7) is insensitive to pepstatin[11]. 1,2-epoxy-3-(p-nitrophenoxy) propane

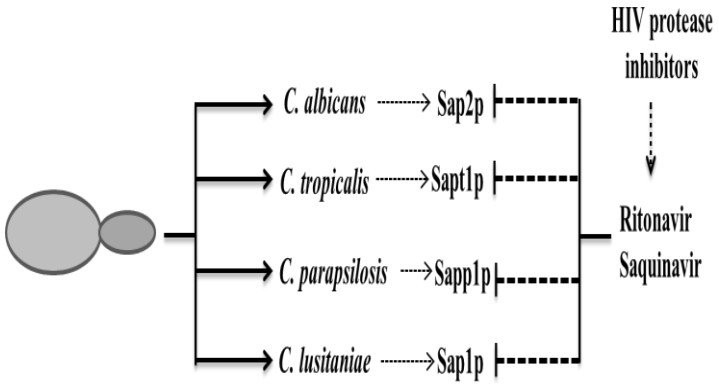


Figure 2: Inhibitory activity of HIV-1 protease against secreted aspartyl protease from pathogenic *Candida* species. HIV protease inhibitor ritonavir and saquinavir inhibit the activity of aspartyl proteases secreted by different *Candida* species.

(EPNP) and diazoacetyl-DL-norleucine methyl ester (DAN) are also known to inhibit aspartyl proteases by binding to their active site[22,32]. Low molecular weight aspartyl protease inhibitor (API) isolated from a thermotolerant *Bacillus licheniformis* show higher inhibiting affinity against pepsin whereas, they show very weak inhibitory activity against other aspartyl proteases. Several HIV-1 protease inhibitors such as saquinavir, indinavir, nelfinavir, and ritonavir are used to improve the clinical outcome of HIV patients[33,34]. Inhibitory activity of HIV-1 protease inhibitors was also tested against aspartyl proteases from *Candida* spp. It has been shown that aspartyl proteases secreted from *C. tropicalis*, *C. parapsilosis*, and *C. lusitaniae* are inhibited by ritonavir and saquinavir but not by other HIV protease inhibitors (Fig. 2)[35].

1.1.5 Regulation of aspartyl proteases

In general, pH is the principal factor behind the regulation of the gene expression of fungal aspartyl proteases**.** Proteolysis of PacC (transcription factor) at alkaline pH generates active form of PacC protein, which, negatively regulate the genes expressed under acidic conditions, e.g. genes encoding aspartyl proteases[36,37]. With this, in pathogenic fungi, such as in *Candida* spp. expression of aspartyl protease encoding genes are also associated with several environmental as well as morphological attributes, including white to opaque switching, yeast to hyphae transition, change in temperature, presence of nitrogen and carbon sources, and with the host response (Fig. 3)[38,39].

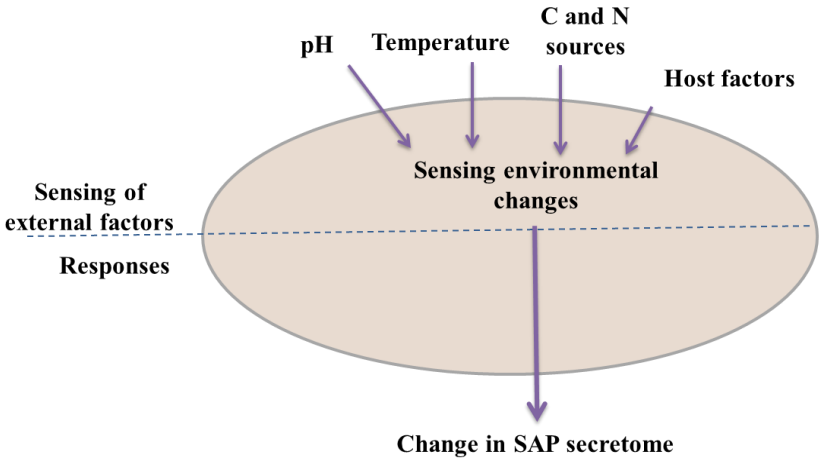


Figure 3: Number of environmental factors affecting fungal secretom. Several factors are reported to activate transcription factors which regulate the expression of genes responsible for aspartyl protease production.

1.2 Introduction of genus *Candida*

Christine Marie Berkhout proposed genus "*Candida*" in 1923 for a group of "thrush" fungi which was originally inaccurately classified as *Monilia*[40]. In 1954, the name *C. albicans* was adopted as *nomen conservandum* (i.e., conserved name). Species *C. parapsilosis* was described first time in 1928 by B. Ashford in samples originated from Puerto Rico, and classified as *Monilia parapsilosis*[41,42]. In 1932, this species has been reclassified as *Candida* by M. Langernon and R. Talice[43,44].

Unlike other fungal representative species, *Candida* belongs to 'CTG-Ser clade' (except *C. glabrata* and *C. krusei*) in which CTG codes amino acid serine instead of leucine[45]. Members of CTG clade are highly diverse in both genotype and phenotype, such as *C. guilliermondii*, *C. lusitaniae*, and *Debaryomyces hansenii* are haploid, whereas, the other members are diploid. Majority of clinically important *Candida* species are part of this 'CTG-Ser clade' (Fig. 4). Series of studies using murine model have shown that *C. albicans* is the most virulent member of this genus, followed by *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. guilliermondii*[46–49].

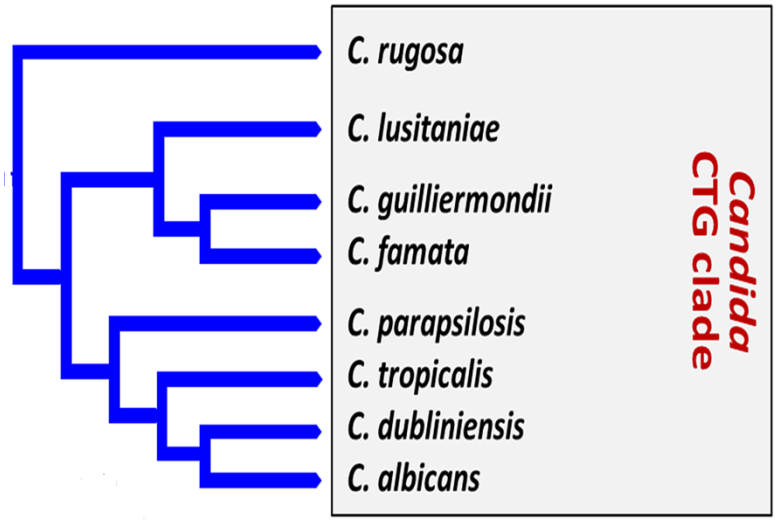


Figure 4: Phylogenetic tree representing closely related *Candida* species belonging to CTG clade[46].

1.2.1 General characteristics of *Candida parapsilosis* genome and morphology

A ubiquitous presence in nature and existence as free-living saprobes, easy transition from being a commensal to a pathogen of humans or animals makes fungus one of the prevalent cause of disease. Members of genus *Candida* reside in healthy hosts without causing any notable damage. Being a commensal pathogen, *C. albicans* easily and quickly adapts to host's environmental changes[50,51]. These ordinarily harmless commensal microorganisms may cause a variety of infections in humans, most commonly called candidiasis. Members of opportunistic pathogenic *Candida* species generally colonize on mucosal surfaces (oral, vaginal or gastrointestinal tract) and disseminate into the organs of the human host. *C. albicans* fits within all six classes of the 'damage-response' framework (DRF) of microbial pathogenesis which mainly defines microbial pathogenesis as an outcome of the interaction between a host and a microorganism[52–54]. Out of approximately 150 known *Candida* species, 95% of infections are caused by only five species: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and recently included *C. auris*[55–57]. The most important representative of pathogenic *Candida* spp. is *C. albicans* which is studied more extensively than any other *Candida* spp. Nonetheless, the clinical significance of non *Candida albicans*

Candida (NCAC) species increased over the past years and more attention is being paid to species like *C. tropicalis*, *C. parapsilosis*, *C. auris*, and *C. glabrata.*

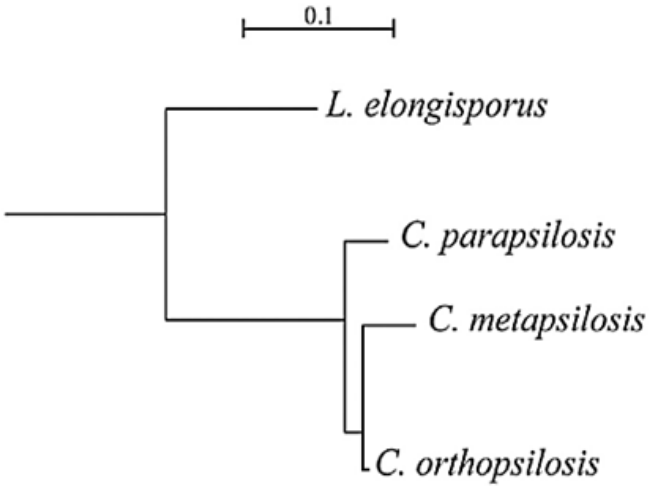


Figure 5: Phylogeny of *C. parapsilosis*. Phylogenetic tree representing relationship of *C. parapsilosis* with other closely related species[70].

C. parapsilosis belongs to a heterogeneous taxon, also referred as *C. parapsilosis sensu lato*. Previously, members of this species are divided into three distinct groups designated I to III but later they were reclassified in three separate "*psilosis*" species. *C. parapsilosis* (clause *sensu stricto*) is classified as a member of group I, *C. orthopsilosis* in group II and *C. metapsilosis* in group III[58] (Fig. 5).

In several clinical studies *C. parapsilosis sensu stricto* remained the more frequently isolated (approximately 90% of all isolates) "*psilosis*" species followed by *C. orthopsilosis* (nearly 10%) and *C. metapsilosis*[59,60]. Recently, it has been shown that *C. orthopsilosis* isolates are capable of causing damage to epithelial cells and epidermal tissues, whereas *C. metapsilosis* is less effective[61]. *C. parapsilosis*

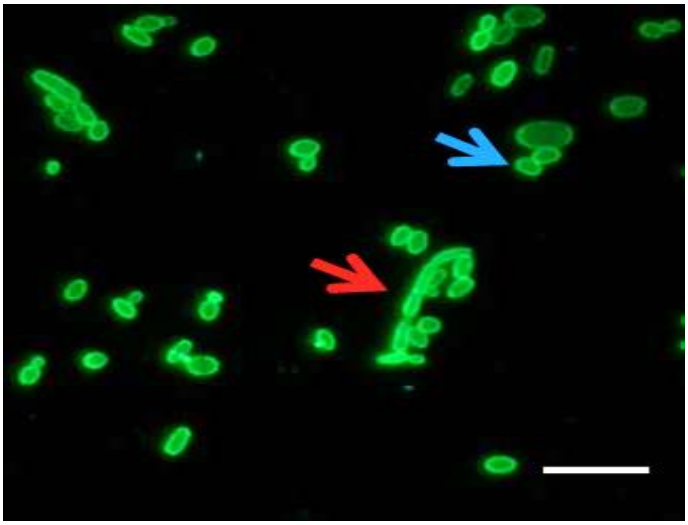


Figure 6: Cell morphology of *C. parapsilosis* (GA1). Bold red-pseudohyphae; and light blue arrows-budding cells. Scale bar 10μm.

classified as a diploid organism and forms small chain of budding cells or pseudohyphae (Fig. 6)[41]. In pseudohyphal form of *C. parapsilosis* elongated mother and daughter cells are separated by septa and form chain like structures.

C. albicans was one of the first eukaryotic pathogens to have its genome sequenced and immediately after that followed by other NCAC species. *C. parapsilosis* possess 13.1 Mb nuclear genome which is smaller than that of *C. albicans* (16 Mb)[62]. *C. parapsilosis* reported to possess 8 chromosomes and 30.9 kb long linear double-stranded mitochondrial (mt) DNA terminating with of a 738 bp of tandem repeats. mt DNA of *C. parapsilosis* is highly compact with more than 90% corresponds to coding sequences for respiratory enzymes, ribosomal RNA, transfer RNA, and other proteins important for mitochondrial functions[63,64]. *C. parapsilosis* also lacks sexual cycle which is present in some *Candida* members such as *C. guilliermondii* and *C. lusitaniae*, which is justified by the presence of mating-type locus (MTLα) as a pseduogene[63]. In contrast to *C. albicans* which forms true hyphae, *C. parapsilosis* forms pseudohyphae (Fig. 6). Hyphal/pseudohyphal form of pathogenic members of *Candida* genus is directly related to its virulence and can be induced by specific environmental and cellular signals. Hyphal/pseudohyphal forms of *C. albicans* and *C. parapsilosis* possesses ability to adhere (ALS: agglutinin-like sequence expressed on hyphal form and critical for adhesion) and penetrate more efficiently in host tissues[65–67].

C. parapsilosis forms white and creamy colonies with variable morphology. Laffey and coworker described four stable and heritable colony phenotypes including concentric, smooth, crater and most common crepe phenotype in *C. parapsilosis* CLIB214[68]. These colony phenotypes are also closely related to cell morphology as crepe and concentric phenotypes are mostly associated

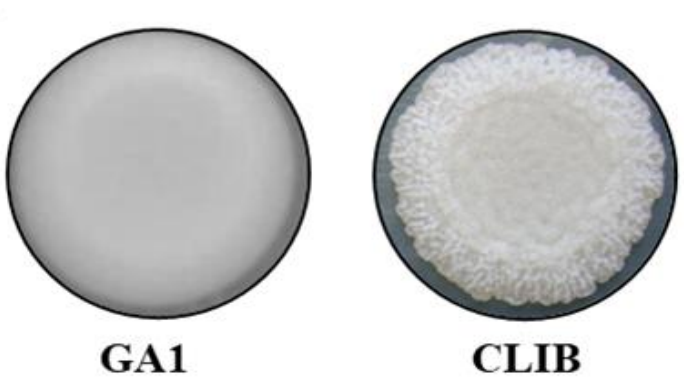


Figure 7: Colony morphology of *C. parapsilosis* in spider media.

with the pseudohyphal form. Uneven distribution of chitin in the cell wall is also reported as characteristic of these phenotypes. In concentric phenotype chitin distribution is present along the length of the cell and at the bud neck, whereas in crater phenotype chitin distribution is around the cell wall as well as in the bud neck. In comparatively smaller smooth phenotype cells chitin predominantly localized at the bud scar[68]. The colony morphology of *C. parapsilosis* is also varies based on strain and media used, such as *C. parapsilosis* strain GA1 forms comparatively smooth colonies in both YPD agar and spider media (pseudohyphae inducing media), whereas CLIB214 forms similar colonies in YPD but morphologically dissimilar in spider media (Fig. 7).

1.2.2 Biofilm formation

A complex assemblage of microbial cells on living or nonliving surfaces (indwelling medical devices) called biofilm. Formation of biofilm attributes in reduced sensitivity of microbes to antibiotics. Reports by National Institutes of Health (NIH) indicates that pathogens forming biofilms are generally responsible for higher (~80%) of microbial infections in humans[69,70]. High mortality rate caused by *C. albicans* is directly associated with its ability to form biofilms and that biofilms are highly resistant to generally used antifungal drugs. This resistance is mainly due to the role of biofilms in physical protection against the drugs and also upregulation of drug pumps in biofilm form *Candida* cells. Recently, six master regulator gene *EFG1*, *TEC1*, *BCR1*, *NDT80*, *ROB1*, and *BRG1* are identified and shown to play crucial role in biofilm development as gene deletion mutants have shown defects in biofilm formation *in vitro* and *in vivo*[71]. In general, NCAC species, such as *C. parapsilosis* forms quantitatively smaller and qualitatively less complex biofilm compared to *C. albicans*[72]. Phenotype of *C. parapsilosis* also affects biofilm formation. Cells of concentric phenotype colonies forms approximately 1.75-fold more biofilm compared to crepe or crater phenotypes whereas smooth phenotype generates 20–60% less biofilm when measured by crystal violet and dry weight method[68].

1.3 *Candida parapsilosis*: commensal to pathogen

C. parapsilosis is a ubiquitous microorganism in natural environment, such as soil, seawater, plants, insects, and domestic animals. *C. parapsilosis* can easily be isolated from mucosal surfaces, skin, and nails of human and mammalian hosts where it resides asymptomatically as a commensal host. Being a skin commensal and its unique ability to adhere on physical surfaces makes it a frequent cause of nosocomial infections in the hospitals. It is also transmitted from mothers to neonates[73]. Among non-*albicans* *Candida* species, infections caused by *C. parapsilosis* increasing worldwide and it became the second or third most common yeast isolated from hospitals of Asian and American countries whereas, the increasing incidence in European countries is also reported[74–76] (Fig. 8). In European hospitals, *C. parapsilosis* causes approximately 25% of total *Candida* infections and in South America the incidence of infections caused by this species increased from 12% to 29% in the last few years[77]**.** It is now the second-most commonly isolated *Candida* species from blood cultures in Europe, Canada, Latin America, moreover in some European hospitals it even outranks *C. albicans*, such as Spain has shown highest prevalence of this species in last few years[78]. *C. parapsilosis* is also second most causative agent of onychomycosis, whereas in some studies it outranked *C. albicans*. Pathogenicity of *C. parapsilosis* is associated with low birth weight (≤1,500 grams) neonates, hospitalized immunocompromised patients and disease such as fungemia, endocarditis, endophthalmitis, arthritis, peritonitis, all of which usually occur in association with receiving parenteral nutrition, invasive surgical intervention and use of intravascular devices[44,74,79]. Despite its clinical significance and higher pathogenicity, less is known about the mechanism of *C. parapsilosis* pathogenicity.

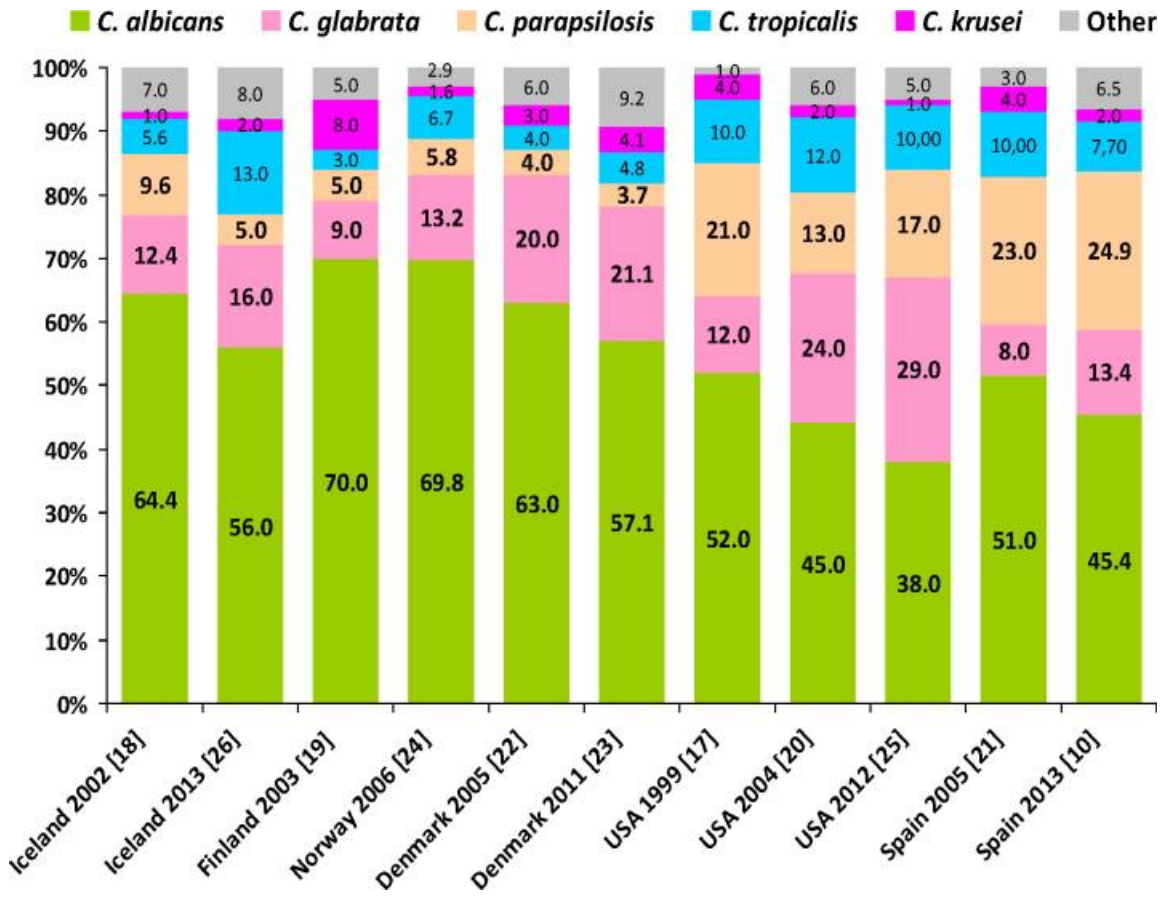


Figure 8: Temporal trend of occurrence of *C. albicans* and other NCAC species during 11-year period (2002-2013). Graph shows the proportion of different *Candida* species isolated in some Europian countries and USA. While *C. albicans* remain most frequently isolated fungi from invasive candidiasis patients, *C. parapsilosis* is the second or third most isolated species[77].

1.3.1 Adherence and invasion

Successful adhesion of *Candida* spp. is proportional to its successful colonization and pathogenicity to host cells. Hyphal cells express higher hyphal-specific cell wall proteins (CWPs) thus show stronger adherence ability with host tissues and with abiotic materials. Several *Candida* adhesion proteins, such as Eap1p (enhanced adhesions to polystyrene), Iff4p, Hwp1p, Int1p, Als3p, GlcNAc (N-acetylglucosamine)-binding protein, and fimbrial adhesin mediate *Candida* binding with epithelial cells[80]. Absence of ALS (agglutinin-like sequence) gene family in nonpathogenic species such as in *Saccharomyces cerevisiae* and presence in pathogenic species indicates their roles in

pathogenesis. *C. parapsilosis* possess 5 possible homologs of *CaALS* genes[63]. Recent study by Bertini et al. highlighted the role of *C. parapsilosis* CPAR2_404800 (an ortholog of *C. albicans ALS7* gene), in determining its phenotyping traits and adhesion to human buccal epithelial cells[65,81]. Study shows deletion of CPAR2_404800 did not alter the growth, morphology and stress tolerance ability of *C. parapsilosis* whereas it did affect its cell adhesion and virulence potential: in knock out mutants significantly reduced *C. parapsilosis* adhesion with human buccal epithelial cells and reduced virulence were detected in *in vivo* urinary candidiasis model. During *C. albicans* infection adhesion of cells is followed by invasion by induced endocytosis, where fungal hyphal associated proteins interacts with host epithelial surface proteins, triggering fungal engulfment into the cell. Detailed molecular events behind the invasion of *C. albicans* are not well known but studies have shown that proteases secreted by *C. albicans* play important role in degradation of hosts epithelial cell junction proteins, thus help *Candida* to easily evade first line of immune surveillance. Recently identified candidalysin (encoded by *ECE1*) from *C. albicans* shown crucial for damage to epithelial cells as *ECE1* null mutant shows attenuated virulence[82]. *C. parapsilosis* is not reported to produce candidalysin.

Using three-dimensional model of the human oral mucosa, Villar et al., have shown that protease secreted by *C. albicans* are responsible for E-cadherin degradation which further attributes in its pathogenicity and infection[83]. In contrast, use of pepstatin A (protease inhibitor) completely inhibits the E-cadherin degradation. *sap1–3Δ/Δ* and *sap4–6Δ/Δ* triple *SAP* deleted mutants show poor invasive capacity signifies the roles of *C. albicans* secreted aspartyl proteases in its invasion. *Candida* secreted proteases have also been shown to degrade several host defense and extracellular proteins, such as mucin (muc 2) and endothelial cell basement membrane proteins[83–85]. However, a clear role and mechanism behind invasion mediated by *C. parapsilosis* secreted aspartyl proteases are not well studied yet.

1.3.2 Virulence factors

Similar to other opportunistic pathogens, *Candida* species pathogenesis starts with host cell surface recognition and adhesion, change in morphology to facilitate further attachment and invasion in host cells, avoiding the host immune recognition by degrading several host defense proteins by secreting various hydrolytic enzymes and further adopting survival defense strategies inside the host cells[86].

1.3.3 Hydrolytic enzymes of pathogenic *Candida* species and their role in virulence

Saprophytic microorganisms secrete proteases to decompose complex nutrients in to the simple materials which can easily and readily be available to the cell. However, pathogenic microorganisms adopted this strategy to combat various host defense mechanism and to fulfill other specialized functions during infection[87]. Bacterial hydrolytic enzymes such as pesticin (*Yersinia pestis*), botulinum B, and tetanus toxins have long been proven as an important player in pathogenesis[88]. Secreted hydrolytic enzymes such as lipases and proteases has been associated with the virulence of bacteria, parasites, and fungi[89].

