**INTRODUCTION**

Candidiasis is a fungal disease with a wide spectrum of clinical entities ranging from mucosal-cutaneous infections to life-threatening invasive disease, associated with high mortality rates. Candida species are the third to fourth most common isolates from bloodstream infections in hospitalised patients, who are neutropenic or immunocompromised, due to haematological malignancies, organ transplantation or immunosuppressive therapy. Patients in intensive care units (ICU) or post major abdominal surgery are at high risk of candidemia, as well. Even under antifungal therapy, in 30-40% of cases the outcome is lethal.

The mucosal-cutaneous forms of candidiasis, although generally, not life-threatening, may still be debilitating and affecting health and well-being. The mucosal-cutaneous forms, particularly vaginal infections, have a high prevalence. Vulvo-vaginitis caused by Candida species is the second most common vaginal infection. These infections are not reportable, however statistics reveal that 70-75% of women during the fertility age will suffer from at least one clinical episode of vulvo-vaginal candidiasis and 5-10% are at risk of recurrent infections.

**Summary**

The interaction of Candida albicans with the host is of a complex nature involving fungal factors and host’s response. In this study, we concentrated on the phenotypic expression of virulence attributes and genotypic characteristics of C. albicans isolates from two distinct clinical entities of candidiasis–bloodstream and vaginal infections, and the possible role of these factors. Hence, we conducted a comparative in vitro assessment of virulence characteristics, including adhesion to epithelial cells and HaCat cell line, biofilm formation, aspartic proteinases and phospholipase activity of 20 C. albicans isolates from patients with C. albicans bloodstream infection and 22 isolates from patients with C. albicans vaginitis. Further, we studied the epigenetic phenotypic switching of the strains and their ploidy, by flow cytometry and CHEF techniques. These studies indicated that although no overall differentiation between the isolates of the two groups (bloodstream infection and vaginitis) could be demonstrated, several characteristics were more specific to one of the groups than the other. While the strains from vaginal infection had higher capacity to adhere, the strains from patients with bloodstream infection had higher activity of phospholipase. Differences were also noted in phenotypic switching, with the strains from bloodstream infection revealing primarily the “white” type colonies, known to be more virulent, and had higher DNA content. This study is unique considering the concurrent comparison of isolates from different clinical entities, at the phenotypic and genotypic level.

**KEYWORDS**

Candida albicans, genotyping, virulence factors
Of the about 200 known Candida species, C. albicans is the most common aetiological agent of infection, both in invasive as mucosal-cutaneous entities.\cite{2,4,7} C. albicans is a human commensal, with a natural habitat in the gastro-intestinal (GI) tract, skin and female genital organ.\cite{8} Although it is believed that the host’s immune status and other human defence systems are the defining factors whether this fungus will be either a benign coloniser, a causative agent of a superficial or invasive infection,\cite{9,11} the interaction of this fungus with the host is more complex, as elegantly described in a recent review of Hofs et al.\cite{12}. The interplay between the expression of the fungal virulence features and the host’s response may play a role, contributing to the end result. To investigate this complex interaction is beyond the scope of our present study.

In this study, we concentrated on the phenotypic expression of virulence attributes and genotypic characteristics of isolates from two distinct clinical entities of candidiasis—blood stream and vaginal infections, and the possible role of these factors.

Preliminary data from an in vitro study indicated\cite{13,14} that such differences may exist. In vivo experimental infections in vertebrate and non-vertebrate models, although not fully conclusive, pointed to such differences,\cite{15} as well.

In this study, we conducted a comparative in vitro assessment of virulence characteristics, including adhesion to epithelial cells and HaCat cell line, biofilm formation, aspartic proteinases and phospholipase activity, as well as phenotypic switching of 20 C. albicans isolates from patients with bloodstream infections and 22 isolates from patients with vaginal disease. In addition, the strains were also analysed with regard to ploidy using flow cytometry and CHEF techniques.

The following manuscript details the data yielded by these investigations and the conclusions thereof. Specifically, we have found that strains isolated from vaginal infection had higher ability to adhere to HaCat cells, while those from invasive disease had higher phospholipase activity. In addition, there were differences as to DNA content and in phenotypic switching.

2 | MATERIALS AND METHODS

2.1 | Candida albicans strains, growth and maintenance

This study included 44 Candida albicans strains:

1. Twenty strains isolated from the blood of hospitalised patients with Candida blood stream infection, as per clinical diagnosis and relevant patient background, which were designated as S (systemic) strains. Specifically, 18/20 were with a central venous catheter (CVC), apparently the source of infection, 6/20 underwent abdominal surgery, four of which received total parenteral nutrition (TPN), 4/20 underwent haemodialysis.

2. Twenty-two strains isolated from patients with vaginal infection in a community clinic, designated as M (mucosal) strains.

3. C. albicans strain CBS 562 (ATCC 18804), which served as control strain throughout this study, is considered as type species strain\cite{16} in the Centraal Bureau of Schimmel Cultures (CBS) collection (Utrecht, the Netherlands) and has been used extensively in our research.