Role of lipases and proteases in *Candida* caused virulence is extensively studied. Secreted aspartyl proteases from different *C. albicans* are reported to play important role in its pathogenesis. Nevertheless, knowledge regarding the virulence properties of NCAC member species including *C. tropicalis*, *C. parapsilosis*, and *C. dubliniensis* secreted proteases limited till date. Role of *C. parapsilosis* associated lipases in virulence is studied in our laboratory. Recent study by Adél Tóth et al. shows that lipase mutants cells ($lip^{-/-}$) are killed more efficiently by primary human peripheral blood mononuclear cells (PBMCs) when compared with the WT strain of *C. parapsilosis*. Interestingly, this study also showed that $lip^{-/-}$ mutant more effectively elicit secretion of proinflammatory cytokines from PBMCs which further highlights their role in inflammation and *C. parapsilosis* mediated virulence[90,91]. Deletion of lipase encoding *CpLIP1* and *CpLIP2* genes also showed attenuated virulence in *in vitro* infection models[92].

Secreted aspartyl proteases (Saps) represent potential virulence factors of *Candida* spp. The role of Saps in yeast pathogenicity has been widely studied in *C. albicans*[87]. It has been anticipated in several studies that secreted proteases from NCAC spp. employ virulence determinants similar to *C. albicans*, although they might possess some specific and unique attributes which differ from *C. albicans*. The numbers of *SAP* gene present in genome of genus *Candida* are variabe among species. *C. albicans* genome contains ten genes encoding Saps, denominated as *SAP1-10*, and protein coded by these genes is also named as candidapepsin. Genome of *C. tropicalis* encompasses four genes, *SAPT1-4*, whereas, *C. parapsilosis* genome has three known *SAPP* genes designated as *SAPP1, SAPP2,* and *SAPP3*. Some of the proteins encoded by these genes, such as Sapp3 from *C. parapsilosis* and Sap7 from *C. albicans*, have not been comprehensively characterized yet, thus their roles in virulence and properties are still remains enigmatic. In general, secreted aspartyl proteases from *C. albicans* are divided into two groups based on their secretion: into the first group Sap1-Sap8 and into the second Sap9-Sap10 proteins belongs. Proteinases Sap1-Sap8 are secreted into the extracellular space (except for Sap7 whose exact localization remains unknown) whereas, Sap9 and Sap10 are GPI-anchored plasma membrane proteins and active near neutral pH. Sap1-Sap3 are optimally active at pH 3-5 and are mostly associated with mucosal infections whereas, Sap4-Sap6 are active between pH 5 and 7 and associated with systemic infections[87]. Role of Sap7 and Sap8 have not been very well studied as compare to the other *C. albicans* Saps. All Saps have characteristic of broad substrate specificity but they preferably hydrolyze bonds between hydrophobic amino acid residues.

1.3.4 Secreted aspartyl proteases of *Candida parapsilosis*

C. parapsilosis genome possesses three genes named *SAPP1*, *SAPP2*, and *SAPP3* encoding aspartyl acid protein designated as secreted aspartyl protease 1 (Sapp1), secreted aspartyl protease 2 (Sapp2) and secreted aspartyl protease 3 (Sapp3), respectively. These proteases are also collectively named as ‘candidaparapsin’. Presence of 14 *SAPP* genes are predicted in *C. parapsilosis* genome by phylogenetic studies whereas, protein level studies are available only for Sapp1p and Sapp2p[26]. Originally, *SAPP1* and *SAPP2* genes were named as ACPR (acid proteinase-related gene) and

ACPL (acid proteinase-like gene)[93]. *SAPP1* encodes a major secreted protease Sapp1p. Previous work by Horvath *et al*. revealed two identical copies of *SAPP1* gene (*SAPP1a* and *SAPP1b*) in the genome of *C. parapsilosis*[94]. In the same work, to further explore the virulence attributes of proteins encoded by these two homologous genes, *C. parapsilosis* Δ/Δ*sapp1a*-Δ/Δ*sapp1b* were generated. These mutants lacking *SAPP1* and *SAPP1b* genes for aspartyl proteases were more susceptible to human serum, have attenuated capacity to damage host-effector cells, were relatively more prone to immune cells attack, phagocytosed and killed more effectively by PBMCs and PBMC-derived macrophages (PBMC-DMs) compared to WT[94]. This study clearly highlighted the role of *SAPPs* in *C. parapsilosis* mediated virulence. In another work, it is demonstrated that the addition of protease inhibitor pepstatin A in culture of *C. parapsilosis* significantly inhibits its epidermal and epithelial damage causing efficiency compared to untreated control cells[95]. Transcriptional study showed that induced expression of *SAPP1* gene can only observe in the presence of exogenous protein as the sole nitrogen source. Even though, Sapp2 requires an alternative source of nitrogen in growth medium for sufficient production of protein for purification[96]. Recently, two homologous sequences with high similarity of *SAPP2* gene were reported in *C. parapsilosis*[97]. Crystal structures and substrate specific properties of Sapp1p and Sapp2p were studied but functional characterization of *SAPP3* needs more scientific attention and for time being it is consider as pseudogene[98,99]. Sapp1p and Sapp2p shares 53.55% sequence identity, which is the highest structural similarity in the whole group of *C. parapsilosis* secreted aspartyl proteases. Sapp1p and Sapp2p both are secreted as soluble enzymes; however, Sapp1p have also been suggested to be attached to the cell wall[97].

Recombinant aspartyl proteases protein Sapp1p and Sapp2p show nearly identical molecular mass of 37 kDa. An optimal pH range for candidaparapepsin proteolytic activity is pH 3-5 which is nearly similar to most of the Saps. However, some candidapepsin (Sap4/6/7/9/10) show optimal activity at less acidic pH compared to candidaparapepsin and remains active in neutral pH as well. *C. parapsilosis* Sapp1p exhibits broad range of substrate specificity in compare to Sapp2p. Broad range of substrate specificity (including immunoglobulin A: IgA; albumin, cell junction proteins,

complement cascade proteins, blood coagulation proteins) of secreted *C. albicans* aspartyl proteases have been reported, however, till now little is known about secreted aspartyl proteases from NCAC species. *C. parapsilosis* Sapp1p can also cleave IgA, which is resistant to several bacterial proteases. *C. albicans* Saps have been shown to activate blood coagulating proteins such as factor XII, factor X and prothrombin, however, *C. parapsilosis* Sapp1p also shown to activate factor X and prothrombin *in vitro*[100]. Sapp1p hydrolyzes bonds with Leu at the P1 position unlike Sapp2p, which cleaves peptide bonds formed by polar residues. Study using reconstituted human oral epithelium (RHOE) showed that *C. parapsilosis* causes significant tissue damage however, damage is reduced in the presence of pepstatin (Sap inhibitor) strongly indicating the involvement of Saps in tissue damage[101].

1.4 Immune recognition and response against *Candida* species

The cell wall of the pathogens is the first point of contact with the host epithelial cells, which is the first-line defense, and immune cells. Recognition of PAMPs (pathogen associated molecular patterns) by cell surface receptors associated with phagocytes (granulocytes, monocytes/macrophages, dendritic cells) as well as non-immune cells including epithelial cells elicit activation of intracellular signaling pathways, thereby, stimulating production of inflammatory mediators including chemokines and cytokines.

Cell wall of *C. albicans* made up of outer and inner layers harboring several PAMP's which are effectively recognized by host PRR's (pattern recognition receptors) present on immune and epithelial cells. Highly glycosylated (with N- and O-linked glycans) mannoproteins form an outer layer of cell wall of the *C. albicans*, while inner cell wall layer composed of chitin and β-1,6- and β-1,3-glucans. By previous studies it is established that N- and O-linked glycans are recognized by the mannose receptor (MR) and Toll-like receptor 4 (TLR4), respectively, while the phospholipomannan is recognized by TLR2, whereas CLR receptor galectin-3 recognizes pathogen associated β-1,2-mannose residues[102]. β-1,3-glucan, the most abundant sugar polymer in the inner layer of the cell wall is recognized by dectin-1 and TLR2. Although, *C. albicans* associated mannan illicit pro-inflammatory responses but recognition of β-1,3-glucan by

PRRs induce the strongest fungal recognition signal[103]. Variability in the composition of mannan and mannoprotein in yeast and hyphal cell also lead to significant differences in secreted cytokines by macrophages and epithelial cells[104]. Yeast form of *C. albicans* shown to induce higher level of interferon-γ (IFNγ) release from human PBMCs compared to hyphal form in TLR-4 dependent mechanism. In contrast, hyphal cells induce higher IL-10 production by a mechanism involving TLR2[104]. Beside TLRs and CLRs, hyphae have also been shown to induce NLRP3 inflammasome activation thus promote higher IL-1β secretion[105]. Study have shown that members of *Candida* genus interact differentially with host epithelial cells thus, clearly implicate that different species may use different mechanisms to avoid recognition[61]. Recently identified novel epithelial receptor, EphA2 (ephrin type-A receptor 2), binds to β–glucan of fungal cell wall and thereby respond with antifungal signals[106]. Mucosal surface IL-17 mediated immunity plays important role against fungal infections. Upon *C. albicans* challenge to skin, intestinal cells or oropharynx infection host innate lymphoid cells (ILCs), Th17, and γδ T cells produce IL-17 which intern induces production of antimicrobial peptides such as β-defensin 3 (DEFB3) from epithelial cells. β-defensin 3 have been shown to play a critical role against *C. albicans* infection as *Defb3*$^{-/-}$ (defensin beta 3) mice are reported to be susceptible to oropharyngeal candidiasis. *C. albicans* associated β-glucan also induces cytokine responses from murine Ly6C^{hi} monocytes and human CD14^{+} monocytes and induces epigenetic and metabolic changes[107–109]. Adaptive immune response against *Candida* species rely on antigen presenting cells (APC) mainly dendritic cells (DCs)[110]. Upon phagocytosis of *C. albicans* yeast cells DCs produce interleukin-12 (IL-12), which drives the polarization to the Th1 subset. In addition, *C. albicans* cells penetrated in the epidermis are sensed by dermal CD11b^{-} CD103^{+} DCs, which intern drives differentiation of Th1 cells. Fungal mediated induction of IL-1β production also drives differentiation of naive CD4^{+} T-cells into Th17 phenotype which is further extended by IL-6.

1.5 Overview of complement system and its activation

The complement system is made up of a more than fifty distinct circulating or membrane bound plasma proteins. These proteins opsonize pathogens to facilitate

phagocytosis by host immune cells, induce a series of inflammatory responses that help to fight infection or kill pathogen[111]. Initiation of complement cascade leads to formation of pore also known as membrane attack complex (MAC) assembly on the cell surface of pathogens resulting in the lysis of target cell by freely diffusing metabolites and small proteins.

Basically, there are three traditional ways in which the complement cascade gets activated and protects host against infection. First, classical pathway (CP) is activated by binding of the collagen-like tail of C1q with fragment crystallizable (Fc) portion of IgM or IgG of antibodies. Binding of C1q to antigen bond antibodies alters its conformation which leads the activation of serine proteases, C1r and C1s. Activated C1s then cleaves C4 and C2 into small inactive fragments (C4a and C2b) and larger active fragments (C4b and C2a). C4b binds to the sugar moieties of cell surface glycoproteins and binds non-covalently to C2a, to generate the classic pathway C3 convertase C4bC2a. C4bC2a is an enzymatic complex which converts the abundant plasma protein and central component of complement cascade into the anaphylatoxin C3a and opsonin C3b. Accumulation of C3b in close proximity to the C4b2a complex leads to the formation of the C5 convertase, $C4b2a(C3b)_n$, which initiates the terminal pathway of complement activation[112]. Terminal complement components (MAC, C5b-9) damage target cell by creating pores in the membrane (Fig. 9).

The second pathway called lectin pathway (LP), is initiated by the binding of collectin family of plasma protein recognition molecules, namely mannan-binding lectins (MBL), collectin (CL) and ficolins to PAMP such β-glucan of fungi and lipopolysaccharides, lipoteichoic acid of bacteria. Binding of MBL or ficolins to sugar molecules on the pathogenic surface leads to activation of MBL-associated serine proteases (MASP-1, MASP-2, and MASP-3). MASP2, associated with MBL or ficolin, activates both C4 and C2 and generates C4bC2a (C3 convertase) in a reaction analogous to the classical pathway.

Thirdly, alternative pathway is constitutively active at low levels in the normal host and referred as “tick-over” mechanism, in which, spontaneous low-level hydrolysis thioester

bond with rate (~1–2%/h) of complement C3 by water leads to the formation of C3(H2O) that functions quite similar to C3b with regard to its ability to bind factor B (CFB). Binding of CFB acts as a substrate for the serine protease factor D (CFD). Cleavage of CFB by CFD results in the formation of the AP C3 convertase [C3(H2O)Bb] which act similar to the classic C3 convertase C4bC2a and cleave C3 into C3a and C3b. Generation of C3 convertase leads to the formation of C5 convertase (C3bBbP) in appropriate circumstances which further progresses to MAC formation on a foreign cell surface, similar to that of the CP. The binding of P to C3bBb on a microbial (or protected) surface stabilizes and protect the convertase from inactivation by regulatory proteins, thereby enhancing the convertase activity. In all pathways upon activation C3 convertase cleaves α-chain of C3 to generate exposed and highly reactive internal thiolester group.

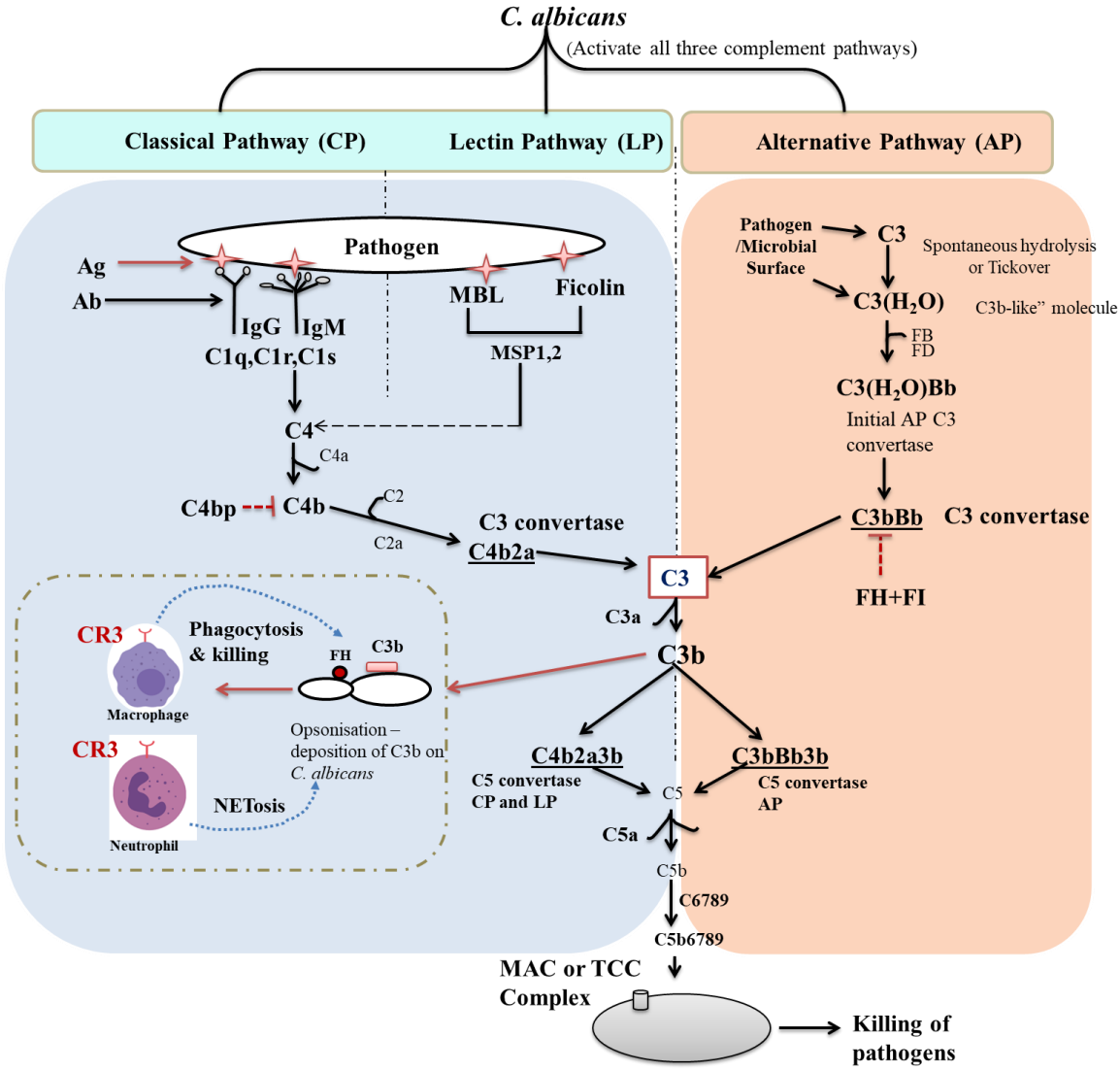


Figure 9: General overview of complement cascade. Complement cascades start with three distinct pathways classical pathway, lectin, and alternative ultimately leading to death of pathogens by the formation of membrane attack complex (MAC) on their cell wall[113]. Intermediate proteins generated by complement cascade can also bind with the cell surface of pathogens which leads to activation of immune cells by binding with complement receptors.

Exposed thiolester group binds covalently to biological surfaces exposing hydroxyl or amino groups and leads to its deposition on the cell surface[114]. Deposition of C3b on the pathogen surface further leads to the opsonization for phagocytosis by polymorphonuclear cells and macrophages. Complement receptor 3 (CR3) and CR4 present on macrophages and neutrophils also facilitate the ingestion and degradation of

pathogens[115]. Mannan specific IgG present in serum also play role and facilitate the binding of C3b to the *Candida* cell wall. To escape the complement attack pathogens adopted several strategies including recruiting the complement regulatory factors (RCA) or cleaving the main complement protein by secretory proteases.

1.5.1 Complement system and regulation

Activated complement components C4b, C3b, and C5b67 can attach to any nearby cell surfaces including host cells and therefore activate complement cascade. To inhibit the unwanted activation of the complement cascade, regulatory mechanisms have evolved. Complement cascade regulation is mainly mediated by several inhibitors of complement pathway called 'regulators of complement activation' (RCA). Two of the main complement inhibitors Factor H (FH) and Factor I (FI) had great attention due to their ability to inhibit alternative complement pathway ("bystander" effect whereby C3b generated in the vicinity becomes attached to a host cell). FH binds through sialic acid, heparin, and sulfated glycosaminoglycans (GAGs) of the host surface thus distinguish between self and non-self cells and prevent complement activation on host surfaces. There are six other proteins related to FH: the product of complement factor H (CFH) via alternative splicing, complement factor H-like protein (FHL-1), and CFH-related proteins (CFHRs : FHR1 to FHR5)[115].

C4-binding protein (C4BP) is a fluid-phase regulator of the CP and LP. C4BP is both a decay-accelerating factor dissociating C2a from the CP C3 convertase and a cofactor, promoting FI-mediated cleavage of C4b into iC4b and further to C4c and C4d. Some other RCA such as CD46, CD55/DAF, CR1 or CD35 and CR2 or CD21 are also important players in complement activation regulation.

1.5.2 The FHL/FHR protein family

The FH like protein (FHL-1) is derived from CFH gene via alternative splicing whereas FHR-1, FHR-2, FHR-3, FHR-4, and FHR-5 proteins encoded by the CFHR1–5 genes present downstream of the CFH gene. Similar to FH, FHR proteins are also composed of SCR/CCP domains (Fig. 10)[116]. Biological functions of FHL-1 and FHR proteins are

under investigation however, scientific evidence till date suggests that they compete with FH to bind C3b and/or glycosaminoglycans (GAGs). Recent evidence also suggests that they are positive regulators of the alternative pathway of complement system. The exact roles of these proteins against pathogens and in several diseases still remain questionable[117–120].

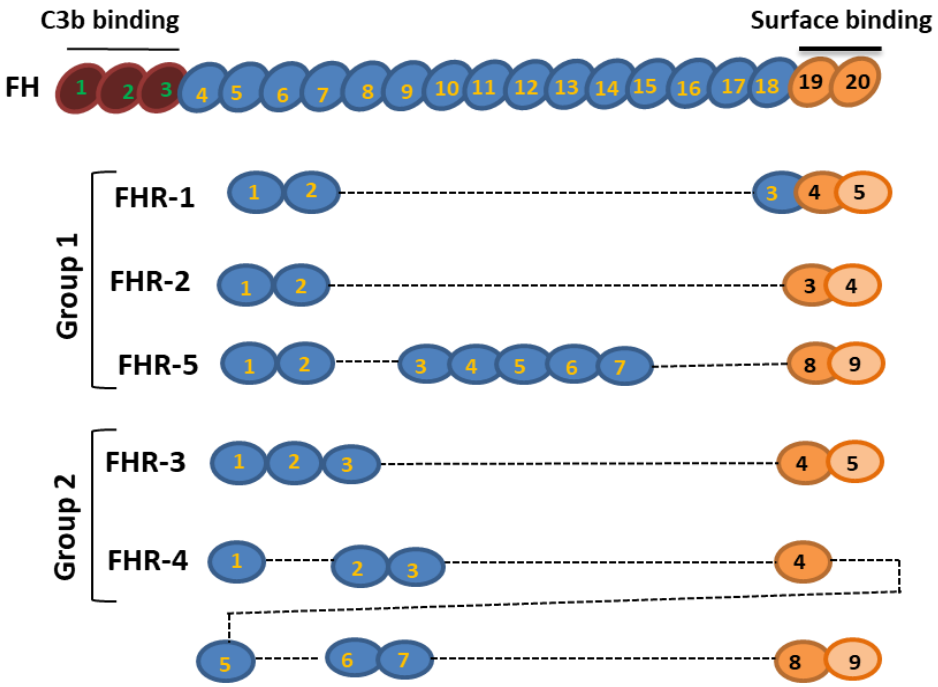


Figure 10: Graphical representation of FH and FH family proteins. FH composed of 20 SCR regions, first three SCR (1, 2 and 3) bind C3b and therefore crucial for complement regulation whereas SCR 19 and 20 bind with pathogens and surface proteins. Complement regulatory regions are not conserved in FH family proteins. The N terminal SCR of FH family proteins share homologies with SCR 6-9, SCR 10-14 and SCR 18-20 of FH. Based on formation of homodimers and heterodimers these proteins are divided in two groups. Group 1 forms the homodimers with each other through N terminal SCR domains whereas group 2 proteins lack this feature. Colors of SCR domains reflect the similarities with SCR domain with FH.

1.5.3 Complement receptors

Immune cells harbor membrane-associated receptors interacting specifically with the complement proteins, named as complement receptors. Binding of complement proteins to these receptors kick-start the signaling and thus modulate the behavior of

complement receptor bearing cells. Based on their binding specificity to the complement proteins, these receptors can be classified in three broad categories. (i) receptors binding to the anaphylatoxins C3a, C5a, and C5a desArg (C3aR, C5aR), (ii) receptors binding to C3 fragment, C3b and its degradation products, iC3b and C3dg (CR1, CR2, CR3, CR4) and (iii) receptors for C1q and related collagenous lectins (cC1qR, C1qRp, gC1qR)[121]. These receptors play important roles in complement-mediated phagocytosis, NO synthesis, leucocyte chemo-attraction, degranulation, and B-cell proliferation[122]. Innate immune cells such as macrophages, neutrophils, and dendritic cells recognize pathogens either by pattern recognition receptors or indirectly by opsonic receptors. Among all complement receptors, CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are the major opsonic receptors. CR3 and CR4 belong to β2 integrin family and possess high affinity towards C3b cleavage product iC3b[123,124]. CR3 can also directly bind to the β-glucan and pH-regulated antigen 1 of *C. albicans*. Interestingly, recently it has been shown that binding CR3/CR4 with the FH coated pathogens presumably facilitate the phagocytosis of pathogens and antifungal response of macrophages. In contrast, *C. albicans* secreted Sap2 can down-regulate the CR3 and CR4 expression on the cell surface of macrophages, therefore, alter the complement-mediated phagocytosis by macrophage[125].

1.6 Interaction of complement cascade proteins with *Candida* species

Pathogenic members of *Candida* species, especially *C. albicans* is strong inducer of all three complement activation pathways resulting in rapid assembly of C3 convertase. Formation of C3 convertase leads to the generation of chemotactic cleavage fragments such as anaphylatoxin C3a, C5a opsonizing fragments C3b which facilitates phagocytosis. Components of complement activation cascade C3a and C5a[126], are crucial against fungal infections, as mice lacking the C3a and C5a are reported to highly susceptible to invasive *C. albicans* infection. Moreover, during fungal infections C5 deficiency is also associated with increased production of pro-inflammatory cytokines, including TNFα and IL-6, and rapid fungal reproduction in many organs. Importance of C5a during *C. albicans* infection is appreciated as C5a is critical for activation of human

monocytes to produce pro-inflammatory cytokines, e.g. IL-6 and IL-1β[127]**.** Aside from C5a, C3a also exerts antimicrobial effects against *C. albicans* presumably by binding to its cell surface and inducing membrane perturbations to release extracellular material. *C. albicans* isolates can bind with complement regulatory proteins such as complement factor H and other members. CFHR-1, CFHL-1, CHR-4 can enhance CR3 mediated antimicrobial activity such as the release of antimicrobial protease lactoferrin from neutrophils and enhance generation of reactive oxygen species by binding to the cell surface of *C. albicans*[128].

1.7 Regulation of host complement cascade by *Candida* species

1.7.1 Binding to complement regulators

C. albicans and NCAC species, such as *C. parapsilosis, C. tropicalis,* and *C. lusitanae* reported to possess several cell wall associated proteins having binding affinity towards host complement factors. Affinity of IgG towards mannan present on the cell surface of fungi and deposition of C3 on the cell surface of pathogens activate classical, alternative and MBL pathways and also facilitate phagocytosis mediated killing of pathogens by host immune cells. Available literature suggests that *C. albicans* mainly uses two mechanisms to evade the complement attack: first by recruiting complement regulators on cell surface and second by secreting proteases to degrade complement proteins[125,129]. Presence of Factor H binding surface protein phosphoglycerate mutase (Gpm1), high-affinity glucose transporter 1 (Hgtp1), glycerol-3-phosphate dehydrogenase 2 (Gpd2), and C3, FH, C4bp, FHL-1 binding pH-regulated antigen 1 (Pra1) of *C. albicans* allow this fungus to efficiently regulate complement system events[130–132]. In addition, *C. albicans* expresses a protein called αvβ3 integrin-like protein which is similar to human αvβ3 integrin. This integrin-like protein acquires a terminal complement pathway inhibitor, vitronectin to the yeast cell surface. Acquired vitronectin inhibits terminal complement complex (TCC) formation and thus presumably help *Candida* to avoid complement attack[133].

In the case of *C. parapsilosis* little is known about complement binding cell surface proteins. With *in silico* experiments we found *C. albicans* orthologous of complement

binding proteins in *C. parapsilosis* genome (Table 1). Function of these proteins in context with binding affinity with host complement proteins is not studied yet. Indeed, higher protein identity can be correlated with function but research need to be done to define exact roles of these proteins.

Table 1: *C. albicans* cell surface proteins important for binding with host complement proteins and percent similarity with their orthologus in *C. parapsilosis*.

C. albicans	Bind with host complement proteins	Orthologous in *C. parapsilosis*	Known functions in *C. albicans*	Amino acid similarity (%)
Gmp1/ CRASP1	FH, FHL-1, and plasminogen	CPAR2_211810	gluconeogenesis, glycolytic process, interaction with host	85%
Pra1/ CRASP2	C3, C4BP, factor H, FHL-1, and plasminogen	No orthologous match found	---------	----------
Hgt1P	FH	CPAR2_108370	glucose transmembrane transporter activity	81%
Gpd2	FH	CPAR2_601770	[NAD+] activity and cell surface localization	80%

1.7.2 Regulation of crucial complement factors by aspartyl proteases

In addition to recruitment of complement regulators on cell surface, *C. albicans* cleave complement proteins by secreting aspartyl proteases. Activation of all three complement pathway leads to the formation of C3 convertase named C4b2b in classical/lectin pathway and C3bBb in alternative pathway. A recent study has shown the high level of Saps specially Sap1, Sap2, and Sap3 blocks classical as well alternative complement pathways by cleaving complement component C3b, C4b, C5, and also inhibit terminal complement complex, whereas other Saps such as Sap9 lacks complement cleavage activity, but cleaves antimicrobial peptide such as histatin 5 in *C. albicans*[129]. C5 plays role in all three complement pathways and cleaved by C5 convertase enzyme to form C5a and C5b. Studies have shown that C5 deficient mice are more susceptible to

Candida infections[106]. C5b is larger fragment further forms complex with C5b-9 to form MAC, which attached to the cell wall of pathogens (especially to gram negative bacteria) to form pores on the cell wall. C5a, the smaller fragment, is a vasodilator, chemotactic, and anaphylatoxin that mediates inflammatory responses at the site of injury by stimulating neutrophils, eosinophils, phagocytes, and endothelial cells[134]. Studies have shown that Saps in the culture supernatant of *C. albicans* as well as recombinant Saps (Sap1p, Sap2p, and Sap3p) degrade host complement component C5 and thus inhibit MAC formation[129]. *C. albicans* secreted Sap2p also cleaves complement inhibitor FH which enhances the antifungal activity of human neutrophils via binding to complement receptor type 3 (CR3)[125].

Even though, researchers have clearly illustrated indispensable role of *C. albicans* secreted aspartyl proteases in evasion of host complement attack. To our knowledge not even a single study has been done till date to find out the role of aspartyl proteases from NCAC species in complement evasion[135].

1.8 Sap mediated modulation of macrophage and neutrophil responses

Macrophages and neutrophils are the main phagocytic cell types of the vertebrate innate immune system[136,137]. Ubiquitous presence of macrophages in the tissues makes them one of the most important warriors against invading pathogens. Macrophages control the infection by inflammatory responses and phagocytosis[106]. Role of macrophages in fungal clearance is described in a recently published article[125], but only few studies deal with the mechanisms behind modulation of macrophage behavior by *C. albicans* secreted aspartyl proateases. Sap1p, Sap2p, Sap3p, and Sap6p from *C. albicans* reported to induce secretion of pro-inflammatory cytokines, such as IL-1β, TNFα, and IL-6, by human monocytes. Additionally, Saps are also able to modulate physiology of monocytes by inducing higher Ca^{2+} influx[138].

Neutrophils kill pathogens either by oxidative stress (reactive oxygen species generated by NADPH oxidase) or by the release of NET (neutrophil extracellular trap)[139]. NETs are consisting of extracellular nucleic acids, histones, and granular proteins, such as calprotectin and pentraxin-3 (PTX3), and crucial for fungal cell killing which are too

large for phago-lysosome mediated killing. Recently, it has been shown that *C. albicans* strains lacking Sap1-Sap3, Sap4-Sap6, Sap9-Sap10, induces significant less NET response compared to WT strain indicating the roles of Saps in regulation of NET formation from neutrophils[138]. Saps (especially Sap2) have reported to exhibit chemoattractant activity towards neutrophils[140]. Saps also stimulate epithelial cells to produce chemokine IL-8 which is a strong chemoattractant for neutrophils. Sap2p and Sap6p are reported to involved in internalization via a clathrin dependent mechanism and therefore possibly stimulate inflammatory process[141]. Despite several studies about the role of *C. albicans* secretory aspartyl proteases in its virulence, not much known about immune-modulatory roles of candidaparapepsins.

1.9 Vaginal Candidiasis

Vaginitis is an acute inflammatory disease, affecting three out of four women worldwide at least once during their fertility life and more than 5% (approximately 150 million) experience subsequent recurrence[142,143]. Although a number of predisposing factors such as oral contraceptive usage, changes in estrogen and progesterone levels during pregnancy, uncontrolled diabetes mellitus, and long-term broad-spectrum antibiotic treatment as well as changes in the composition of the vaginal microbiota have been identified to increase the risk of vaginitis; still mechanism of pathogenesis behind *Candida* caused vaginitis (vaginal candidiasis) is not well studied[144,145].

Similar to the skin and oral cavity, vagina harbours various microbes. These microbes help to maintain the adequate pH, hinder the growth of pathogens, stimulate the local inflammation and decrease pregnancy complications[146,147]. A slight change in the composition of resident microbial community and host defense encourages the emergence of opportunistic infections caused by *C. albicans* which is highly abundant in vaginal mycobiome during vaginitis[148]. Studies have been done to investigate on the mechanisms of pathogenesis of *C. albicans* caused vaginitis by elucidating the roles of fungal virulence factors, such as change in morphogenesis, secreted factors, and biofilm formation as well as host immune responses against *Candida* during infection[149,150].

Secreted aspartyl proteases of *Candida* species have long been reported as a virulence-associated trait of these pathogenic fungi and higher secretion of these proteases are reported in vaginitis[151,152]. Various studies have shown that members of *C. albicans* Sap family have variable abilities to induce pro-inflammatory cytokine secretion mediated by Akt/NF-κB activation[153,154]. A recent study shows that *C. albicans* Saps, particularly Sap2, Sap6, and chemo-attractive chemokines, such as IL-8 and MIP-2 released from Sap treated vaginal epithelium, have ability to mediate neutrophil chemotaxis[150]. Even though significant studies have shown higher production of Saps in vaginal infection and their role in vaginal inflammation, no clear scientific evidence has been shown for the mechanisms involved. Additionally the role of proteases secreted from NCAC species in vaginal candidiasis is also not well studied.

2. Objective of the present work

Aims

The specific aims of the present work were to generate the *SAPP* reintegrant mutant strains (in *sapp1/2/3*$^{-/-}$ background) in order to understand their role in *C. parapsilosis* caused infection, additionally, to understand the regulation of hosts complement cascade by *C. parapsilosis* secreted aspartyl proteases Sapp1p and Sapp2p.

Experimental strategies

- Generation of *SAPP* reintegrant mutant strains.
- Delineation of the role of *C. parapsilosis* secreted aspartyl proteases Sapp1, Sapp2 and Sapp3 in *C. parapsilosis* biofilm formation, cell wall stability, and epithelial adhesion and damage.
- Determination of the role of individual aspartyl proteases of *C. parapsilosis* in modulation of macrophage inflammatory responses.
- An in depth study to understand the role of *C. parapsilosis* secreted aspartyl proteases in the evasion of host complement attack.

3. Material and methods

3.1 Media used in this study

Liquid and solid media were prepared according to the following protocols:

LB: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl in ddH_2O. For selection of bacterial colonies antibiotics were added to the media after autoclaving and cooling: ampicillin (100 µg/ml), kanamycin (50 µg/ml) or chloramphenicol (20 µg/ml) depending on the selection marker gene harbors by the plasmid.

YPD: 0.5% (w/v) yeast extract, 1% (w/v) peptone, 1% (w/v) glucose in ddH_2O. Nourseothricin (NTC) in 100 µg/ml concentration was added for selection of NTC resistant colonies.

YNB: 0.67% (w/v) yeast nitrogen base (without amino acids, with $(NH_4)_2SO4$), 2% (w/v) glucose in ddH_2O.

YCB: 2.34% yeast carbon base (YCB), 0.2% BSA (pH 4.0) in ddH_2O.

For the preparation of plates, the described LB and YCB media broths were supplemented with 1.5% (w/v) agar and yeast media with 2% (w/v) agar.

Spider medium: 1% peptone, 1% yeast extract, 1% mannitol, 0.5% NaCl and 0.2% K_2HPO_4.

3.2 *C. parapsilosis* and *E. coli* strains used in the study and cultivation conditions

List of *C. parapsilosis* strains used in this study are described in supplementary table 1. Yeast strains used in the present study were maintained on YPD plates supplemented with 1% 100 unit/ml penicillin-streptomycin solution (Sigma-Aldrich) at 4 °C. One day prior to the experiments *C. parapsilosis* wild-type GA1 strain[155], reintegrant mutant strains of aspartyl protease coding genes (*RI_SAPP1, RI_SAPP2,* and *RI_SAPP3*) and *sapp* null mutant (*sapp1/2/3*$^{-/-}$) were grown in YPD or in yeast carbon base (YCB) medium supplemented with 0.2% BSA at 30 °C on an orbital shaker at 180 rpm for 18-20 h. Cells were harvested by centrifugation at 900 g for 5 min, washed twice with sterile 1x PBS (phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), further diluted in 1x PBS and counted in a Bürker chamber chamber and diluted to the appropriate concentration prior to the experiments. Unless

otherwise stated, all experiments were performed using living *C. parapsilosis* cells and without serum treatment.

E. coli 2T1 cells were stored on LB agar plates in 4 °C and routinely cultured at 37 °C in LB liquid medium before competent cell preparation.

3.3 Generation of *sapp1/2/3*$^{-/-}$ and reintegrant mutant strains

SAPP mutant strains *C. parapsilosis sapp1/2/3*$^{-/-}$ and *C. parapsilosis RI_SAPP* were generated as described previously with minor modifications[94,135]. Briefly, to generate *C. parapsilosis sapp1/2/3*$^{-/-}$, 500 bp upstream and downstream regions of *SAPP2* and *SAPP3* were PCR amplified and cloned into the pSFS2a plasmid with a recyclable NAT cassette. Further, the *SAPP2* deletion cassette was introduced in the *ΔΔsapp1a ΔΔsapp1b* deletion mutant *C. parapsilosis* strain to generate *ΔΔsapp1a ΔΔsapp1b ΔΔsapp2* mutants. Finally, the *SAPP3* deletion cassette was generated similarly to *SAPP2*, and *C. parapsilosis ΔΔsapp1a ΔΔsapp1b ΔΔsapp2* mutant strain was transformed with the construct to generate the *sapp1/2/3*$^{-/-}$ strain as described before[92].

Mutant *C. parapsilosis* strains expressing the individual *SAPP* genes were generated in the *sapp1/2/3*$^{-/-}$ mutant background. Solely *SAPP1*, *SAPP2*, and *SAPP3* expressing mutants were established broadly using Gateway cloning technology (Invitrogen) which takes advantage of the site-specific recombination reactions and rely essentially on the BP and LR clonase reactions (Fig. 11). In brief, the *SAPP* genes from WT (GA1) *C. parapsilosis* strain were amplified with the help of *attB* tagged primer pairs. The *attB* containing amplified *SAPP* genes were cloned in to a donor vector pDONR221 includes *att*P sites resulting in the formation of an entry clones. The resulting entry clone containing the *SAPP* genes flanked by *att*L sites further recombined with *att*R containing destination vector to form expression clones containing *SAPP* genes under *CaTDH3* promoter. Fragement containing *CaTDH*3 and *SAPP* genes were digested and cloned in pNRVL vector. Finally, pNRVL vetor was linearised using *stu*I restriction enzyme and transformed in to the *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strain targeting the

neutral locus, *Cp*NEUT5L (large intergenic region facilitates the integration and expression of ectopic genes)[156].

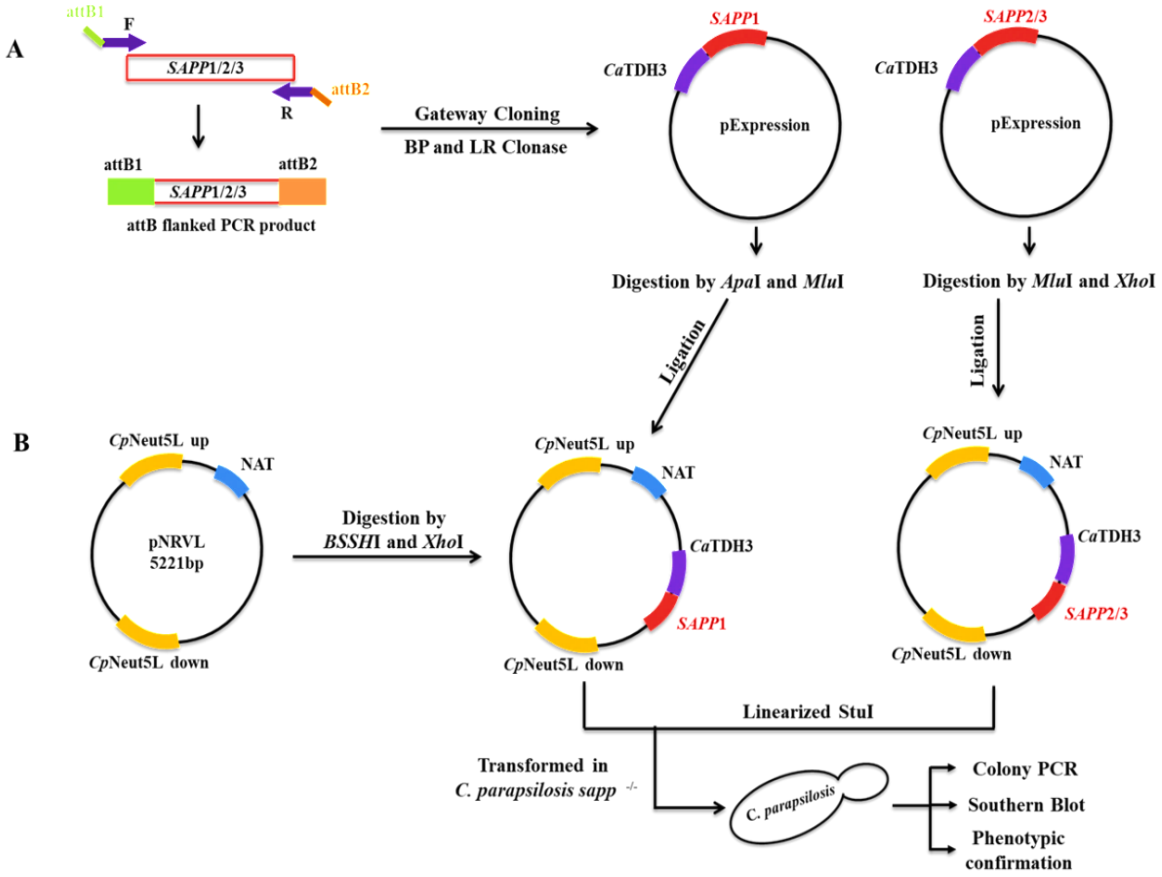


Figure 11: Schematic overview of Gateway cloning strategy for reintegration of *SAPP* genes. (A) *SAPP* genes were amplified by using *att*B primers and the amplified fragments were used to generate expression vectors containing *SAPP* genes under the control of *Ca*TDH3 promoter. (B) Expression vectors were further double digested and fragment containing *Ca*TDH3_*SAPP1/2/3* were ligated into pNRVL vector. pNRVL vectors were linearized and transfected into *C. parapsilosis sapp1/2/3*$^{-/-}$. Colonies grown on NTC containing agar plates were checked using colony PCR, Southern blot, and phenotypic analysis.

3.4 Validation of reintegrant mutant strain

The successful reintegrations were identified by PCR screening and Southern blot analysis.

3.4.1 Fast track DNA isolation

For fast track DNA extraction, a small amount of *C. parapsilosis* cells were picked by sterile tooth pick and mixed in 1.5 ml centrifuge tube with 80 µl of lysis buffer (1% SDS, 200 mM LiAc). Tubes were gently vortexed and incubated at 70 °C for 5 min. 240 µl of 96% ethanol was subsequently added to the mixture, vortexed and centrifuged for 5 min at 16,200 g in microcentrifuge. The pellet was washed with 70 % ethanol above mentioned g force. Pellet was air dried and DNA was resuspended in 50 µl Milli-Q water. Samples were stored at -20 °C for further analysis.

3.4.2 PCR conditions

For PCR amplification, each reaction contained 1x PCR buffer, 1 unit of Taq DNA polymerase (Thermo Scientific), 10 µM of each forward and reverse primers (Table S2), 2.5 mM of each deoxynucleoside triphosphate and 1 µl of DNA sample up to a total reaction volume of 15 µl. The following PCR conditions were used to amplify *C. parapsilosis SAPP1/2/3*: 94 ^{o}C for 1 min, followed by 35 cycles of 15 s at 94 °C, 20 s at 52 °C, and 2.5 min at 72 °C with a final extension of 5 min at 72 °C.

3.4.3 Detection of PCR products

2 µl of amplicons were analysed with agarose gel electrophoresis [0.8% agarose gel containing EtBr dissolved in 1xTAE buffer (40mM Tris, 20mM Acetate and 1mM EDTA)] and visualized under UV irradiation.

3.5 Southern blot analysis

Southern blot analysis was performed to verify integration of the *SAPP* containing cassette to the proper genomic region (at *Cp*NEUT5L locus) as desribed[155].

3.5.1 Hybridization of the DIG-labeled probe to DNA

Hybridisation was performed using the DIG-probe prepared from downstream sequence of the *C. parapsilosis* neutral locus (*Cp*NEUT5L). 10 µl (250 ng) of the DIG-probe was diluted in 5 ml of hybridization buffer (5x SSC, 0.1% N-lauryl-sarcosine, 0.02% SDS,

1% Blocking reagent) and boiled in water bath for 10 min and immediately chilled on ice for 5 min. The mixture was added to the nitrocellulose membrane followed by incubation at 65 °C for 18-24 h. The membrane was washed twice with 100 ml pre-warmed washing solution 1 (20xSSC, 10% SDS) for 5 min at 65 °C. The third and fourth washing was done for 15 min at 65 °C with 100 ml pre-heated washing solution 2 (2x SSC, 2% SDS).

3.5.2 Detection of the hybridized DIG-labeled probe

A peroxidase labeled anti-digoxigenin-AP Fab fragments (Roche) was added to the membrane to detect the DIG labeled probes for 30 min at RT. Afterwards the membrane was washed with 1x washing buffer 2 (3M NaCl, 0.2M NaH_2PO_4-H_2O, 0.02M EDTA, 1% SDS) for 2 times 15 min each at RT and once for 2 min with buffer 3 (0.1M Tris, 0.1M NaCl, 50mM $MgCl_2$). NBT-BCIP substrate was prepared by mixing 120-150 µl of NBT-BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, Roche) substrate in 10 ml of buffer 3. The solution was vortexed, poured in the transparent polyethylene bag contain nitrocellulose membrane and kept in dark until bands appeared. With probes used a 2,800bp fragment for *SAPP1*, 2791bp fragment for *SAPP2* and *SAPP3* were detected in overexpression *C. parapsilosis* strains, whereas these bands were absent in WT and *sapp1/2/3*$^{-/-}$ strains.

3.6 RNA extraction

To extract RNA from *C. parapsilosis* WT, *RI_SAPP* and *sapp1/2/3*$^{-/-}$ strains grown in YCB+0.2% BSA medium we used Ribopure Yeast RNA isolation Kit (Ambion) and followed the standared protocol provided by manufacturer. Purity of RNA samples was also checked by real-time PCR.

3.6.1 cDNA synthesis

cDNA synthesis from RNA samples was performed with RevertAid First Strand cDNA synthesis kit (Thermo Scientific) according to the protocol provided by the manufacturer.

3.6.2 Quantitative real-time PCR

Real-time qPCR amplification and detection were carried out in 96-well optical plates on a BioRad thermocycler with CFX96 detection system. Reaction mixture waswas set up as follows: 1x SYBR Green qPCR Master Mix (Applied Biosystems), 0.4 µM concentration of each primer, and 1 µl cDNA in a final volume of 20 µl per reaction. The amplification conditions were as follows: denaturation for 3 min at 95 °C, followed by 49 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 65 °C for 30 s, with a final extension step at 72 °C for 30 s. TUB4 gene was used as a housekeeping gene for an internal control. The primers used in real-time qPCR experiments are given in Table S2.

3.7 Phenotypic characterization of mutants by protease assay

For semiquantitative detection of proteinase production, 18 h culture of *C. parapsilosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains were grown in YPD. Cells were collected, washed two times with 1x PBS and resuspended in 500 µl of 1x PBS. 10^6 cells in 10 µl 1x PBS were spotted onto YCB+BSA containing plates and incubated for 3 days at 30 °C. After cultivation plates were stained with Amido Black for 3-4 min and washed with methanol-acetic acid-water (3:1:6) solution. The experiments were repeated 4 times. The diameter of the clearing zones around the colonies was determined with ImageJ software.

3.8 Growth curve analysis

C. parapsilosis strains grown in YPD and YCB media for 18 h were washed two times with 1x PBS. Concentrations were adjusted to 5×10^5 cells/ml diluting either in YPD or YCB + 0.2% BSA medium. 100 µl of suspension was plated onto 96-well flat-bottom microtitration plates (Biologix). The plates were incubated at 30 °C for 24 h in a plate reader (SPECTROstar Nano, BMG labtech). The optical density (OD) at 600 nm was recorded for each well automatically after every 30 min and post shaking. O.D. measurements were performed assuming that O.D. values at 600 nm obtained are proportional to the fungal cell numbers.

3.9 Serum sensitivity assay

Serum sensitivity assay of *C. parapsilosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains was performed based on previously published protocol with minor modifications[94]. Briefly, 1×10^5 cells of *C. parapsilosis* were cultured into 1 ml YPD medium supplemented with 20% intact or heat-inactivated human serum and incubated at 30 °C with continuous shaking. Aliquots were collected at 0,6,24 and 48 h postinoculation and plated to YPD plates to determine colony forming units (CFU).

3.10 Biofilm formation assay

Biofilms were developed on commercially available pre-sterilized, polystyrene, flat-bottom 96-well microtiter plates. Wells were coated with heat inactivated human serum one day before the experiment. Thereafter wells were washed three times with sterile 1x PBS. 10^6 *C. parapsilosis* cells in 100 µl RPMI or YNB media was transferred into each well of a microtiter plates. Plates were sealed with parafilm and incubated for 48 h at 37 °C to allow yeasts to develop biofilm. After biofilm formation, medium was aspirated carefully and wells were washed three times with 200 µl 1x PBS. Level of biofilm formed was measured by XTT reduction assay and CV staining as discribed[135].

3.11 Pseudohyphae growth assay

Effect of *SAPP* gene deletion and reintegration on pseudohyphae formation was also investigated. For that, *C. parapsilosis* cells were either stained with wheat germ agglutinin-tetramethylrhodamine (WGA-TRITC) and visualized with Olympus light microscope, examined with Amnis Flow Sight (Luminex) and colony morphology investigated after spotting the *C. parapsilosis* cells on pseudohyphae inducing media (YPD+10% fetal bovine serum and spider medium) as described before[135].

3.12 Analysis of cell wall composition with microscopy imaging technique

For cell wall content analysis *C. parapsilosis* cells were fluorescently labeled with CCW fluorescent dye mix [8 µl 2.5 mg/ml Canavalia A-fluorescein isothiocyanate conjugated (ConA-FITC), 1 µl 1 mg/ml Calcofluor White (CW), 1 µl 1mg/ml WGA-

TRITC, 90 µl 1% BSA] as described before[135]. The fluorescently labeled cells were washed three times with 1x PBS and resuspended in 100 µl of 1x PBS and visualized with fluorescence microscope (Zeiss).