An additional control strain used in some experiments was SC 5314, a well-studied strain. All original S and M isolates were frozen at -80°C. For experimentation, strains were moved to -20°C or 4°C and sub-cultured on Sabouraud’s dextrose agar (SDA) to be grown at 28°C, or as required for the assays described below.

2.2 | Growth rate measurements

For measurement of growth rates and hyphae-formation, the strains were grown in YPD broth supplemented with adenine, uridine and histidine, in 96-well microtitre plates. The plates were incubated at 30 and 37°C for 24 h. Measurements were done every 15 min at 600 nm using the Tecan microplate reader (Sunrise model).\cite{17} Six tests were done per strain and the experiments were repeated at least twice.

2.3 | In vitro adhesion to epithelial buccal cells (BEC)—microscopic evaluation

The method used was as described previously.\cite{18} Briefly, human buccal epithelial cells (BEC) were collected from healthy donors (five students—young adults; consent was given) by sterile cotton swabs, and suspended in phosphate-buffered saline (PBS) as pool, washed three times and re-suspended to 1 × 10⁶ cells/mL. C. albicans cultures were grown in liquid yeast extract peptone dextrose (YPD) under constant shaking (400 rpm) at 28°C. The yeasts were collected, washed in PBS and suspended at a concentration of 10⁵/mL. Equal amounts (0.2 mL) of BEC and C. albicans cells were incubated at 37°C on a rotator for 2 h. Evaluation of adherence was done microscopically after filtration through a Millipore filter (12 micron pore size) to remove non-adherent yeast, on slides stained by crystal, by counting 3 × 100 cells (by the same person). Percentage of adherence was defined as the number of BEC which had ≥20 adhering C. albicans/cell.

2.4 | In vitro adhesion of C. albicans to HaCat Cell line—fluorometric evaluation

The assay was based on previously described methods.\cite{19} Human keratinocyte cell line from adult human skin (HaCat) was grown in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C under CO₂ (7%) and adjusted to a concentration of 2.5 × 10⁶ cells/mL. A quantity of 0.1 mL of cell suspension was added to each well of a 96 microtitre plate and grown at 37°C for 48 h to full confluency. C. albicans was grown as described in previous paragraph. The C. albicans cultures were labelled by Fluorescein isothiocyanate (FITC). The FITC labelled C. albicans were kept at 4°C until use (up to 1 month). FITC labelled C. albicans (0.1 mL) were added to each well with the HaCat cells and the microtitre plates were incubated at 37°C under CO₂ for 2 h. The microtitre plates were rinsed twice with saline and the adherence was measured by a fluorimeter (Fluorescence plate reader -FL 600) at a
530/20 and 485/20 nm wave length. The percentage of adherence was calculated by dividing the fluorescence signal of labelled C. albicans in the test by the signal of the initial sample of C. albicans.

Confocal laser scanning microscopy (CLSM) was carried out using a modification of a method described in previous studies.\textsuperscript{20,21} The cells were stained by MitoTracker red and C. albicans by FITC.

For visualisation of the cells, the fluorescence measurements were done at the excitation of 560 nm and emission at 620 nm wave length; visualisation of C. albicans was achieved by measurements at the excitation of 488 nm and emission at 520 nm wave length.

2.5 | Biofilm formation assay

Biofilm formation by the C. albicans strains was carried out in flat-bottom polystyrene 96-well microtitre plates. C. albicans cultures grown in YPD at 28°C under shaking for 18 h, were washed and suspended in RPMI 1640 at a concentration of 10^6/mL. The yeast suspension (100 μL) was placed in each well and the plates were incubated at 37°C for 48 h. The biofilm formation was evaluated by the XTT assay using spectral readings at 490 nm wave length (ELISA Reader Ceres 900).\textsuperscript{22,23}

2.6 | Secreted acid proteinases (SAP) activity

Activity of secreted acid proteinases (SAP) was assayed based on the method described by Arias et al.\textsuperscript{24} with slight modifications, on a medium containing bovine serum albumin (BSA). C. albicans strains were grown on Sabouraud’s dextrose agar (SDA) at 37°C for 48 h. An inoculum of 10^5 C. albicans cells were inoculated on the plates containing BSA and incubated at 37°C for 5 days. The SAP activity was determined by measurement of the hydrolysis area (clearing of the opacity) around the yeast colonies. The following evaluation scheme was used:

- No lysis = no activity of SAP enzymes
- A lysis zone of 1-2 mm around the colony = 1 = mild activity
- A lysis zone of 3-5 mm around the colony = 2 = for strong activity