3.13 Cultivation conditions of TR146 and A-431 cell lines

The human buccal epithelial squamous cell carcinoma line TR146 was kindly provided by Dr. Julian Naglik, Kings College London, UK and cultured as described[157].
The human A-431 vaginal epithelial cell line, obtained from ATCC (LGC Standards) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated (HI) foetal calf serum and 100 unit/ml penicillium/streptomycin (Sigma-Aldrich) at 37°C as described[151].

3.14 Adhesion assay

The cell adhesion assay was performed by slightly modifying the protocol published previously[158]. $1x10^7$ *C. parapsilosis* cells were allowed to adhere in 96-well flat bottomed, non-tissue culture plates (Biologix) in 200 µl RPMI medium (Gibco) at 37 °C, for 120 min. Non-adherent cells were removed by washing the wells two times with 200 µl 1xPBS. The attached *C. parapsilosis* cells were then vigorously resuspended in water, diluted (1,000 fold) and plated onto YPD plates. Colonies were counted after two days of incubation at 30°C and data was represented based on CFU[158]. Experiment was performed three times in triplicates.

Adhesion to epithelial cells was performed as described previously[159]. 3×10^5 TR146 or A-431 cells were grown to confluence in 24-well plates for 24 h in DMEM-F12 (Gibco) medium. After washing with 1x PBS three times, 1 ml serum-free DMEM containing *C. parapsilosis* cells in MOI 5 (TR146 cells:*C. parapsilosis* = 1:5) or MOI 10 (A-431 cells:*C. parapsilosis* = 1:10) was added to the epithelial cells and incubated for 90 min at 37 °C with 5% CO_2 in cell culture incubator. The non-adherent *Candida* cells were aspirated and wells were washed three times with 1xPBS. Wells were filled with 1 ml of double distilled water containing 0.1% triton x-100 at 37 °C for 1 h until epithelial cells were lysed. *Candida* cells were collected, diluted, plated onto YPD plates and incubated

at 30 °C for 2 days. After incubation colonies were counted. Three independent experiments were carried out in triplicates

3.15 Isolation and differentiation of PBMCs

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by Ficoll Paque PLUS (GE Healthcare) density gradient centrifugation as described previously[90]. The isolated cells were cultured for 7 days in the presence of X-VIVO 15 medium (Lonza) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, Sigma-Aldrich).

3.16 Cell damage assay

LDH activity in cell culture supernatants was measured by using the Cytotoxicity Detection Kit (LDH; Roche) according to the manufacturer's instructions. Human peripheral blood mononuclear cells derived macrophages (PBMC-DMs) were stimulated with *C. parapsilosis* cells at a PBMC-DMs:*C. parapsilosis* ratio of 1:5 for 24 or 48 h, while A-431 cells were stimulated at a ratio of 1:10 (A-431: *C. parapsilosis*) or left unstimulated (negative control). During analysis, the LDH activity measured in cultures containing yeast cells alone was subtracted from the values measured in stimulated samples. LDH released from lysed PBMC-DMs were used as a positive control. Experiments were performed three times in triplicates.

3.17 Macrophage killing assay

PBMC-DMs were coincubated in 24-well plastic cell culture plates with *C. parapsilosis* WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ strains at MOI of 5 (PBMC-DM cells:*C. parapsilosis* = 1:5). As a control, the same numbers of yeast cells were incubated in the appropriate cell culture medium without macrophages. After 3 h of incubation, macrophages were lysed using 0.1% triton x-100 in PBS. The lysates were then serially diluted, plated onto YPD agar plates and incubated at 30 °C for 2 days. The number of CFU was determined and multiplied by the dilution factor to calculate the original living cell number. The

killing percentage was calculated as follows: [(number of living *Candida* cells in control plates – number of living *Candida* cells in cocultures) / number of living *Candida* cells in control wells] x 100. Macrophages derived from 4 healthy human blood donars used for killing assay.

3.18 Whole blood killing assay

For whole blood killing assay, fresh heparinized human blood from healthy donar was mixed with 10^7 *C. parapsilosis* cells (total volume of 1 ml, 900 µl of blood and 100 µl of *Candida* cells). The mixture was incubated at 37 °C for 24 h. 50 µl of 1000 times diluted suspension was plated onto YPD agar plates in triplicates and incubated at 30 °C for 2 days. Number of surviving fungal cells was counted and the killing efficiency of whole blood was calculated as described above. Experiments were repeated three times in triplicates.

3.19 Phagocytosis assay

Phagocytic activity was examined as previously described[160], with minor modifications. *C. parapsilosis* cells were labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester (Invitrogen) and co-cultured with PBMC-DMs at MOI 5 for 3 h in 12-well tissue culture plates (VWR). Cells were then washed two times with 1x PBS, gently resuspended to a single cell suspension by pipetting, transferred to 1.5 ml microcentrifuge tubes, harvested by centrifugation and resuspended in 200 µl 1x PBS. Samples were measured using a Amnis Flow Sight (Luminex) and analysed using the IDEAS software.

3.20 Phagosome-lysosome fusion

To examine the phagosome-lysosome fusion, *C. parapsilosis* cells were labeled with fluorescent dye pHrodo® (Invitrogen). For labeling, first the yeast cell suspension (10^9 cells in 100 µl 1x PBS buffer) was treated with 11 µl of 1M Na_2CO_3 (pH=10) and then 2 µl pHrodo (1 mg/ml in DMSO) was added, and was incubated for for 1 h at room temperature in dark. Next, cells were washed 4 times with 1x PBS and adjusted to the

proper concentration. Human PBMC-DMs were infected with labeled *Candida* cells at 1:5 ratio (PBMC-DMs: *Candida*) and kept for 2 h at static condition at 37 °C to allow phagocytosis. Then the non-phagocytosed *Candida* cells were washed with 1x PBS and macrophages were detached from cell culture plates by TrypLE™ Express solution (Gibco). Finally, the macrophages were collected in FACS buffer (0.5% FBS in 1x PBS), and suspended in 1x PBS, followed by measurement. Data were analyzed by the IDEAS Software (Amnis).

3.21 Cytokine measurements

PBMC-DMs were infected with 2.5×10^6 *C. parapsilosis* WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ cells and coincubated for 24 h at 37 °C. Concentration of IL-1β, Il-6, TNFα and IL-8 was determined from supernatant by commercial ELISA kits (R&D Systems) in according to the manufacturer's instructions. Concentration of cytokines represented in pg/ml. The experiments were performed with macrophages derived from PBMCs from at least six healthy donors.

3.22 Detection of proteolytic activity of Sapp1p and Sapp2p

The proteolytic activity of purified Sapp1p and Sapp2p (1 µg each) was assayed by incubating them with purified human complement proteins C3b, C4b, and factor H (FH) (Merck) or with recombinant factor H-like protein 1 (FHL-1) (expressed and purified as described previously)[161] or FHR-1 or FHR-5 (Novoprotein) for 3 h or 15 h at 37 °C. Aliquots were taken at the indicated time points, separated by SDS-PAGE, and analyzed by Western blotting. C3b was identified by the use of polyclonal goat anti-human C3 (Calbiochem, Quidel) in combination with a horseradish peroxidase (HRP)-conjugated goat antibody (DAKOCytomation). C4b was detected with a monoclonal anti-C4c antibody (Quidel) and with HRP-conjugated goat anti-mouse Ig (Dako). To detect cleavage of FH, FHL-1, FHR-1, and FHR-5, polyclonal goat anti-FH (Calbiochem, Merck), mouse monoclonal anti-FH (A254; from Quidel), and polyclonal goat anti-FHR-5 (R&D System) and the corresponding HRP-conjugated secondary antibodies rabbit anti-goat Ig and goat anti-mouse Ig (Dako) were used. In addition, cleavage of C3b and C4b by the natural, complement-specific protease factor I in the

presence of the cofactors FH and C4BP (Hyphen Biomed) was assayed to compare with the cleavage patterns generated by the Sapp proteases.

3.23 Staining of macrophage CD11b/CD11c receptors

Isolation of CD14+ monocytes and macrophage differentiation was carried out as described previously[162]. To investigate the effect of Sapp proteins on cell surface receptors, 1×106 PBMC-DMs were incubated with Sapp1p or Sapp2p (5 µg each) for 30 minutes at 37 °C. In control experiments PBMC-DMs were incubated in 1x PBS buffer without addition of any proteins. PBMC-DMs were washed with 1x PBS containing 1% FCS and 0.1% NaN_3. 100 µg/ml monoclonal antibodies were added to the cells (against CD11b monoclonal mIgG1 clone ICRF44 (Biolegend), against CD11c monoclonal mIgG1 clone BU15 (ImmunoTools GmbH) and against CD18 monoclonal mIgG1 clone TS1/18 (Biolegend) and isotype control (mouse IgG1 clone MOPC-21, Biolegend) for 20 min at 4 °C followed by the addition of goat anti-mouse IgG-Alexa Flour 488 secondary antibody (1:200, Molecular Probes) for 20 min at 4 °C. 20,000 cells were measured by Cytoflex flow cytometer (Beckman Coulter) using CytExpert software for data acquisition and Kaluza Analysis software for data analysis.

3.24 *Galleria mellonella* fungal burden and survival

Galleria mellonella larvae were purchased from TruLarv™ (Biosystems, Netherlands). Upon arrival, the larvae were stored at 4 °C in dark and subsequently used within a maximum of 7 days. Before infection, larvae were taken from cold storage, kept nearly 20 min at room temperature and proleg was sterilized gently with cotton swab dipped in 70% ethanol.

Initially the sub-lethal *C. parapsilosis* inoculum concentration was determined by injecting larvae with serial dilutions of *C. parapsilosis* WT cells: 10 µl of cell suspension containing 10^8, $5x10^7$, $2.5x10^7$ cells/ml were injected into *G. mellonella* by sub cutaneous micro-injection by inserting needle of Hamilton syringe into the proleg of larvae. In survival experiments larvae were infected with 10 µl of cell suspension containing 10^8 *Candida* cells /ml). As a control groups, non-injected larvae and injected

with 10 µl of sterile 1x PBS were included. Infected larvae were incubated at 30 °C for 7 days and monitored daily for survival. Groups of twenty larvae were used per strain with two experimental replicates.

For CFU determination, 0.25-0.30 gram larvae were infected with 10 µl of cell suspension containing 10^7 *C. parapsilosis* cells /ml, sacrificed at 24 h post-infection and fungal burden of each individual larvae was determined following the next protocol: larvae was homogenized in 5 ml 1x PBS in 50 ml plastic canonical tube with homogenizer at 3000 rpm for two cycles of 1 min. 50 µl of homogenate was plated onto YPD plates containing penicillin and streptomycin. Plates were incubated at 30 °C for 2 days and colonies were counted.

3.25 *Drosophila melanogaster* infection

For the infection of flies, pricking method was used. Pricking method of infection is proven to be more reliable and triggers comparatively lower induction of damage caused antimicrobial proteins compared to other infection modes[163].

OregonR (OrR) flies (WT) and MyD88 mutant strain (females or males, 15 per experimental group) were infected with a thin sterile needle dipped in the suspension of *C. parapsilosis* cells (2×10^7 cells/ml diluted in sterile 1x PBS). Flies were than incubated at 25 °C on regular fly medium. 25 flies were infected with each mutant strains and experiment was at least twice

3.26 Statistical analyses

Statistical analyses were performed either by t-test or by one way ANOVA using the software GraphPad Prism 6 (GraphPad Software, Inc.). Mantel-Cox (Log-rank) tests were used for survival data evaluation. Differences were considered statistically significant at $p \leq 0.05$ (*$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$)

3.27 Ethical statement

For isolation of PBMCs, blood samples were taken from healthy donors. This procedure and the respective consent documents were approved by the Institutional Human

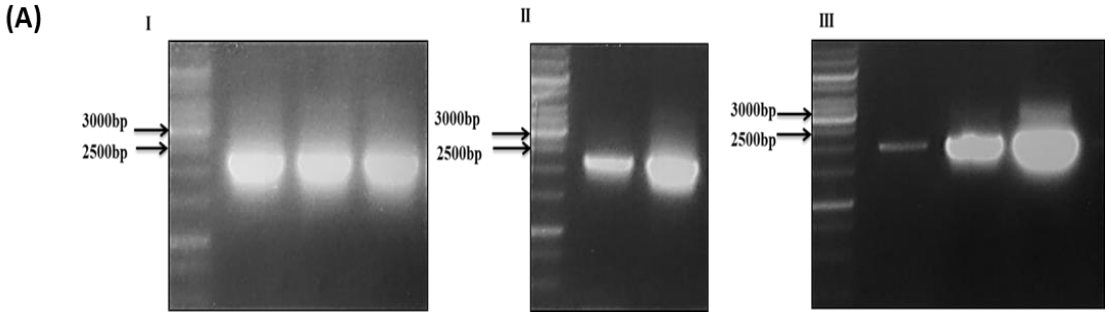
Medical Biological Research Ethics Committee of the University of Szeged. All healthy donors provided written informed consent. All experiments were performed in accordance with guidelines and regulations of the Ethics Committee of University of Szeged, and experimental protocols were approved by institutional committee.

4. Results

4.1 Reintegration of the *SAPP1*, *SAPP2*, and *SAPP3* genes *C. parapsilosis sapp1/2/3*$^{-/-}$ strain

Previous work from our laboratory clearly demonstrated that *C. parapsilosis SAPP* deletion mutants, i.e. *C. parapsilosis Δ/Δsapp1a-Δ/Δsapp1b* and *Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2* strains are more sensitive to human serum and *in vitro* less virulent compared to the donor strain (WT)[94]. To further analyze the role of *SAPP* genes, we overexpressed *SAPP1*, *SAPP2*, and *SAPP3* genes individually in *C. parapsilosis sapp1/2/3*$^{-/-}$ *strains*. pNRVL vector (Fig. S1) containing the *SAPP1*, *SAPP2*, and *SAPP3* genes under the control of constitutive *Ca*TDH3 promoter was linearized by enzyme *Stu*I and transfected into *C. parapsilosis sapp1/2/3*$^{-/-}$. Colonies were selected on NTC plates. Further we performed colony PCR (Fig. 12A) and Southern blot to identify mutant strains and to validate the proper integration.

For Southern blot, *C. parapsilosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains genomic DNA was digested with *Hin*dIII restriction endonuclease and transferred onto nylon membrane. Hybridisation was performed with high stringency using the probe prepared from downstream sequence of the *C. parapsilosis* neutral locus (*CpNeut5l*). Only one band was detected in case of *C. parapsilosis* WT and *sapp1/2/3*$^{-/-}$ strains whereas mutant having correct integration of *SAPP* gene resulted 2 bands indicating that *SAPP* genes are integrated to the correct locus (Fig. 12B).



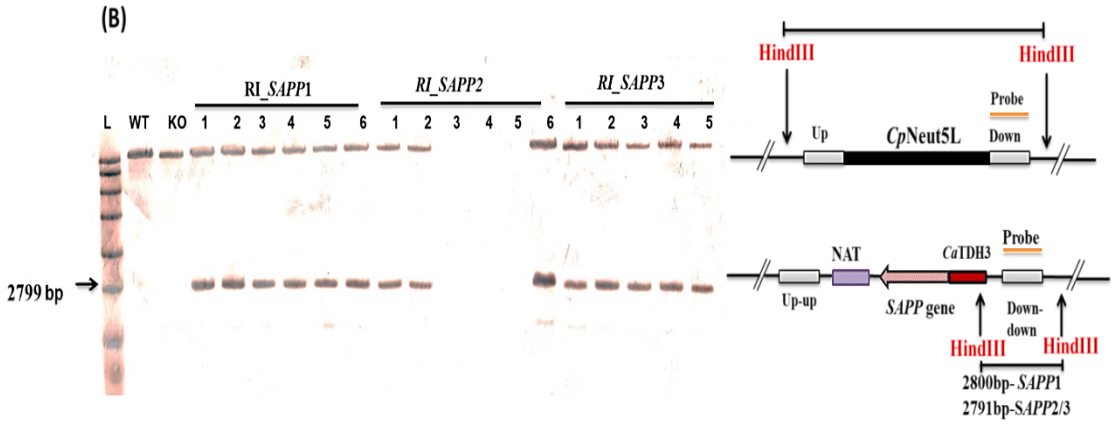


Figure 12: Confirmation of mutant strains. (A) Colony PCR: PCR amplification of *SAPP* genes in mutant strains resulted for different length fragments such as for *SAPP1* expected fragment size was 2215 bp (I), for *SAPP2* was 2243bp (II) and for *SAPP3* was 2000bp (III). In all gel pictures left lane represents the ladder. (B) Southern hybridization analysis of *Hind*III-digested genomic DNA isolated from *C. parapsilosis* WT (lane 1), *sapp1/2/3*$^{-/-}$ (lane 2), *RI_SAPP1* (lane 5 to 10), *RI_SAPP2* (lane 11 to 15) and *RI_SAPP3* (lane 17 to 21) reintegration mutants. Molecular size marker is indicated by L (left lane).

4.2 Relative transcription of *SAPP* genes in reintegrant *C. parapsilosis* strains

The transcription level of the *SAPP* genes in the reintegrant strains was determined by using qRT-PCR. The qRT-PCR analysis revealed that transcription of *RI_SAPP1* and *RI_SAPP2* were nearly similar to transcription of the *SAPP1* and *SAPP2* genes in wild-type *C. parapsilosis*, whereas increased by four fold in case of *RI_SAPP3* strain in comparison with the control when culturing the cells in YCB+0.2% BSA medium (Fig. 13).

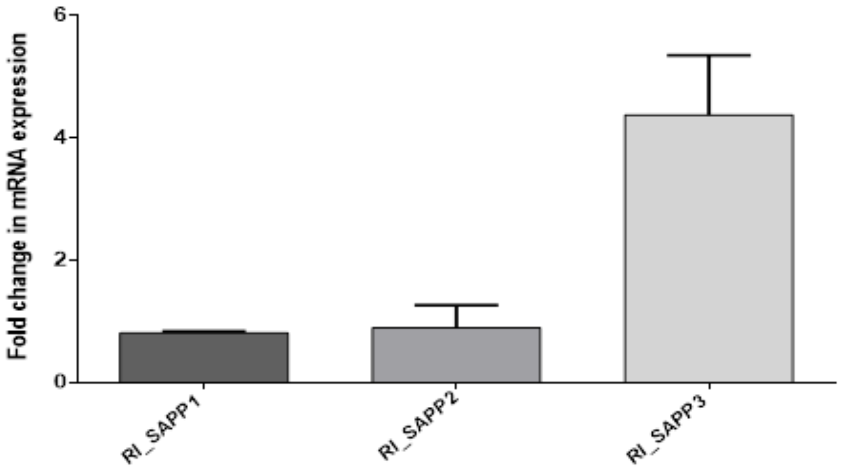


Figure 13: Transcription of *SAPP* genes in the mutant *C. parapsilosis RI_SAPP1, RI_SAPP2* and *RI_SAPP3* strains. Fold change of *SAPP* gene transcription in *RI_SAPP* mutants compared to the wild-type strain corresponding genes after growth in YCB + 0.2% BSA medium. Transcription of *SAPP1* and *SAPP2* genes in reintegrant strains *RI_SAPP1* and *RI_SAPP2* was restored while *SAPP3* gene's transcription was upregulated in the *RI_SAPP3* strain by ≥4 fold. The experiment was performed in triplicates**.**

4.3 Detection of protease activities of Sapp in WT and mutant strains of *C. parapsilosis*

Candida secreted aspartyl proteases can hydrolyze BSA. To test if reintegrant strains restored protease production capability, WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP1, RI_SAPP2, RI_SAPP3 C. parapsilosis* strains were spotted onto YCB + 0.2% BSA containing plates and plates were stained with amido black. The widths diameter of clearance zones were measured after three days of incubation. Wild-type *C. parapsilosis* showed a clear hallow zone (7.3 mm) in BSA containing plates whereas, *RI_SAPP1* (5.78 mm) and *RI_SAPP2* (5.76 mm) also showed proteolytic activity. In contrast, *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strains did not show any proteolytic activity, although the growth of the colonies was nearly similar to WT (Fig. 14A and B).

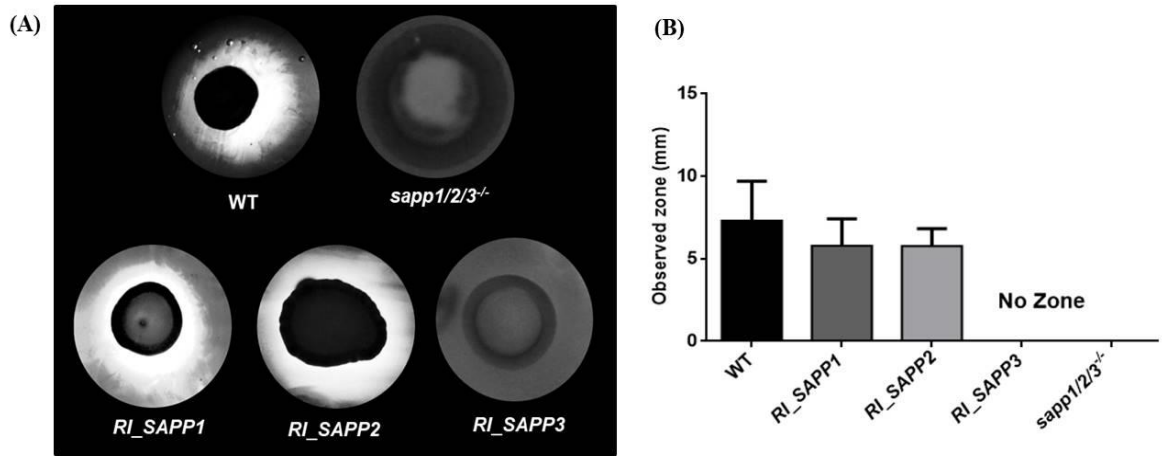


Figure 14: Protease activity of WT and *SAPP* mutant strains were detected by BSA degradation assay. (A) 10 µl of 18 h WT and mutant *C. parapsilosis* cultures were spotted onto agar plates containing YCB + 0.2% BSA. Halo zone around the colonies of wild-type, *RI_SAPP1*, and *RI_SAPP2* depicts protease activity. *sapp1/2/3*$^{-/-}$ and *RI_SAPP3* mutant strain did not form halo zone around colonies indicating no protease secretion. Images are representative of 4 independent repeats. (B) The radius of clear white zone was measured and plotted in the graph.

4.4 *C. parapsilosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains grow at similar rate

Estimation of fungal growth by continuous monitoring the change in the OD has been used to check the growth of WT and mutant *C. parapsilosis* strains. To monitor the effect the deletion and reintegration of *sapp* genes in growth rate of *C. parapsilosis* first, we examined growth of WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* mutant strains in normal YPD and YCB+0.2% BSA growth medium for 24 h. Deletion or reintegration of *SAPP* genes had no effect on the growth of *C. parapsilosis* at 30 °C either in YPD or in YCB+BSA medium (Fig. 15A and B). Growth curve results indicate that *SAPP* gene in *C. parapsilosis* do not influence its growth.

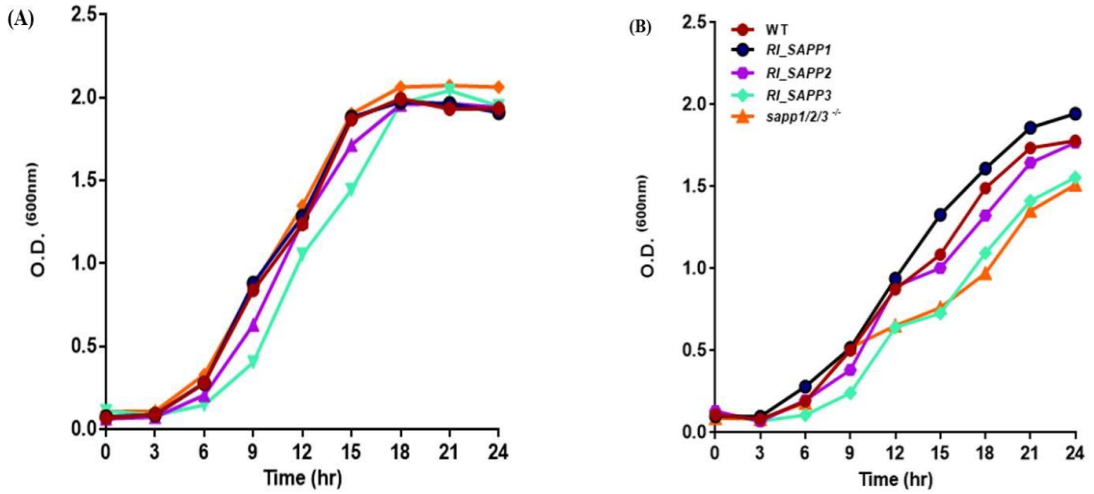


Figure 15: Kinetic growth curve analysis. Growth of *C. parapsilosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains (5×10^5 cells/ml as initial concentration) was examined in YPD (A), and YCB (B) at every 30 min time interval by measuring OD at 600 nm. WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains grown in YPD and YCB media with a similar growth rate at 30 °C. Experiments were performed two times in triplicates.

4.5 *C. parapsilosis SAPP3* reintegrant and sapp1/2/3$^{-/-}$ mutant strains are more sensitive to normal human serum

C. albicans Saps are reported to cleave human complement proteins and other microbicidal plasma proteins thus providing additional advantage of avoiding host immune response. To investigate the effect of the fungicidal effect of human serum, yeast cells were cultivated in 20% normal human serum and heat inactivated serum; then, CFU determinations were performed at different time intervals. Whereas all *C. parapsilosis* strains grown similarly in 20% heat-inactivated serum, the viability of the *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strains in intact serum was reduced significantly after 18 and 24 h incubation, respectively (Fig. 16A and B). There were no significant differences between the growth of WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ mutant strains in heat inactivated human serum at any time point of treatment.

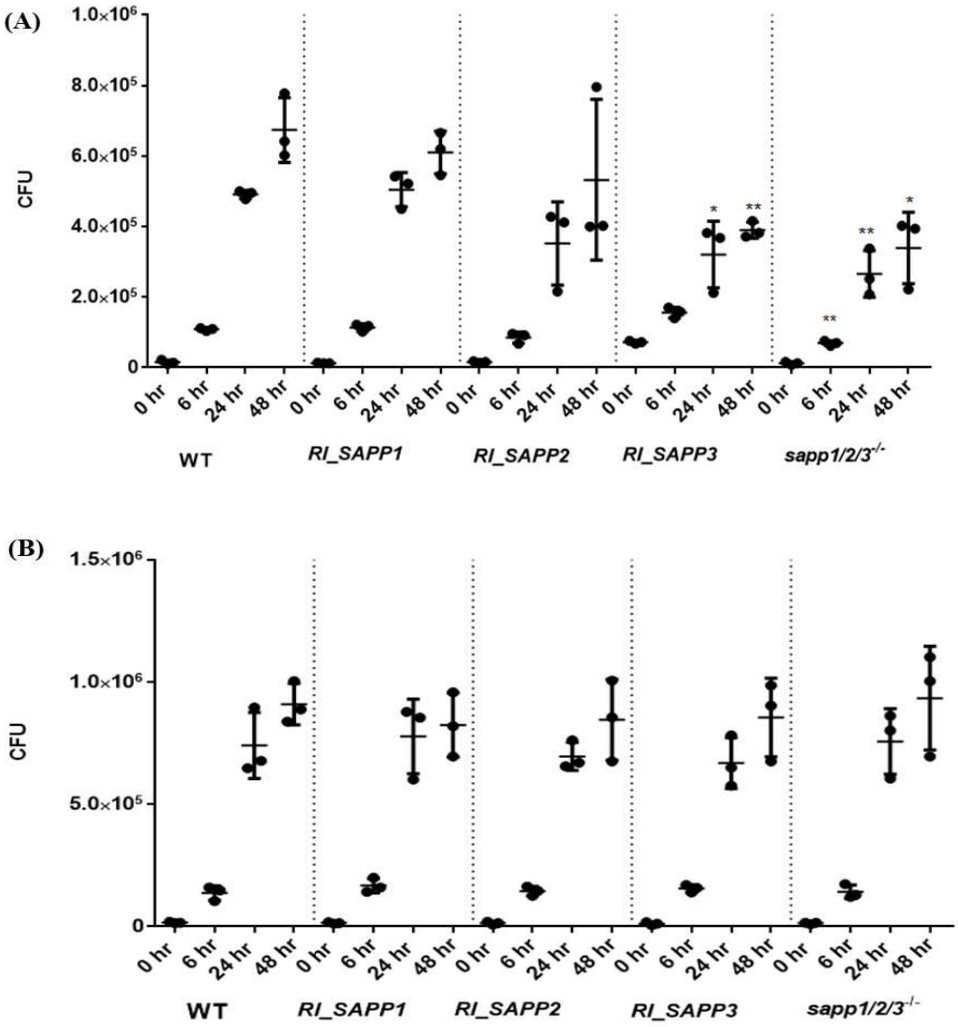


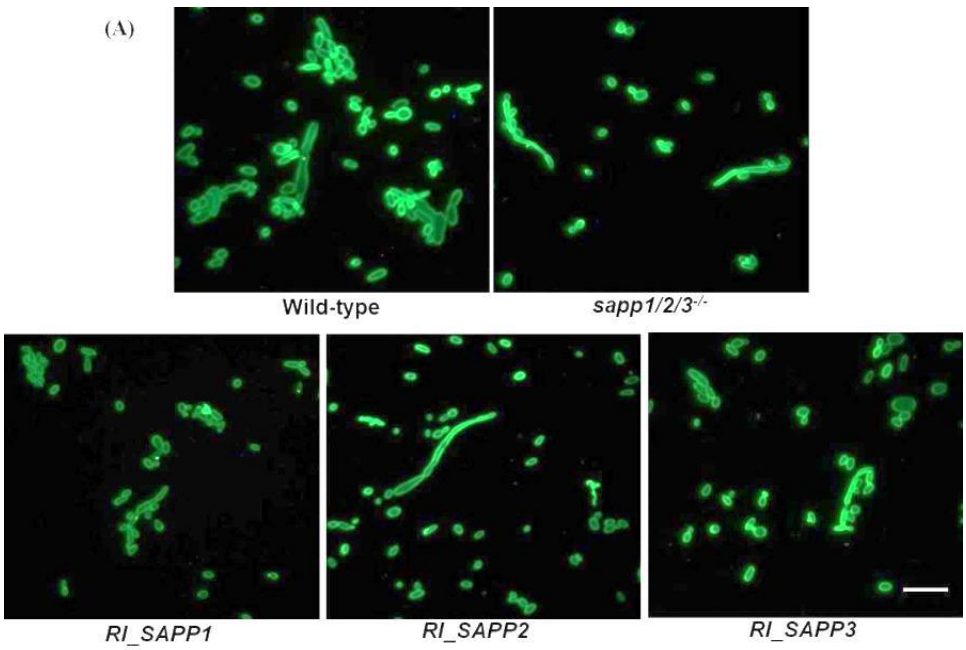
Figure 16: Plasma sensitivity assay. (A) Growth of *C. parapsilosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains in 20% normal human serum (NHS) and heat inactivated (HiS) examined by CFU determination at 0, 6, 24 and 48 h. Results show that NHS significantly inhibits the growth of *sapp1/2/3*$^{-/-}$ strain at 16 and 24 h when compared with WT. (B) HiS did not show any sensitivity against *sapp1/2/3*$^{-/-}$ compared to WT.

4.6 Deletion or reintegration of *SAPP* genes did not alter the pseudohyphae formation abilities of *C. parapsilosis*

Hyphae formation play important role in pathogenesis of *C. albicans*. Rather than forming true hyphae *C. parapsilosis* forms pseudohyphae. It has been reported that pseudohyphal form of *C. parapsilosis* express more adhesion proteins than yeast form,

thus it is more virulent. Therefore, we examined that deletion of *SAPP* genes has any effect on pseudohyphal formation of *C. parapsilosis*. Interestingly, deletion of the *SAPP* genes and reintegration of the genes did not result any significant change in pseudohyphae formation either on solid or in liquid media compared to WT (Fig. 17A-D). Effect of gene deletion and reintegration on phenotypic variation was also examined by observing the wrinkling and peripheral filamentation. *C. parapsilosis* WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ mutants exhibited indistinguishable colony morphologies at 30 and 37 °C (Fig. S2). Additionally, to investigate on the effect of deletion and reintegration of *SAPP* genes on cell wall composition, differential staining with Concanavalin A (dye for mannoproteins, Calcofluor white (dye for chitin content), and Wheat germ agglutinin (fluorescence dye for β glucan) was used. No differences in cell wall composition was observed in WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strains (Fig. S3).

Further, we also investigated stress tolerance of WT and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strains at several conditions (Table S3). Surprisingly, we did not found any differences in stress tolerance of *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strain compared to WT (Fig. S4). Thus, results indicate that *SAPP* did not play significant role in pseudophyphae formation, stress tolerance, and cell wall composition in *C. parapsilosis*.



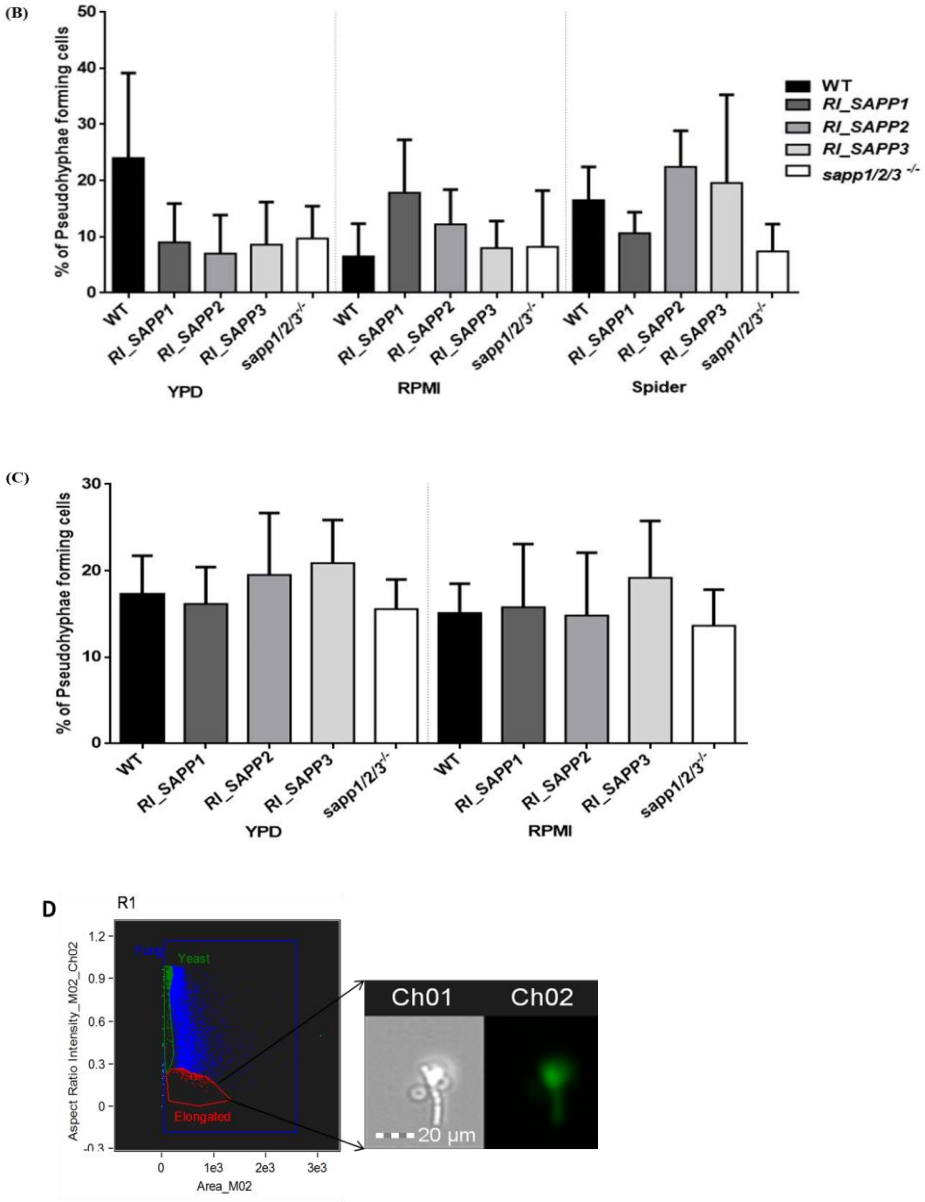


Figure 17: Pseudohyphae formation of *C. parapsilosis* WT and mutant strains. *C. parapsilosis* WT and mutant strains were grown in pseudohyphae inducing conditions (YPD or RPMI 1640 + FBS, 37 °C in CO_2 incubator). Number of pseudohyphae forming cells was counted and percentage of the pseudohyphae forming cells was calculated by dividing pseudohyphae forming cells by the total number of cells. (A) Representative picture of *C. parapsilosis* WT and mutant strains stained with concanavalin A to visualize the pseudohyphae. (B) Percentage of pseudohyphae forming cells was calculated from three independent microscopic pictures by dividing number of pseudohypha forming cells to total number of cells and multiplied by 100. (C) Percentage of pseudohyphae forming cells analyzed by flow cytometer. (D) Representative image shows yeast cell and pseudohyphal cell. Scale bar 10µm.

4.7 Deletion and reintegration of *SAPP* genes did not alter the biofilm formation in *C. parapsilosis*

Previous study has shown a significant reduction in biofilm formation capabilities of the *sap5Δ/Δ*, *sap6Δ/Δ*, and *sap5/6 ΔΔ/ΔΔ* deletion strains of *C. albicans* compared to the WT reference strain[164]. Thus to further understand whether deletion and reintgration of *SAPP* genes affect biofilm formation of *C. parapsilosis* strains were inoculated in YNB and RPMI media and allowed to form biofilm for 48 h at 37 °C; next MTT reduction assay and crystal violet (CV) staining method was used to determine the biofilm formation level. Growth assay was performed previously to rule out the possibility of variation in the biofilm level due to the difference growth rate. No significant differences were observed in biofilm formation of WT and mutant *C. parapsilosis* strains (Fig. 18A and B).

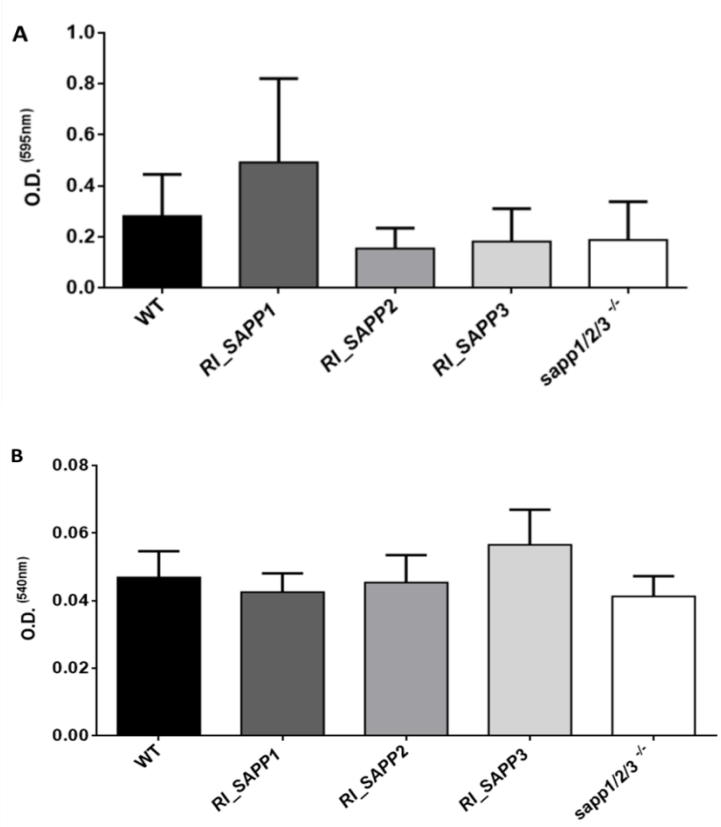


Figure 18: Biofilm formation by different *C. parapsilosis SAPP* mutant strains. Biofilm was quantified by crystal violet stain (A) and MTT colorimetric assay (B). *C. parapsilosis* WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ form similar biofilm either grown in RPMI or in YNB media.

4.8 Secreted aspartyl proteases of *C. parapsilosis* affect its adhesion

In *C. albicans SAP* (primarily *SAP5 and SAP6*) deletion mutants have pronounced reduction in the number of adhered cells[164]. Therefore, we examined weather reintegration or deletion of *SAPP* genes in *C. parapsilosis* affect adhesion to artificial surface and epithelial cells. Results have clearly shown that all three reintegrant *C. parapsilosis* strains exhibited significant reduction in number of cells (CFU) adhered to the wells when compared to the WT. Highest reduction in adhesion was observed in the case of *SAPP* null mutant *C. parapsilosis* strains (nearly 60% of total cells were not adhered), whereas lowest reduction was observed in *C. parapsilosis RI_SAPP1* mutant strain (20%) followed by *RI_SAPP*2 and *RI_SAPP3* (25-25%). However, comparison of the adhesion efficiency of reintegrant strains clearly showed that *C. parapsilosis RI_SAPP1* exhibited highest adhesion efficiency when compared with the *RI_SAPP2* and *RI_SAPP3* reintegrant *C. parapsilosis* mutant strains (Fig. 19A). A significant reduction in adhesion to oral epithelial cells (TR146 cells) of *RI_SAPP3*, and moderately lower adhesion in case of *sapp1/2/3*$^{-/-}$ and *RI_SAPP2 C. parapsilosis* strains were also observed. At the same time, *RI_SAPP1 C. parapsilosis* strain restored adhesion capacity to epithelial cells nearly to WT (Fig. 19B).

We also monitored the adhesion efficiencies of WT and *SAPP* mutant *C. parapsilosis* strains to vaginal epithelial cells (A-431 cells). Our result suggest that *C. parapsilosis* WT and *RI_SAPP1* adhere significantly more to A-431 cells as compared to *RI_SAPP2*, *RI_SAPP3* although without reaching significant difference to *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strain (Fig. 19C). These finding clearly indicate the role of *SAPP1* and *SAPP2* in adhesion of *C. parapsilosis* to epithelial cells.

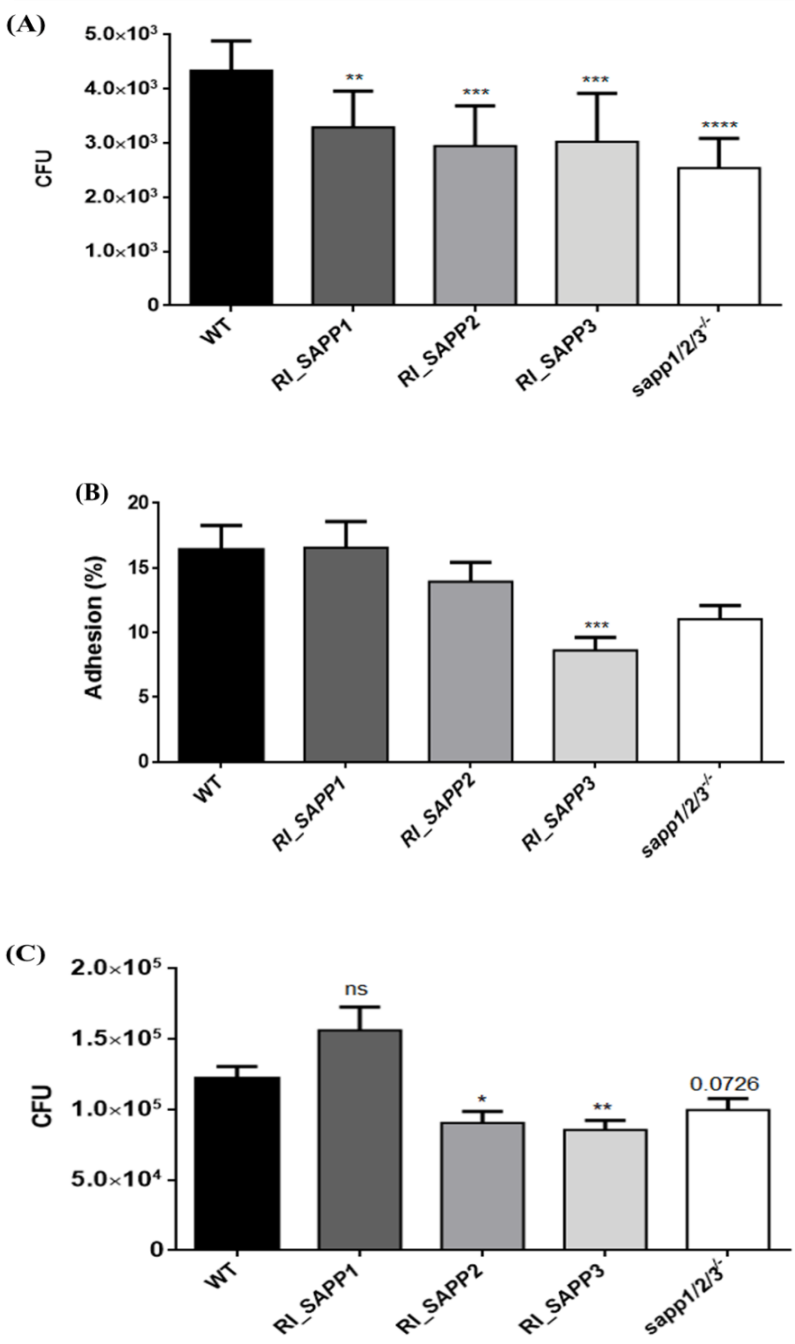
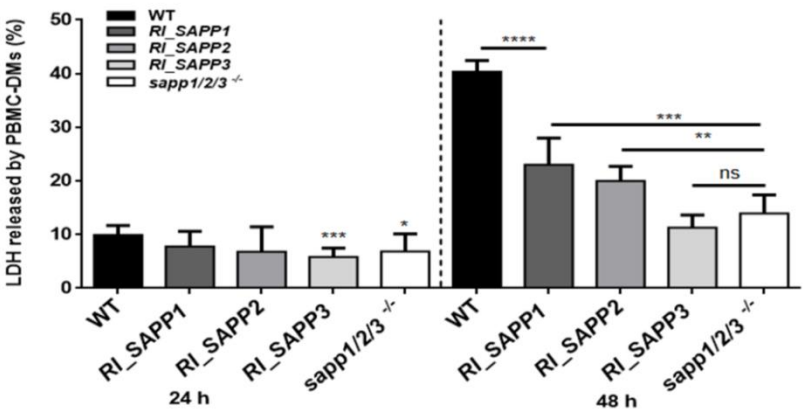


Figure 19: *In vitro* cell adhesion assay. WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains were allowed to adhere either solid surface (A), to oral epithelial cells (B), or to the vaginal epithelial cells (C), for 90 min. All experiments are repeated three times in triplicate and a significant difference is considered in compare to WT.

4.9 *SAPP* null mutant *C. parapsilosis* is less capable of causing host-cell damage whereas reintegration of *SAPP1* and *SAPP2* genes restored cell damage causing capabilities

Damage of PBMC-DM cells in the presence of WT, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strains was monitored with lactate dehydrogenase (LDH) release assay after 24 and 48 h coincubation. WT, *RI_SAPP1*, and *RI_SAPP2 C. parapsilosis* strains cause nearly similar damage to those seen with PBMC-DMs (7.779% ± 1.001% and 6.807% ± 1.642%, respectively) whereas, *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ caused significantly less damage (5.843% ± 0.5715% and 6.862% ± 1.340%, respectively) than the wild-type strain (9.944% ± 0.6143%) after 24 h coincubation with PBMC-DMs. Differences between the examined strains became more evident following 48 h of coincubation. Host cell damage was least severe in macrophages infected with the *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ strains (11.28% ± 0.8304% and 13.95% ± 1.153%, respectively), followed by *RI_SAPP2* (19.98% ± 1.238%) and *RI_SAPP1* (23.04% ± 1.661), compared to that seen with the wild-type strain (40.36% ± 0.6912%) (Fig. 20A). Additionaly, in the case of PBMC-DMs, *C. parapsilosis* WT reported to cause significantly higher damage at both 24 h and 48 h (Fig. 20B). LDH released by A-431 cells infected with *C. parapsilosis* WT (9.774 ± 1.618) was significantly higher compared to *RI_SAPP2* (4.937 ± 0.8536), *RI_SAPP3* (4.109 ± 0.9633), and *sapp1/2/3*$^{-/-}$ (3.243 ± 0.3529) strains indicating that mainly *SAPP1* and *SAPP2* are crucial in *C. parapsilosis* to cause host cell damage.

(A)



(B)

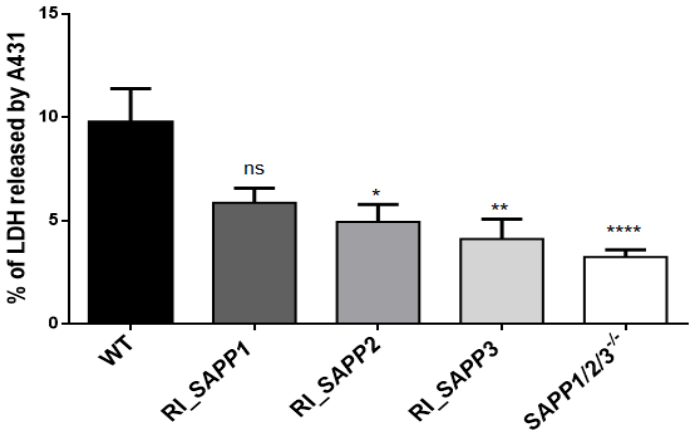
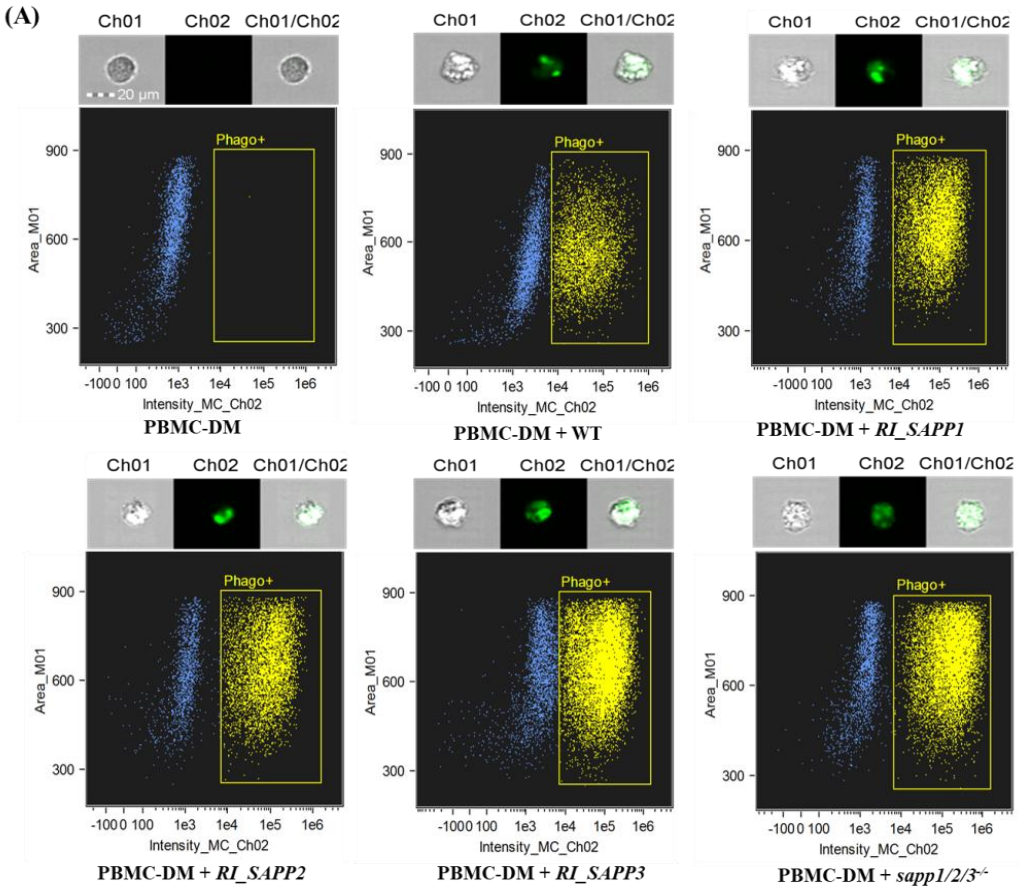


Figure 20: Host cell damage by WT and mutant strains: Host cell damage was measured by the amount of lactate dehydrogenase (LDH) released by human PBMC-DM (A), and A-431 cells (B). Cells were stimulated with *C. parapsilosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* strains with MOI 5 in case of PBMC-DMs and MOI 10 in case of A-431 cells. LDH released was measured after 24 h and 48 h of post infection and expressed as % of positive control. Results clearly showed that WT causes more damage either to PBMC-DM or A-431 cells compared to *sapp1/2/3*$^{-/-}$. In A-431 cells WT and *RI_SAPP1* strains causes similar damage however all reintegrant mutant strains showed attenuated damage causing capacities to PBMC-DMs.

4.10 Macrophages phagocytosed *sapp1/2/3*$^{-/-}$ mutant *C. parapsilosis* more efficiently than WT, *RI_SAPP1, RI_SAPP2, and RI_SAPP3* cells

Human PBMC-DMs were used to characterize the immune modulatory properties of *SAPP* genes in *C. parapsilosis*. We first examined the phagocytic activity of PBMC-DMs by fluorescence-activated cell sorting (FACS). *C. parapsilosis* yeast cells were labeled with the fluorescent dye Alexa Fluor 488 (succimidyl ester), washed, and then coincubated with PBMC-DMs for 2 h. Our results indicated that PBMC-DMs ingested *RI_SAPP3* (60.63 ± 5.430) mutant strains more efficiently compared to the wild-type (46.70 ± 2.041) *C. parapsilosis* (Fig. 21), highlighting the importance of *SAPPs* in modulating phagocytotic behavior of macrophages. Additionaly, *sapp1/2/3*$^{-/-}$ (59.03 ± 2.675) was also significantly more phagocytosed by PBMC-DMs compared to WT *C. parapsilosis.*



(B)

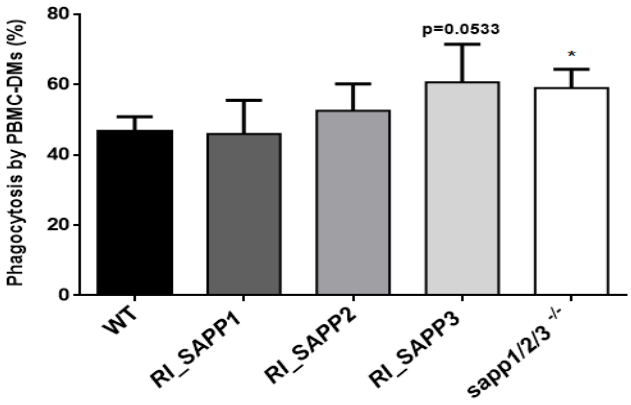
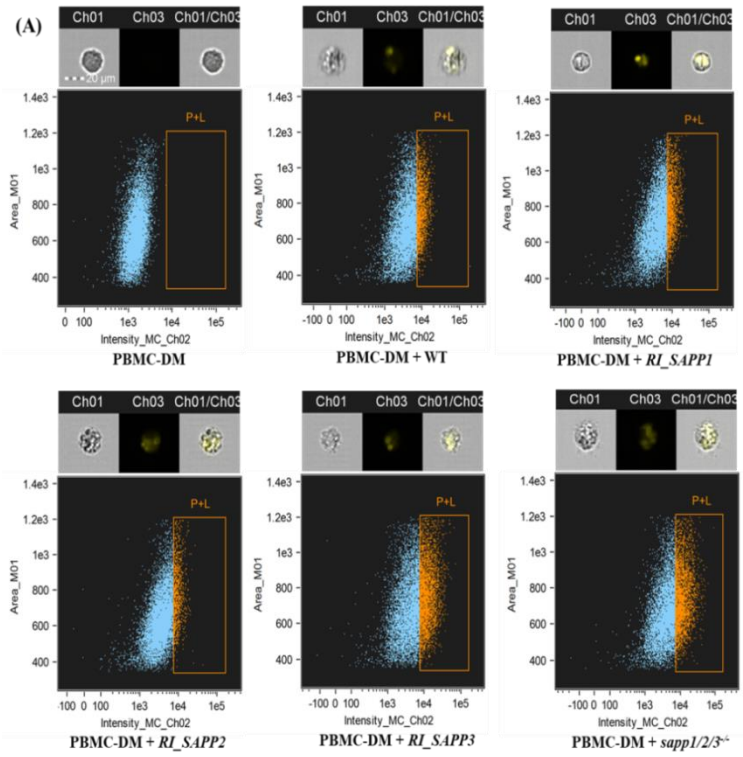


Figure 21: Phagocytosis of *C. parapsilosis* strains by human macrophages. Alexa Fluor 488 labeled yeast cells were co-incubated with PBMC-DMs. (A) Representative dot plots (gated cells) show the PBMC-DMs phagocytosed *Candida* Ch1: brightfield image, Ch2: fluorescence lebelled *Candida*, Ch1/Ch2: merge image. (B) Graph shows the difference between phagocytosis in case of the wild-type and the mutant strains infected PBMC-DMs.

4.11 *SAPP2* influences the phagosome-lysosome fusion

Next, we examined whether lack or reintegration of *SAPP* genes into *C. parapsilosis* genome has any effect on phagosome-lysosome fusion in human PBMC-DMs. We examined co-localization of phagosome-lysosome after coincubation with pHrodo (Invitrogen) stained *Candida* cells with PBMC-DMs for 2 h. WT ($16.66\% \pm 0.5732\%$) and *RI_SAPP2* ($13.78\% \pm 1.216\%$) infected PBMC-DMs showed less phagolysosome formation compared to *RI_SAPP1*, *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ ($20.76\% \pm 0.7194\%$, $29.52\% \pm 2.719\%$, $28.70\% \pm 2.025\%$ respectively) *C. parapsilosis* strains, suggesting that similar to *C. albicans SAP2*, *C. parapsilosis SAPP2* gene's influence on phagosome maturation (Fig. 22A and B).



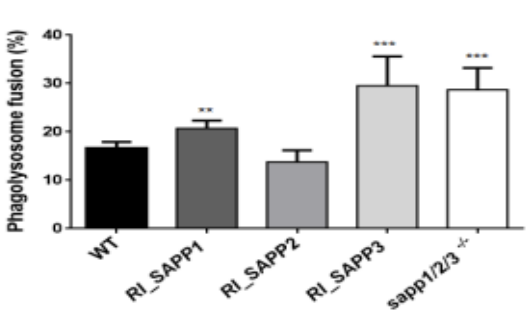
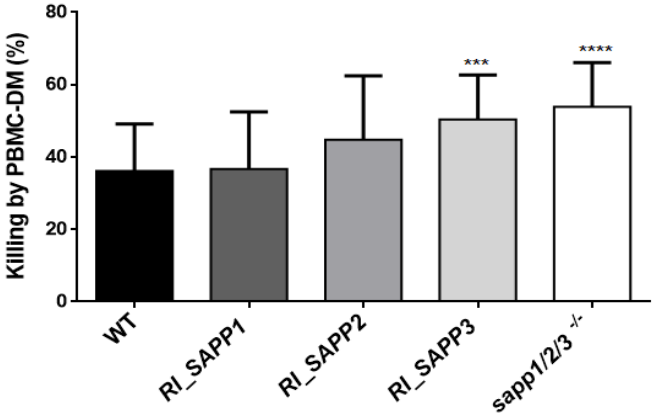


Figure 22: Phagosome-lysosome fusion in response to WT and *SAPP* mutant *C. parapsilosis.* Phagosome-lysosome co-localization in human PBMC-DMs following the phagocytosis of pHrodo labeled *Candida* cells was investigated. (A) Representative plots of a phagocytosing macrophage during quantitative imaging analysis. Ch1: brightfield image, Ch3: green fluorescence channel, Ch1/Ch3: merge image. (B) Graph shows the difference between the extent of phago-lysosome fusion in case of the wild-type and the mutant strains.

4.12 *SAPP* null mutant and *RI_SAPP3 C. parapsilosis* cells are killed more efficiently by PBMC-DMs and in human whole blood compared to *RI_SAPP1/2* strains

Phagocytosis of *Candida* cells by macrophages and subsequent formation of phagolysosome in general leads to the killing of pathogen. *C. albicans* mediated alteration of phagosome-lysosome fusion is well reported[165]. In our above mentioned results we observed that several *C. parapsilosis* mutant strains (*RI_SAPP1*, *RI_SAPP3*, and *sapp1/2/3*$^{-/-}$) alter phagolysosome fusion compared to WT strain. Therefore, to further examine if alteration of phagolysosome formation is related with yeast cell killing efficiency of PBMC-DMs we compared fungal killing by recovered fungal CFUs after coincubation PBMC-DMs with WT and mutant *C. parapsilosis* cells. Our data showe that PBMC-DM cells were able to kill *C. parapsilosis RI_SAPP3* mutant cells with significantly higher efficiency compared to WT cells. Notably, PBMC-DM killed significantly more [32% WT vs 53.9% *sapp1/2/3*$^{-/-}$ and 50.39% *RI_SAPP3*] (Fig. 23A). However, killing efficiency of macrophages was nearly similar against WT, *RI_SAPP1*, and *RI_SAPP2 C. parapsilosis* strains indicating significant contribution of *SAPP1* and *SAPP2* in modulation of killing capacity of macrophages. In addition to PBMC-DMs, we also examined the killing efficiency of whole human blood. Our results show more efficient killing of *C. parapsilosis RI_SAPP3* and *sapp1/2/3*$^{-/-}$ by human whole blood indicating protective roles of *SAPP1* and *SAPP2* (Fig. 23B).

(A)



(B)

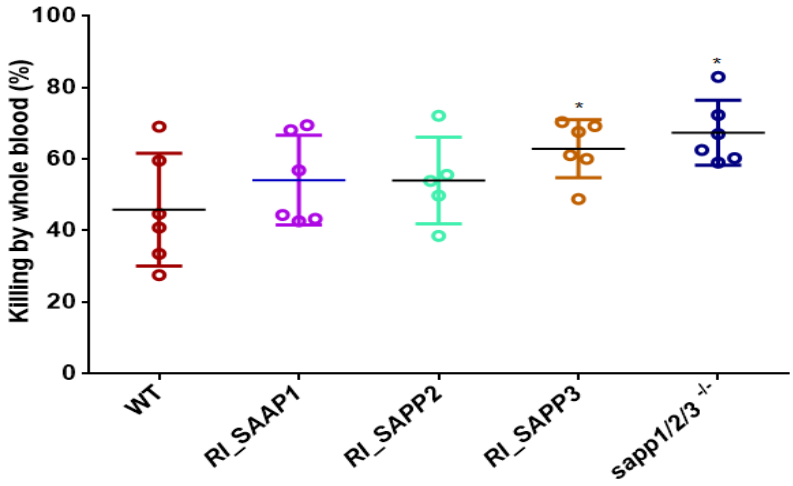


Figure 23: Killing efficiencies of human PBMC-DMs and whole blood. The efficiency of killing by human macrophages (A), and human whole blood (B) was analyzed by CFU determination. Experiments were performed in triplicates. The obtained data represents the killing efficiency of macrophages gained from 5 healthy donors. Blood from three different donors was used.

4.13 *SAPPs* regulate the cytokine response of host macrophages

Aspartyl proteases of *Candida albicans* effectively regulate inflammatory responses[140,141]. Recent studies have shown that sap2 and sap6 effectively induce the activation of NLRP3 inflammasome and thus leading to the secretion of interleukin-1β (IL-1β) and IL-18 via caspase-1 activation[141]. These candidapepsins also induce secretion of chemoattractive chemokine's such as IL-8 and MIP-2 by vaginal epithelial cells[140]. But, role of candidaparapesins in modulating the immune responses is still enigmatic thus, in order to examine the immunological responses triggered by *RI_SAPP* and *sapp1/2/3*$^{-/-}$ mutant *C. parapsilosis*, we stimulated human PBMC-DMs for 24 h with each strain and measured IL-1β, TNFα, IL-6 cytokine and IL-8 chemokine release. The obtained results indicate that PBMC-DMs stimulated with either the wild-type or *RI_SAPP1* and *RI_SAPP2* strains produced similar IL-1β, IL-8 and TNFα levels. In contrast, macrophages stimulated with *C. parapsilosis sapp1/2/3*$^{-/-}$ released significantly less IL-1β and IL-6, and moderately but not significantly less IL-8

compared to the WT (Fig. 24). PBMC-DMs stimulated with *RI_SAPP3* mutant *C. parapsilosis* cells produced significantly lower IL-8 and moderately low IL-6, however, no significant differences were observed in the secretion of IL-1β and TNFα compared to WT.

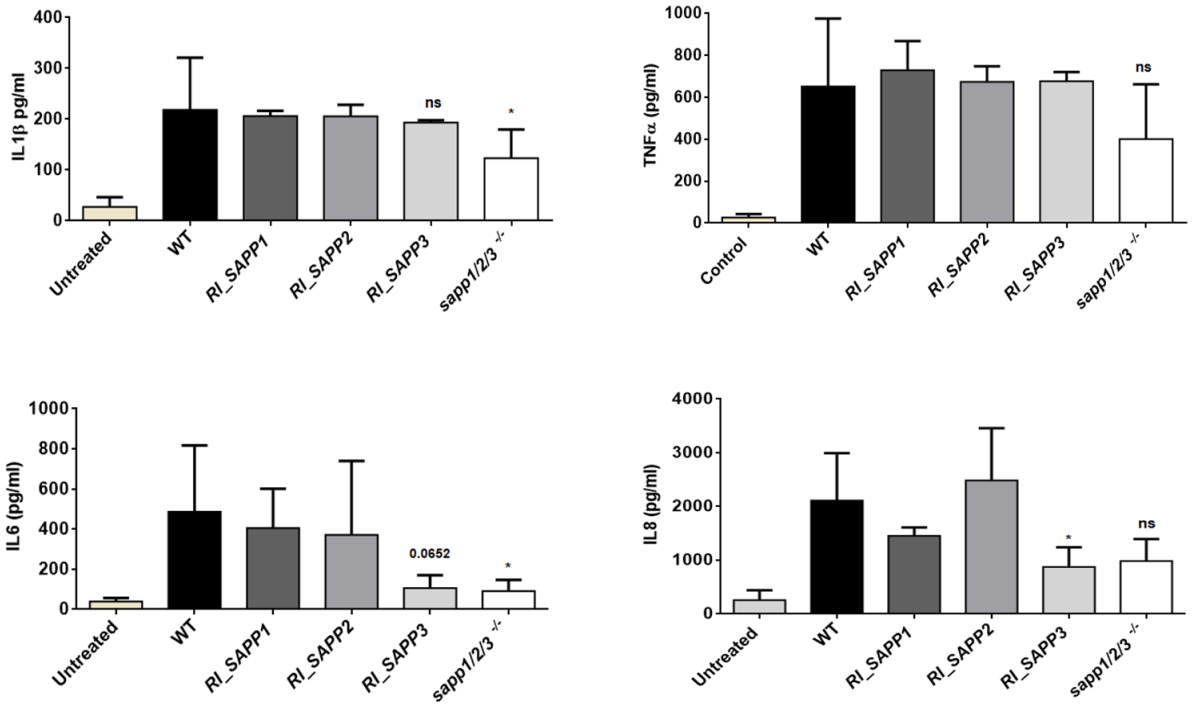


Figure 24: Cytokine secretion of human PBMC-DMs in response to WT and *SAPP* mutant *C. parapsilosis* strains. IL-1β, TNFα, IL6, and IL-8 levels were measured by ELISA after stimulation of PBMC-DMs with WT or *SAPP* mutant *C. parapsilosis* strains for 24 h. Data represent cytokine production ± SEM from 5 donors.

4.14 Sapp1 and Sapp2 have differential cleavage capacity against human complement proteins

Fungal proteases are known to inactivate various host factors including complement proteins such as C3b and C4b thus provide fungus an alternative mechanism to avoid host complement attack. Therefore, to test if *C. parapsilosis* Sapp proteins can also cleave human complement proteins we incubated human complement proteins C3b and C4b with the purified Sapp proteins[96,166]**.** Result indicated that Sapp1p and Sapp2p can

efficiently cleave the human C4b (Fig. 25A and B), whereas cleavage efficiency of Sapp1p is higher against C3b (shown with strong cleaved band) compared to Sapp2p (light cleaved band) (Fig. 25B). As a control, only C3b and C4b without Sapp proteins were incubated for the same period of time.

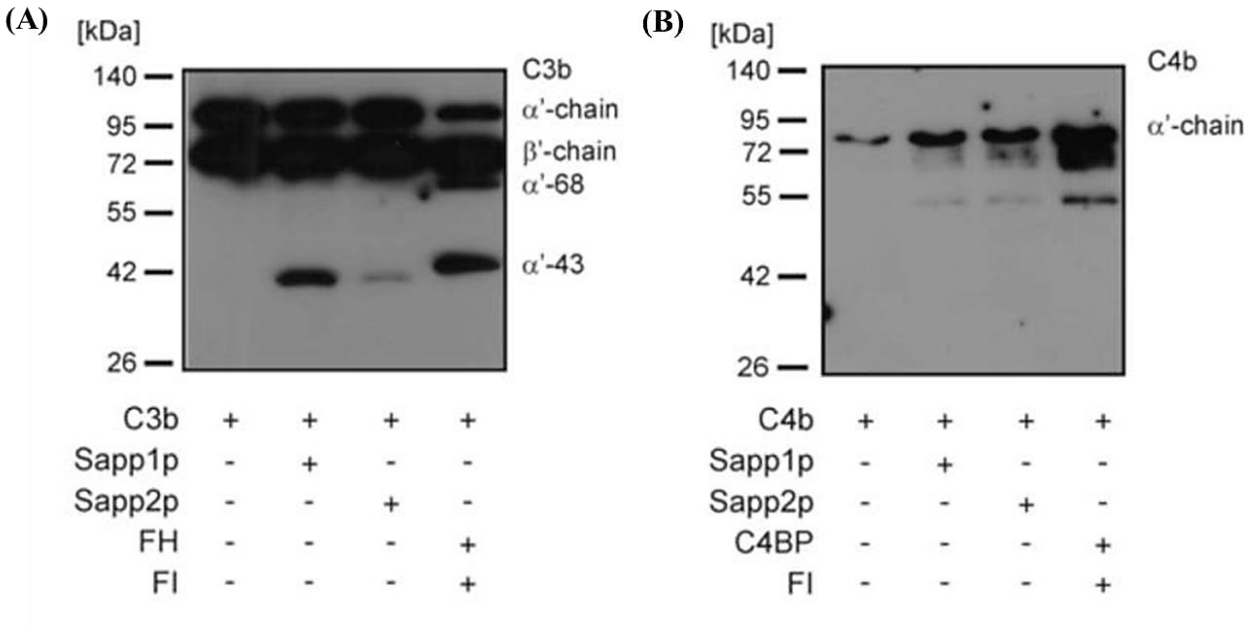


Figure 25: Sapp mediated cleavage of human complement proteins. Sapp1p and Sapp2p were incubated with various human complement proteins; after incubation, the mixture was separated by SDS-PAGE, transferred to a membrane and C3b fragments were identified by Western blot. (A) C3b incubated with Sapp1p (lane 2), Sapp2p (lane 3). C3b cleavage products obtained by the host specific serine protease Factor I which cleaves C3b in the presence of the cofactor Factor H are indicated in lane 4 and untreated C3b in lane 1. (B) Cleavage pattern of C4b by Sapp1p and Sapp2p. In the gel pictures above, extreme right lanes in the blots representing endogenous cleavage pattern of C3b and C4b.

4.15 Sapp2p but not Sapp1p can cleave FHR-5

In addition to the above mentioned complement factors, FH and CFH family proteins CFHL-1, CFHR-1, CFHR-5 play important role in modulating neutrophil behavior by binding to *C. albicans* and to neutrophils. Furthermore, CFH, CFHL1, and CFHR1 are also reported to increase attachment of neutrophils to *C. albicans* and recently proven to be positive regulators of the complement cascade[167]. Therefore, to delineate if *C. parapsilosis* Sapp1p and Sapp2p can modulate this relationship by cleaving FH and

CFH family protein we measured the capacity of Sapp1p and Sapp2p to degrade FH, CFHL1, CFHR1, and CFHR5. Sapp1p and Sapp2p both cleaved FH after 15 h but not 3 h of incubation (Fig. 26A). Interestingly, Sapp2p but not Sapp1p cleaved FHR5, indicating differential proteases activities of *C. parapsilosis* Sapps against human complement proteins (Fig. 26B). Coincubation of Sapp1p or Sapp2p with FHL-1 or FHR-1 revealed that the proteases cannot cleave these human complement proteins as visualized by Western blot (Fig. 26C and D).

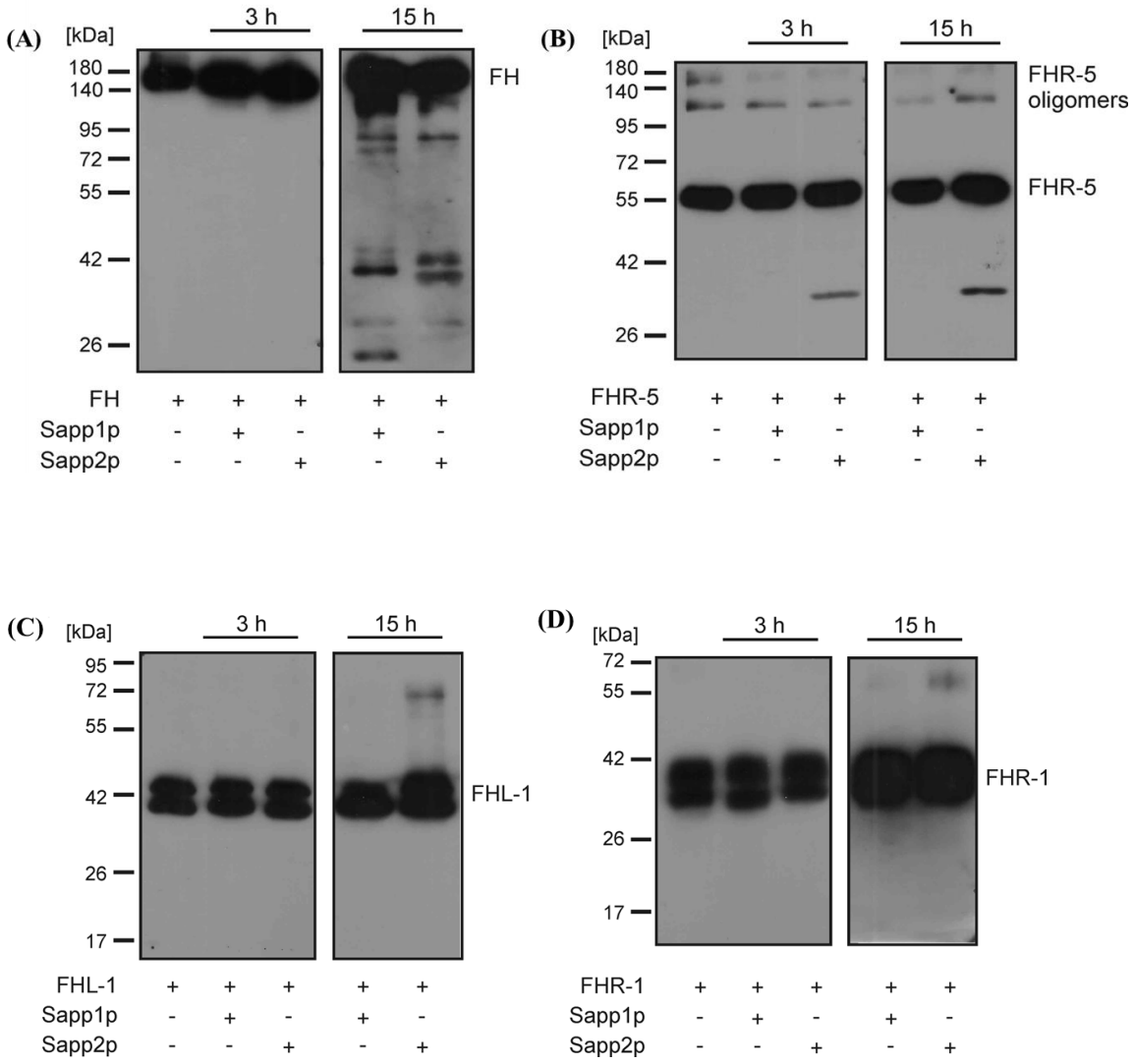
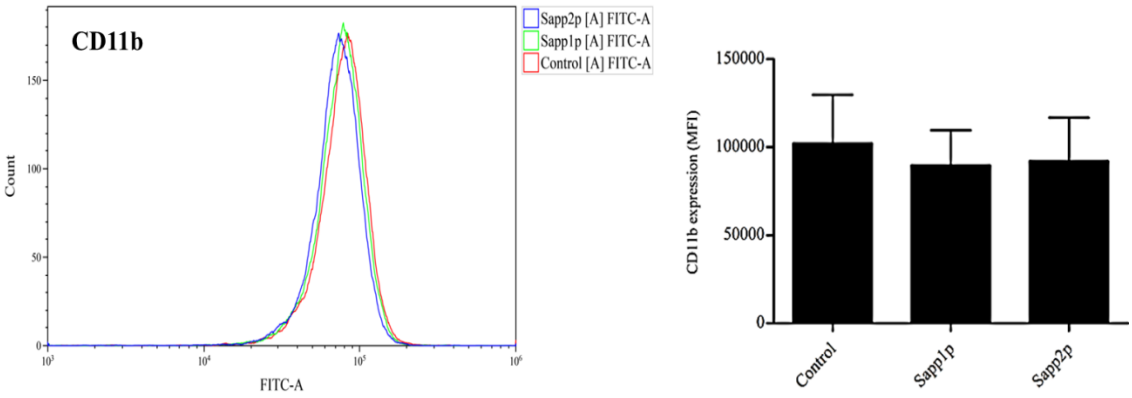


Figure 26: Sapp mediated cleavage of human complement like or related proteins. (A) Cleavage of FH by Sapp1p and Sapp2p. (B) Cleavage of FHR5 by Sapp2p. (C&D) Sapp1p and Sapp2p cannot cleave FHL1 and FHR1 either after 3 h or after 15 h of coincubation.

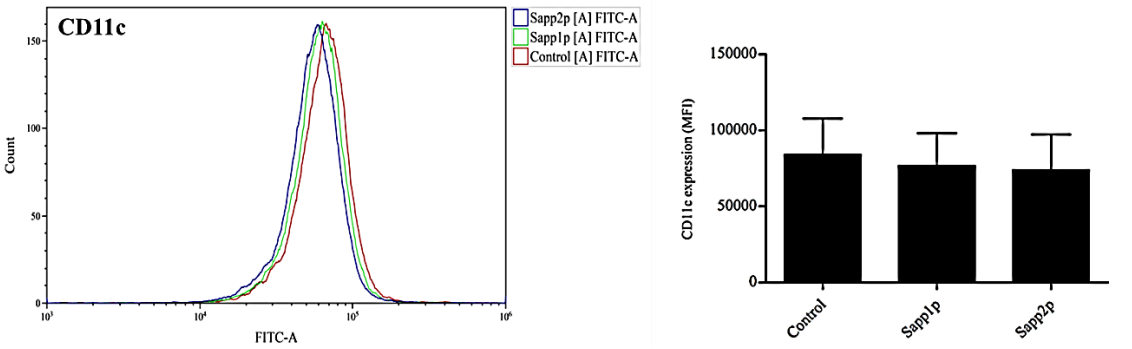
4.16 Sapps cannot alter the expression of CR3 and CR4 on macrophage surface

CR3 and CR4 are membrane bound receptors with multiple functions. CR3 (CD11b/CD18) plays important role in phagocytosis of opsonized pathogens, activates NK cells, acts as adhesion molecule for leukocytes and induces NETosis from neutrophils. Several bacterial proteases and *C. albicans* Saps are shown to modulate their expression on macrophages[125]. Thus, to understand the effect of *C. parapsilosis* Sapp1p and Sapp2p proteins on the cell surface expression of CR3 and CR4, PBMC-DMs were treated with these proteases. Unexpectedly, we did not found any substantial differences in the CR3 and CR4 expression on macrophages either after Sapp1p or Sapp2p treatment indicating in contrast to *C. albicans*, *C. parapsilosis* Sapp proteins cannot alter CR3 and CR4 expression on macrophages (Fig. 27 A-C).

(A)



(B)



(C)

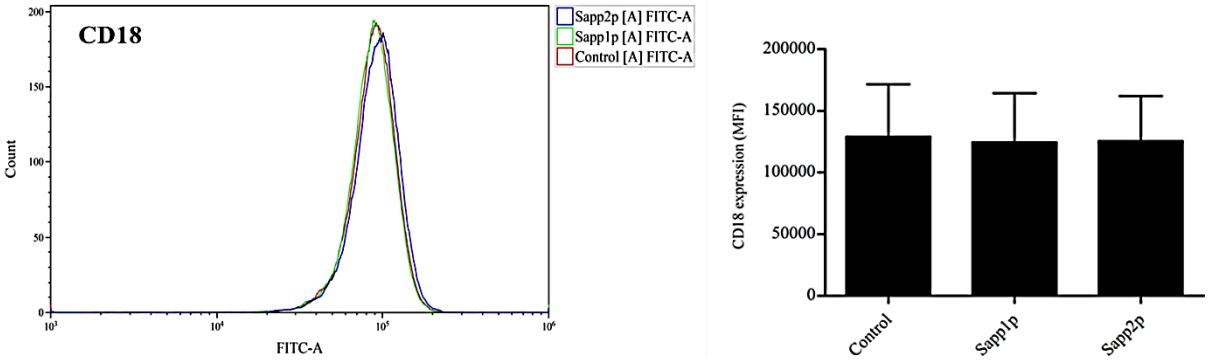


Figure 27: Sapps of *C. parapsilosis* cannot inactivate CR3 and CR4. PBMC-DMs were incubated with Sapp1p (5 µg of each) and Sapp2p for 30 mint. The expression of CR3 and CR4 was measured after the indicated time using mAbs specific to the receptor chains. Data represent mean ±SD of three experiments. No differences have been seen in the receptor expression.

4.17 *Galleria mellonella* infection

Galleria mellonella (wax moth) larvae have been proven to be effective non-mammalian infection model due to the easy maintenance, cost-effectiveness and most importantly similarities of innate immune system of *G. mellonella* with the innate system of mammals[168–170]. We have pursued this notion to use *G. mellonella* as a model to provide a robust assay system to assess *C. parapsilosis* virulence. To this context, *G. mellonella* was infected with WT and *SAPP* mutant *C. parapsilosis* strains. Additionally, for control *G. mellonella* injected with PBS and without any injection was also monitored for survival. The course of infection was followed for 24 h for determination of fungal load and seven days for survival. *G. mellonella* infected with WT show higher fungal burden compared to *RI_SAPP2*, *RI_SAPP3*, and *sapp1/2/3*$^{-/-}$ mutant *C. parapsilosis*, however, moderate but not significant decrease in CFU in larvae was obtained in *RI_SAPP1* (Fig. 28A). We recovered nearly similar CFU from *Galleria* infected either with WT or with *RI_SAPP1 C. parapsilosis* strains.

Survival rate of larvae infected with the WT decreased moderately but not significantly compared to larvae infected with *sapp1/2/3*$^{-/-}$ mutant *C. parapsilosis*. In addition,

larvae infected with *RI_SAPP* mutant *C. parapsilosis* strains showed no any significant difference in survival compared to WT (Fig. 28B).

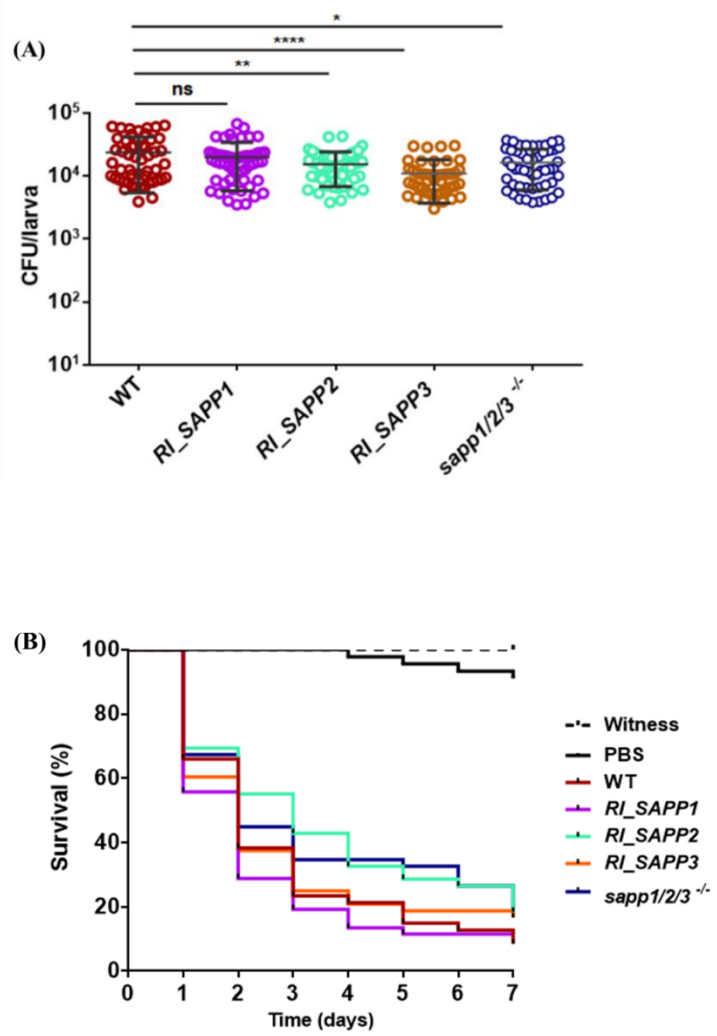
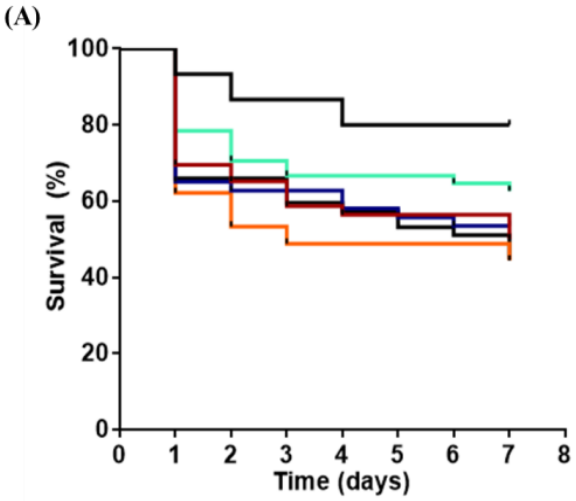


Figure 28: Fungal burden and survival of *Galleria mellonella* post infection. (A) Fungal burden WT, *sapp1/2/3*$^{-/-}$ mutant strain, and *RI_SAPP* strains were determined using *G. mellonella* larvae. For each individual larvae, 10^6 *Candida* cells were injected and the fungal burden was determined by number of CFU. Each mutant was tested four times using at least five larvae each time. The mean and SD of each data is represented by horizontal and vertical bars, respectively. (B) Survival curves of *G. mellonella* infected with *C. parapsilosis* WT, *RI_SAPP1*, *RI_SAPP2*, *RI_SAPP3*, and *sapp1/2/3*$^{-/-}$ mutant with 10^6 *Candida* cells/larvae. No significant difference in survival of larvae was observed after infection with different *C. parapsilosis* strains.

4.18 Survival of *Drosophila melanogaster* after *C. parapsilosis* infection

The host innate immune response appears to be of major player of host defense against invasive *Candida* infections in mice[171]. Consequently, *D. melanogaster*, which depend exclusively on an innate immune response to protect against pathogens, have been considered appropriate models to study *Candida* infections[172]. Therefore, we used *Drosophila* as a model to provide a robust assay system to assess *C. parapsilosis* virulence. We did not find any significant difference in survival either in wild-type Oregon R-S (OreR) flies or in MyD88 (an adapter protein involved in the Toll-like receptor and IL-1 receptor signaling pathway) knock out flies (MyD88$^{-/-}$) mutant flies infected with *C. parapsillosis* WT, *sapp1/2/3*$^{-/-}$, and *RI_SAPP* cells. Additionally, comparing the survival of OreR and MyD88$^{-/-}$ flies clearly showed that MyD88$^{-/-}$ flies were approximately two times more susceptible to the infection of WT *C. parapsilosis* during the first day than the wild-type flies (Fig. 29A and B). Moreover, killing of MyD88 mutant flies by *C. parapsilosis* was more efficient at whole examination period than killing of OreR flies, which clearly indicates that MyD88 signaling pathway plays important role in protection against *C. parapsilosis* infection in *D. melanogaster*.



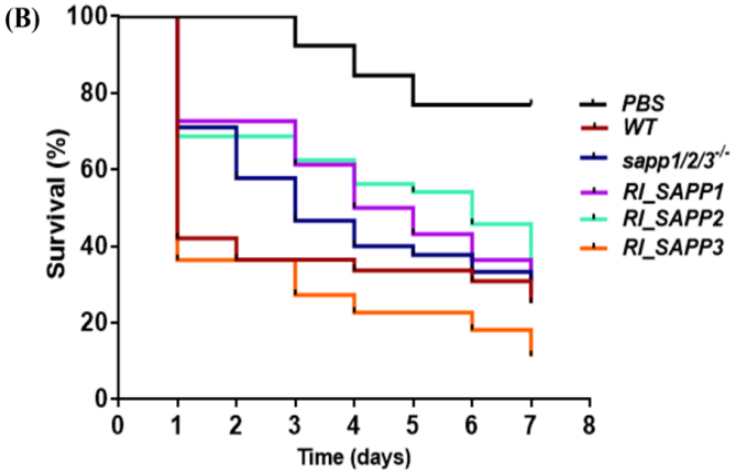


Figure 29: Estimated survival curves for OreR and MyD88$^{-/-}$ flies infected with *C. parapsilosis* WT and mutant strains. OreR (A), and MyD88$^{-/-}$ (B), flies (n=40) infected with WT and different mutant strains. For control, flies were injected with PBS to monitor the physical effects of the injection *per se*. Flies were checked on every day after post infection over 7 days of period. No significant difference was observed in the survival of the flies infected either WT or mutant *C. parapsilosis* strains.

5. Discussion

Aspartyl proteases are present in diverse range of microorganisms. These proteases play crucial role in nutrition and pathogenesis. Presence of aspartyl protease gene family in pathogenic non *Candida albicans Candida* species (NCAC) and absence in some non-pathogenic fungal species such as in *Saccharomyces cerevisiae* suggests its role in pathogenicity. The human fungal pathogen *C. parapsilosis* genome contains three aspartyl protease genes, named *SAPP1*, *SAPP2,* and *SAPP3* respectively. *SAPP1* can be found in duplicate (*SAPP1a, SAPP1b)* whereas, some sequences with high similarity with *SAPP2* was also identified in *C. parapsilosis* genome. The proteins level similarity among the *C. parapsilosis* Sapps and also with *C. albicans* Saps is quite low, thus, we presumed functional differences among Sapps in *C. parapsilosis*. Previously, in our laboratory *SAPP* null mutant *C. parapsilosis* strains *(*Δ/Δ*sapp1a*, Δ/Δ*sapp1b*, Δ/Δ*sapp1a/b*) were generated and it had been shown that *sapp* mutant strains are less virulent compared to wild-type *C. parapsilosis*. However, the study did not clearly highlight the role of individual *SAPP* genes in *C. parapsilosis* virulence. Thereof, to identify the roles of each aspartyl acid proteases in its virulence, first we generated mutant strain lacking all aspartyl protease coding genes (*C. parapsilosis sapp1/2/3*$^{-/-}$) and then integrated individual genes under the control of a strong constitutive *C. albicans* (*Ca*) TDH3 promoter (*Ca*TDH3) to *Cp*Neut5L locus. Further, we analyzed the virulence properties of wild-type, *RI_SAPP*, and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strains. Further, we also analyzed the protease activity of *C. parapsilosis* Sapp1p and Sapp2p against human complement proteins.

Cell wall composition of *Candida* spp. and their associated proteins play important role in determination of its morphology and also its recognition by host immune cells. Sapp proteins, such as Sap9 and Sap10 are associated with cell wall of *C. albicans* and reported to play role in cell wall integrity, biofilm formation, and interactions with hosts immune cells[173]. Therefore, first we tested role of *SAPP* genes in *C. parapsilosis* pseudohyphae, biofilm formation, and cell wall composition. Surprisingly deletion or reintegration of *SAPP* genes did not affect these properties of *C. parapsilosis*. We also

tested cell wall integrity of *C. parapsilosis* mutant strains by providing cell wall stress our results have shown that even providing higher amount of cell wall stress *sapp1/2/3*$^{-/-}$ grow similar to WT, indicating *SAPP* in *C. parapsilosis* did not play role in its stress tolerant abilities.

Efficient microbial adhesion to the epithelial cells is a crucial step for mucosal colonization and infection. Several *C. albicans* and *C. parapsilosis* cell wall associated proteins are engaged in attachment of *C. albicans* yeast to the host epithelium[174]. Once attached to epithelium pathogenic *Candida* spp. such as *C. albicans*, *C. dubliniensis*, and *C. tropicalis* undergo morphological switching from yeast to hyphae, which is a major virulence trait. *C. parapsilosis* and *C. glabrata* do not form true hyphae but still adhere to epithelial cells however, the attachment of non-hyphae/pseudohyphae forming cells are not as efficient as in the case of *C. albicans*. Apart from adhesion proteins such as Hwp1p (hyphal wall protein 1, Als (agglutinin-like sequence) proteins, Hyr1p (hyphal regulated protein, Eap1p (enhanced adherence to polystyrene), Sap4-6p bind to host integrins, Sap6p presumably serve as ligands for several cell surfaces receptors independently of its proteolytic activities in *C. albicans*. Interestingly, Sap9p and Sap10p have contrary roles in epithelial adhesion as deletion of the *SAP9* gene from *C. albicans* increases adhesion, while deletion of the *SAP10* gene reduces adhesion[173]. Thus, to understand the roles of *C. parapsilosis* Sapp proteins in adhesion to human epithelial cells we performed adhesion experiment using human TR146 and A-431 cell lines. Our results highlighted that deletion of all aspartyl protease genes of *C. parapsilosis* moderately, but not significantly reduce the adhesion of *C. parapsilosis* to epithelial cells. Of note, highest reduction was noted in case of *RI_SAPP3* strain whereas, reintegration of *SAPP1* and *SAPP*2 genes restored the adhesion efficiencies of *sapp1/2/3*$^{-/-}$ strain. These results indicate the involvement of *SAPP1* and *SAPP2* in attachment of *C. parapsilosis* to oral and vaginal epithelial cells.

We also investigated the interactions of WT and mutant strains with PBMC-DMs. Macrophages are central players of the immune system, connecting the innate and adaptive arms of the host's immune responses. Immune cells respond against pathogens

either by phagocytosis mediated killing or activating partner cells by secreting cytokines. Phagocytosis starts by ingeston of pathogens. Internalized *Candida* cells get enclosed in phagosomes, which further fused with lysosome. Fusion leads to discharge of the lysosomal enzymes into the resulting phagolysosomes. Notably, some bacterial pathogen such as *Mycobacterium tuberculosis*, *Toxoplasma gondii* and fungus *C. albicans* and *C. glabrata* evade phagolysosome mediated killing by altering phagosomal pH, hyphae mediated rupturing of phagosomal wall and by utilizing amino acids inside the phagosomes[175]. Of note, acidic pH (4.5-5) of phagolysosome also favors the activity of secreted aspartyl proteases. Expression of Sap genes of *C. albicans* and *C. tropicalis* after ingestion by immune cells suggest their role in fungal adaptation to the intracellular environment[176]. Presumably, acidic environment of phagosome-lysosome intensifies the activity of acid pH induced pathogenic factors, including secreted aspartyl proteases which in turn benefit microbial intracellular survival. Here, in present study interestingly we also reported less phagolysosome fusion in macrophages infected with *C. parapsilosis* WT compared to *sapp* null mutant strain.

Flow cytometric analysis revealed that primary human monocyte-derived macrophages efficiently ingest and kill *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ mutant *C. parapsilosis* strains compared to WT *in vitro*. In addition to the intracellular killing of microbes, the secretion of inflammatory mediators by macrophages is essential for the efficient eradication of pathogens. TNFα, IL-1β, IL-6, and IL-8 are the potent inflammatory cytokines with pleiotropic effects, including the enhancement of antimicrobial capacity of neutrophils and macrophages, modification of vascular permeability, and recruitment of leukocytes to the site of inflammation, whereas the chemokine IL-8 is the most potent activator of neutrophil recruitment. Release of several inflammatory mediators were analysed in macrophages in response to WT and mutant strains of *C. parapsilosis.* We found that macrophages stimulated with wild-type and *RI_SAPP1* or *RI_SAPP2* strains of *C. parapsilosis* produced more IL-1β and IL-6 and mild but not significantly higher IL-8 and TNFα after 24 h compared to *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ mutant strains. Interestingly, we also observed that Sapp1 and Sapp2 play important role in

adhesion and cause damage to vaginal epithelial cells (A-431), indicating important role of *C. parapsilosis* Sapps in vaginitis. However, further studies are required to get clear understanding of *C. parapsilosis* aspartyl proteases role in vaginitis.

With nearly 50 serum and cell membrane-anchored proteins complement system plays an essential humoral part of innate immunity. These complement proteins act in a systemic myriad to opsonize the microbes and facilitate their phagocytosis, activate cellular responses, initiate inflammation, or directly lyse certain microbes by forming the pores in their cell wall[177]. C3, as a crucial component of complement pathways and plays a central role in activation of all three complement pathways. Factor H (FH) which is composed of 20 individually folding complement control proteins (CCP) or short complement regulatory (SCR) domains, act as regulator of complement pathway by binding with C3b through its N-terminal CCP1–4 domains, C3b/C3d, pentraxins, and sialic acid/ glycosaminoglycans through C-terminal CCP19–20. Thus binding of FH on host surfaces under complement attack restricts complement activation. FH is reported to induce secretion of IL-1β from human monocytes and chemokine IL-8 from neutrophils[125]. Recently, FH family proteins FHR and FHL are also reported to be crucial for complement cascade regulation but exact roles of these proteins are uncertain[178]. FHR-1 was reported to inhibit C5 and the terminal pathway whereas FHR-2 inhibits the alternative pathway and activation of the terminal pathway and FHR-3 and FHR-4 enhances the cofactor activity of FH. FHR-5 was to display weak cofactor activity and inhibit the C3 convertase in fluid phase. In general, FHR proteins appear to lack significant complement inhibitory activity, but further studies are needed to clarify if any of the FHRs possess some form of such activity[128]. Prevailing evidence suggest that bacterial and fungal pathogens can effectively bypass or alter host complement attack either by recruiting complement regulator protein on their cell surface or by cleaving them by secreting aspartyl proteases.

We evaluated the growth inhibitory efficiency of human serum against *C. parapsilosis* wild-type and mutant strains. Congruent with the previous study, we found that *RI_SAPP3* and *sapp1/2/3*$^{-/-}$ mutant strains are hypersensitive towards human serum

compared to wild-type, *RI_SAPP1*, and *RI_SAPP2 C. parapsilosis* strains. Heat inactivation of serum did not show any growth inhibitory effect either on wild-type or mutant strains. Heat inactivation of serum reported to inhibit the activity of complement component C2 and factor B (FB) of complement system. C2 is the crucial component in the activation of classical and lectin pathways, whereas FB is important for alternative pathway[179]. Inactivation of C2 and FB cannot lead activation of complement pathways and that can be one of the possible reasons why heat inactivated plasma did not alter the growth of *C. parapsilosis* wild-type or mutant strains.

To further determine whether *C. parapsilosis* Sapps can cleave of these complement proteins and regulators, we incubated human complement proteins with Sapp1p and Sapp2p for different period of time. Interestingly, our results demonstrated divergent cleavage activity of Sapp1p and Sapp2p on complement proteins. Sapp1p and Sapp2p both can cleave C3b, C4b, and FH whereas, Sapp2p but not Sapp1p cleaved FHR-5. Interestingly, both of the protease cannot cleave FHL-1 or FHR-1. The cleavage of FHR-5 but not FHL-1 and FHR-1 clearly demonstrates that the protease activity of Sapp2p presumably lies somewhere near to SCR 3,4,5,6 or 7 SCR domains which are absent in FHL-1 and FHR-1 but present in FHR-5 and FH, however, further detailed studies are needed to confirm the Sapp cleavage sites in complement proteins. Taken together, here we demonstrated for the first time that Sapp1p, Sapp2p both can cleave complement proteins C3b, C4b and FH but not FHR-1 and FHL-1 whereas only Sapp2p can cleave FHR-5. With this, the roles of these proteases on complement receptors such as CR3 and CR4 were largely unknown thus, we aim to characterize the expression of these receptors mainly on Sapp1p and Sapp2p treated human macrophages. Unexpectedly, we did not find any change in expression of either on CR3 or CR4 receptor on Sapp treated macrophages. However, this does not exclude the possibility that CR3 and CR4 functions are not affected by these proteases as they can efficiently bind with complement proteins C3b, iC3b and FH which are efficiently cleaved by these proteases.

In conclusion, our data show that secreted aspartyl proteases of *C. parapsilosis* promotes the survival of fungal cells in macrophages and mitigate the inflammatory response of the host, thereby interfering with the efficient clearing of the pathogen. As per our best knowledge in present study first time we have reported that Sapp1p and Sapp2p have differential cleavage activities against human complement proteins.

Even though, the finding of present study made a considerable progress to understand the role of *C. parapsilosis* aspartyl proteases in modulation of the behavior of macrophages and host complement cascade further understanding of the signalling cascades induced by *C. parapsilosis* aspartyl proteases in immune cells undoubtedly provide a great insight into the mechanisms that underpin during its virulence and could lead us towards new therapeutic interventions against *C. parapsilosis* caused pathogenesis.

6. Summary

Candida parapsilosis is one of the most frequently isolated, non *Candida albicans Candida* (NCAC) species from preterm infants and neonates, skin and nails of healthcare personnel. Being a skin commensal it has unique abilities to adhere on physical surface and possess potent abilities to form biofilm on indwelling devices, such as central venous catheters. Among NCAC species, infections caused by *C. parapsilosis* increasing worldwide and it became the second or third most common yeast isolated from hospitals of Asian and American countries whereas, the increasing incidence in European countries is also reported. Several factors adhesion to host surfaces, switching from yeast to pseudohyphal form, biofilm formation and secretion of hydrolytic enzymes such as lipases, phospholipases and aspartyl proteases are considered as crucial virulence factors. Historically *C. parapsilosis* are considered to possess three aspartyl protease genes designated as *SAPP1*, *SAPP2*, and *SAPP3* but recently 14 more potential aspartyl protease encoding genes were identified. Proteins coded by these aspartyl protease genes considered important for nutrient acquisition and critical for penetration of host epithelial barrier. Crystal structures of Sapp1p and Sapp2p are known but their virulence properties remain enigmatic. Therefore, present study was designed to get deeper insight of virulence properties of *C. parapsilosis* Sapps.

To identify the virulence properties of individual Sapp isoenzymes in present study we generated reintegrate strains of *SAPP1*, *SAPP2*, and *SAPP3* under a strong promoter in a *SAPP* null (*sapp1/2/3*$^{-/-}$) mutant of *C. parapsilosis*. Further to demonstrate if reintegration of *SAPP* genes alter the physical attributes of *C. parapsilosis*, growth rate, pseudohyphae formation, stress tolerance, alteration in cell wall composition, and biofilm formation of mutant strains were examined. Interestingly, these mutant strains did not show any difference either in cell wall composition or in biofilm formation however *sapp* null (*sapp1/2/3*$^{-/-}$) and *RI_SAPP3* mutant *C. parapsilosis* showed reduced adhesion to human epithelial cells compared to the wild-type strain. Further, virulence properties of the mutant strains were also examined using *in vitro* and *in vivo* models. The *sapp* null (*sapp1/2/3*$^{-/-}$) and *RI_SAPP3* mutant *C. parapsilosis* caused less damage to human PBMC-DMs, phagocytosed and killed more efficiently compared to

wild-type. In addition, *sapp1/2/3*$^{-/-}$ mutant strain was less capable to induce inflammatory cytokine release, mainly IL-1β, compared to the wild-type *C. parapsilosis*. More colony forming units (CFU) was recovered from *Galleria mellonella* infected with either wild-type or *RI_SAPP1* strain compared to other mutant strains, which clearly indicate that *SAPP1* play important role in *C. parapsilosis* virulence.

C. parapsilosis activate all three pathways of complement system. Activated complement system act as host weapon against pathogens. Thus, to avoid complement attack pathogen either cleaves complement proteins or regulates their activation by recruiting complement regulator proteins on their cell surface. Till today, role of *C. parapsilosis* secreted aspartyl proteases in regulation of complement cascade is largely unknown. Here, using purified Sapp1p and Sapp2p we showed that these proteases can efficiently cleave human complement proteins C3b, C4b. Complement regulators control the spontaneously activated complement cascade and thus prevent unwanted self-tissue destruction and autoimmune disease. Interestingly, factor H (FH), a potent complement regulator of alternative complement pathway is also cleaved by both Sapp1p and Sapp2p. Complement factor H-related/like proteins (FHRs/FHLs) are recently proven to be positive complement regulators. Surprisingly, FHR-5 is cleaved by Sapp2p but not by Sapp1p whereas Sapp1p and Sapp2p cannot cleave FHR-1 and FHL-1.

Taken together, results of the present study suggested that even though deletion and reintegration of *SAPP*s did not affect physical and morphological attributes of *C. parapsilosis* much extent, it significantly affects the virulence properties of *C. parapsilosis* including its adhesion properties to epithelial cells, damage causing efficiency, phagosomes-lysosome fusion, and inflammatory responses from human blood-derived macrophages. In addition, as per best of our knowledge first time we have shown that Sapp1p and Sapp2p can cleave human complement proteins, thus; presumably alter the complement mediated immune responses.

We believe that findings of this study could potentially lead to provide an additional rationale for Sapp blockade as a therapeutic alternative in *C. parapsilosis* caused pathogenesis.

7. Hungarian summary

A *Candida parapsilosis* az úgynevezett „nem-*Candida albicans*” *Candida* fajok (NCAC) körébe tartozó élesztőgomba, melyet legtöbbször koraszülöttekből, újszülöttekből és a kórházi dolgozók bőr- és köröm felszínéről izolálnak. Bőrfelszíni kommenzalista fajként, egyedi tulajdonságai révén képes adhézióra, továbbá biofilm képzésére különböző beültetett orvosi eszközökön, például centrális vénás katéterek felületén. A „nem-*albicans*” *Candida* fajok közül a *C. parapsilosis* okozta egészségügyi megbetegedések száma egyre nagyobb számban fordul elő világszerte. Több ázsiai és amerikai ország kórházaiban a második vagy harmadik legtöbbször izolált élesztőgombának számít, emellett egyre több európai esetről is beszámoltak már. Számos faktort megemlíthetünk, melyek virulencia faktorként funkcionálnak és meghatározzák a faj fertőzőképességét. Ilyen például az adhéziós képesség a gazdaszervezet sejtjeinek felületén, morfológiai váltás (élesztő formából pszeudohifás forma), biofilm képzés és hidrolitikus enzimek (lipázok, foszfolipázok, aszpartil proteázok) szekréciója. Eredetileg úgy tartották, hogy a *C. parapsilosis*nak három aszpartil-proteáz kódoló génje van, melyet az alábbi módon jelöltek: *SAPP1, SAPP2* és *SAPP3.* A közelmúltban viszont további 14 aszpartil-proteázt kódoló gént azonosítottak filogenetikai módszerekkel. Ezen gének által kódolt fehérjék fontos szerepet játszanak a tápanyagfelvételben és a gazdaszervezet hámrétegébe történő bejutás során. Míg a Sapp1p és Sapp2p fehérjék kristályszerkezete ismert, addig a virulenciában betöltött szerepük továbbra is kérdéses. Ezért a jelen kutatási munkában célul tűztük ki a *C. parapsilosis* Sapp fehérjék virulenciában betöltött szerepének részletesebb megismerését.

A jelen kutatási munkában az egyes Sapp izoenzimek virulencia tulajdonságainak azonosítása érdekében létrehoztunk *SAPP1, SAPP2* és *SAPP3* reintegrált törzseket erős promóter szabályozása alatt, *sapp* null *(sapp1/2/3*$^{-/-}$) mutáns *C. parapsilosis* törzsben. További célunk volt bemutatni ezen reintegrált *SAPP* gének lehetséges hatását a *C. parapsilosis* fiziológiai tulajdonságaira, növekedési sebességére, pszeudohifa képzésére, stressztűrő képességére, sejtfal összetételére és biofilm képzésére. Érdekes módon ezen mutáns törzsek nem mutattak változást sem a sejtfal összetételében, sem a biofilm

képzésében a vad típussal összehasonlítva. Azonban a *sapp1/2/3*$^{-/-}$ és *RI_SAPP3* mutáns *C. parapsilosis* törzsek kisebb mértékű humán hámsejtekhez történő adhéziós képességet mutattak, mint a vad típusú törzs. Ezenkívül vizsgáltuk az említett törzsek virulencia tulajdonságait *in vitro* és *in vivo* modellek bevonásával is. A *sapp1/2/3*$^{-/-}$ és *RI_SAPP3* mutáns *C. parapsilosis* törzsek esetében az emberi PBMC-DM sejtek kisebb mértékű károsodását és hatékonyabb fagocitózist figyeltünk meg, szemben a vad típusú törzzsel. A *sapp1/2/3*$^{-/-}$ mutáns törzs a vad típusú törzshöz viszonyítva kisebb mértékben indukálta gyulladásos citokinek szekrécióját, főként az IL-1β-t. Nagyobb számú kolóniaképző egységet (CFU) határoztunk meg a vad típusú és *RI_SAPP1* törzsekkel fertőzött *Galleria mellonella* lárvákban, mint a többi mutáns törzsben. Ez alapján megállapítható, hogy a *SAPP1* fontos szerepet játszik a *C. parapsilosis* virulenciájában.

A *C. parapsilosis* a komplementrendszer mindhárom útvonalát képes aktiválni. Az aktivált komplementrendszer a gazdaszervezet védelmét biztosítja a kórokozók ellen. Ahhoz, hogy ezt a védelmi vonalat a kórokozó elkerülje, képes hasítani a komplement fehérjéket vagy úgy szabályozza ezen fehérjék aktivációját, hogy az őket szabályozó fehérjéket a saját sejtfelszínére toborozza. Napjainkig a *C. parapsilosis* által szekretált aszpartil-proteázok szerepe a komplementkaszkád aktiválásban nagyrészt ismeretlen. Tisztított Sapp1p és Sapp2p alkalmazásával kimutattuk, hogy ezen proteázok képesek hatékonyan hasítani a C3b és C4b humán komplement fehérjéket. A komplement szabályozók a spontán aktivált komplementkaszkádot vezérlik, megakadályozva ezzel a nem kívánatos szövetpusztulást és autoimmun betegség kialakulását. A Sapp1p and Sapp2p képes hasítani a H-faktort (FH) is, mely a komplementrendszer alternatív útvonalának szabályozója. A H-faktorral rokon és H-faktor-szerű fehérjék (FHR és FHL) bizonyítottan pozitív komplementszabályzók. Meglepő módon az FHR-5-t a Sapp2p hasítja, de a Sapp1p nem, valamint a Sapp1p és Sapp2p egyike sem tudja hasítani az FHR-1-t és FHL-1-t sem.

Összegezve a jelen kutatási munkánkban elért eredményeket elmondható, hogy bár a *SAPP* gének deléciója és túlzott expressziója sem befolyásolta nagymértékben a *C. parapsilosis* fizikai és morfológiai tulajdonságait, addig virulencia képességére

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Úgy gondoljuk, hogy a jelen kutatási munkánk eredményei hozzájárulhatnak egy jövőbeni, a *C. parapsilosis* okozta fertőzések kezelésére a Sapp-blokádot alternatív terápiaként alkalmazó szer kifejlesztéséhez.

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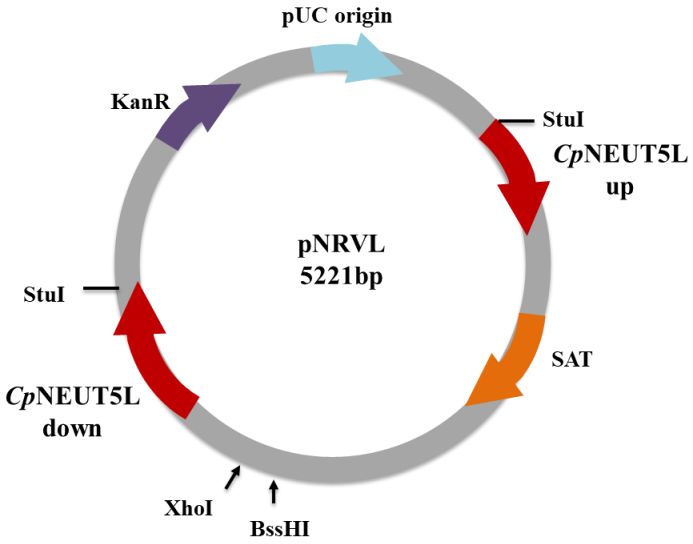
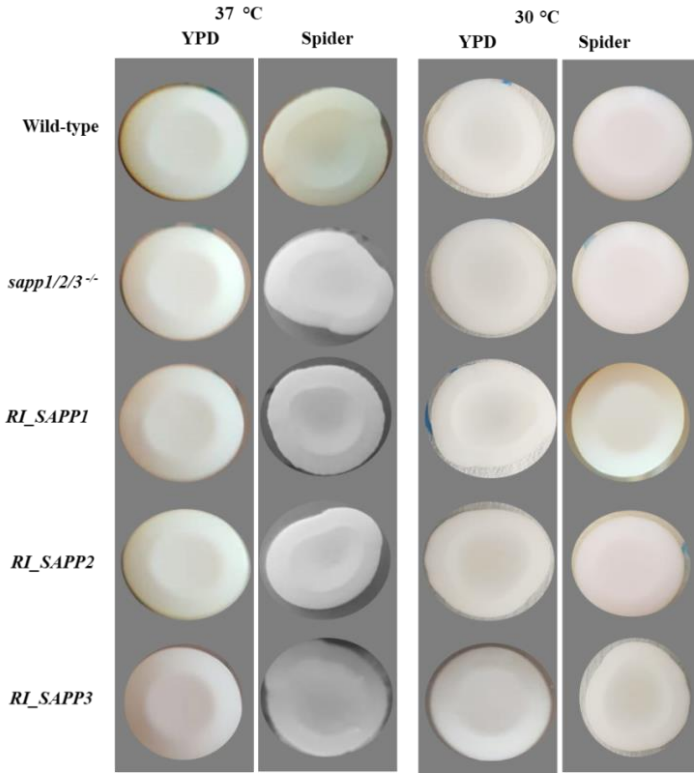
11. Supplementary Information

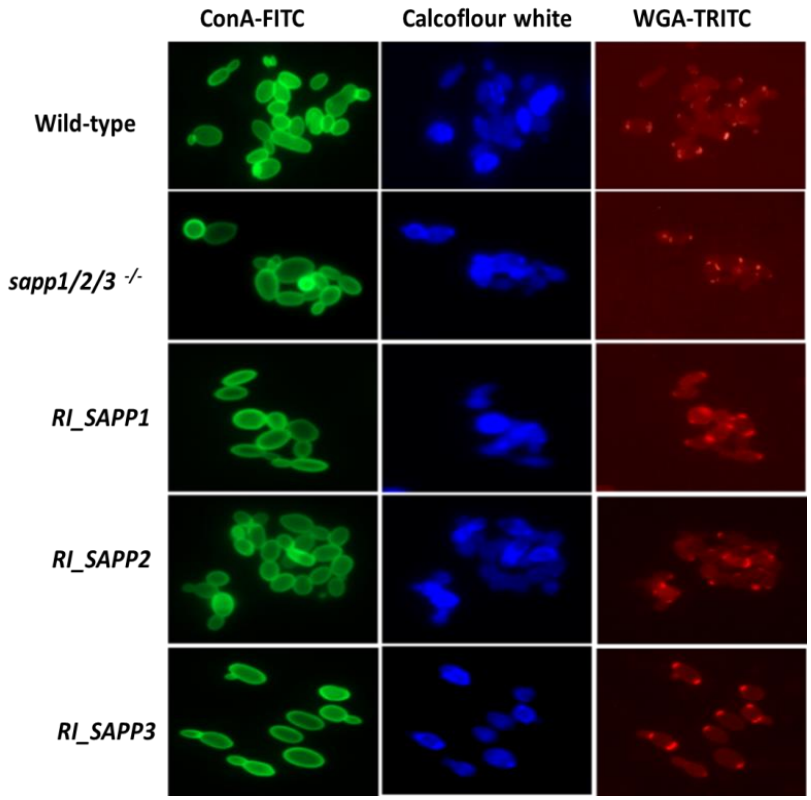


Supplementary Figure 1: Diagrammatic representation of pNRVL plasmid.

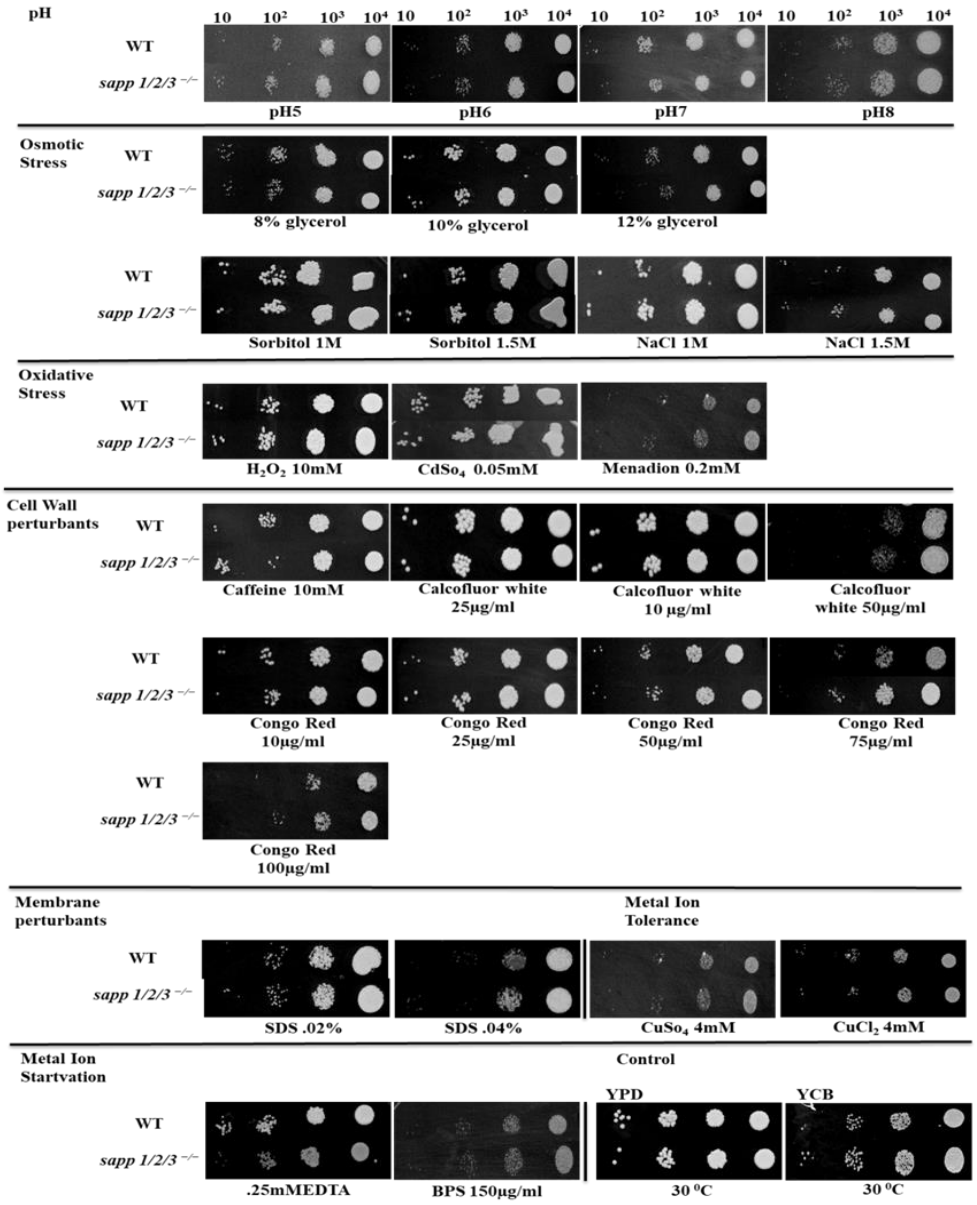
Kan- Kanamycin, *Xho*I, *BssH*I, *Stu*I – restriction enzymes



Supplementary Figure 2: Deletion and reintegration of *SAPP* gene in *C. parapsilosis* did not alter morphology. Change in morphology was examined by spotting wild-type and mutant strains onto different media. No alteration in colony morphology in mutant strains were observed after 5 days of incubation on spider and YPD media at 37 and 30



Supplementary Figure 3: Deletion and reintegration of *SAPP* gene did not affect cell wall content in *C. parapsilosis*. Cells were stained with calcofluor white (CFW), wheat germ agglutinin (WGA) and Concanavalin A-FITC to visualize the cell wall content. No difference in cell wall content was observed in deletion or reintegration of *SAPP* genes.



Supplementary Figure 4: Phenotype screening of *C. parapsilosis* wild-type and *sapp1/2/3*$^{-/-}$ strains. Overnight strains grown in YPD were diluted and 5µl of this dilution was plated onto YPD medium, without or with the addition of a variety of stressors as described in *Materials and Methods*. Deletion of *SAPP* genes in *C. parapsilosis* did not result in any growth defect compared to WT when grown at 30 °C at various stress conditions.

Supplementary Tables

Supplementary Table 1: List of strains used in present study.

Strain name	**Parent strain**	**CpAR gene ID**	**Genotype**	**Reference**
GA1				175
RI_SAPP1	*sapp1/2/3*$^{-/-}$	CPAR2_102410	*CpNEUT5L/Cpneut5l : : CpTDH3-SAPP1-NAT1*	This study
*RI_SAPP*2	*sapp1/2/3*$^{-/-}$	CPAR2_102580	*CpNEUT5L/Cpneut5l : : CpTDH3-SAPP2-NAT1*	This study
*RI_SAPP*3	*sapp1/2/3*$^{-/-}$	CPAR2_102420	*CpNEUT5L/Cpneut5l : : CpTDH3-SAPP3-NAT1*	This study
sapp1/2/3$^{-/-}$	GA1		*Δ/Δsapp1a-Δ/Δsapp1b-Δ/Δsapp2-Δ/Δsapp3*	

Supplementary Table 2: List of primers used in the present study

***Cp* Primers (Colony PCR)**	**Sequence**
SAPP1 (F)	5’-TAAACTGCTTCATTGCTGGTGT-3’
SAPP2 (R)	5’-TGTACCAATCAGGGTTAGTGACC-3’
SAPP2 (F)	5’-GTCATATGGGGGATTTGCAC-3’
SAPP2 (R)	5’-TGTACCAATCAGGGTTAGTGACC-3’
SAPP3 (F)	5’-CTGGGTCATTGATGCAAATTC-3’
SAPP3 (R)	5’-TGTACCAATCAGGGTTAGTGACC-3’
qRT-PCR	
***Cp* Primers (Real time)**	**Sequence**
*SAPP*1 (F)	5’-ACTGGACAACAAATTGCAGATG -3’
*SAPP*1 (R)	5’-TAAACTGCTTCATTGCTGGTGT-3’
SAPP 2 (F)	5’-GTCATATGGGGGATTTGCAC-3’
SAPP 2 (R)	5’-CGCTTTGCTGATGTTACCAG -3’

SAPP 3 (F)	5'-CTGGGTCATTGATGCAAATTC-3'
SAPP 3 (R)	5'-AGGTTGAGGTGTCTGGATCG-3'
TUB4 (F)	5'-GAACACTTATGCCGAGGACAAC-3'
TUB4 (R)	5'-ACTCTCACCACTGACTCCTTGC-3'

Supplementary Table 3: Stress conditions used for screening of stress tolerance of wild-type and *sapp1/2/3*$^{-/-}$ *C. parapsilosis* strain. Growth of the mutants was determined in 31 different stress conditions; YPD or YCB used as a base media.

Condition	**Reagent**	**Concentration/percent**
pH	5,6,7 and 8	
Osmotic stress	Glycerol	8%, 10%, 12%
	Sorbitol	1M, 1.5M
	Nacl	1M, 1.5M
Oxidative stress	H_2O_2	10mM
	$CdSo_4$	0.05mM
	Menadione	0.2mM
Cell wall perturbant	Caffeine	10mM
	Calcoflour white	10, 25,50µg/ml
	Congo Red	10, 25,50, 75, 100µg/ml
Membrane perturbant	SDS	0.02%, 0.04%
	$CuSo_4$	4mM
	$CuCl_2$	4mM
Metal ion starvation	EDTA	0.25mM
	BPS	150µg/ml