2.7 | Phospholipase activity

Phospholipase activity of the C. albicans strains was assayed on a medium containing egg yolk, as described by Price et al.\textsuperscript{25}. The C. albicans strains grown as in the previous paragraph were inoculated as colony on plates containing egg yolk and incubated at 37°C for 7 days. Phospholipase activity was indicated by a zone of precipitation around the colony. Evaluation was based on the ratio between the diameter of the colony and the total diameter of the colony + precipitation zone = the Pz. The scheme of evaluation used was as follows:

\[
P_Z < 0.70 - 0.79 (++) = \text{moderate activity}
\]

\[
P_Z \leq 0.69 (++++) = \text{high activity}
\]

2.8 | Phenotypic switching

\textit{Candida albicans} has the ability to undergo an epigenetic switch expressed in different morphology of colonies: opaque—white, and different cellular morphology.\textsuperscript{26,27} The phenotypic switching of the C. albicans strains in this study was assessed, as described by Anderson and Soll\textsuperscript{28}, on Lee medium following incubation during 7 days at 28°C.

2.9 | Flow cytometry for ploidy measurements

Flow cytometry was used to assess the DNA content of the different isolates, providing an estimate of the ploidy state of each strain.\textsuperscript{26,29} Analysis of the data yields an estimate of DNA copy number with ±10% accuracy.\textsuperscript{28} Importantly, the CBS 562 strain, which was used in other assays, fell within the expected diploid range.

Flow cytometry was performed essentially as described by Hickman et al.\textsuperscript{29} Cells were washed with 50:50 TE and treated with 1 mg/mL RNAse A and then 5 mg/mL Proteinase K. Cells were then washed with 50:50 TE, re-suspended in Sybr Green I (1:85 dilution in 50:50 TE) and incubated overnight at 4°C. Stained cells were collected and re-suspended in 50:50 TE and analysed using a FACScalibur. Whole genome ploidy was estimated by fitting DNA content data with a multi-Gaussian cell cycle model that assumes the G2 peak has twice the fluorescence of the G1 peak and that minimises S-phase cell contribution to the error function. Ploidy values were calculated by comparing the ratio of peak locations in experimental samples to those of diploid and tetraploid controls.

2.10 | CHEF analysis

CHEF analysis of C. albicans chromosomes was performed using a BioRAD DRII CHEF gel electrophoresis apparatus, essentially as described previously\textsuperscript{30} on arbitrary chosen strains from the two groups (S and M). The gels were stained with ethidium bromide and photographed by short-wave ultraviolet trans-illumination.

2.11 | Statistical analysis

All tests for comparison between S and M groups underwent statistical analysis by Student t-test. Significance was determined by a \( P \) value of <.05.

3 | RESULTS

3.1 | Growth rates of S and M C. albicans strains

Growth rates were measured by the doubling time at 30 and 37°C. These assays revealed no notable differences between the S and M strains (Figure 1).
3.2 | Adhesion and Biofilm formation of S and M C. albicans strains

Figure 2 summarises the adherence values to buccal epithelial cells (BEC) of all S and M C. albicans strains and the control strain CBS 562, which is a cut-off point for comparison. It can be noted that both among the S and M strains there is a gradient of the adherence ranging from 44-65% and 43-60% respectively. It can also be noted that six out of the 20 S strains (30%) reveal higher values than CBS while among the M strains four out of 22 (16.6%) demonstrate higher values. The data shown in Figure 2 are means from three independent experiments, each including two samples. Statistical analysis by Student t-test of the S vs M groups did not show significant differences.

Figure 3 shows the data obtained in assays evaluating the adherence of the strains to the tissue culture cell line HaCat. In these assays, it is obvious that the M strains have higher capacity to adhere than the S strains. The analysis by Student t-test showed a statistical difference: $P$ value=.02.

The different adherence ability of strains could be visualised by comparing strongly adherent (M33) and less adherent strain (S14) using confocal laser microscopy—Picture 1.

As adherence is generally considered an initial step in the infectious process of candidiasis followed by biofilm formation, we assessed also the ability of the two groups of strains to form biofilms,
as expressed by metabolic activity measured by the XTT test. This is shown in Figure 4: both groups included strains with higher or lower capacity to form biofilm than the control strain—CBS 562. Statistical analysis did not reveal a significant difference between the groups. However, it can be noted that some strains are heavy producers, eg M44 as compared to a low biofilm producer, eg S9.

### 3.3 Secreted aspartyl proteinases (SAPs) and phospholipases activity of C. albicans S and M strains

Hydrolytic activity of C. albicans, including production of proteolytic enzymes, such as the secreted aspartyl proteinases (SAPs), or phospholipases are considered virulence attributes of the fungus, taking part in the pathogenesis of Candida infections.

Hence, we tested these characteristics in our sets of C. albicans strains, isolated from patients with invasive and vaginal Candida infections. The results of these tests are presented in Table 1 (phospholipases) and Figure 5 (SAPs).

Based on the criterion of Pz, as defined in the Methods chapter, low Pz values indicate high phospholipase activity. Table 1 reveals that using this criterion 13/20 S strains (65%) have lower Pz values than CBS-562 (0.55) which means high activity of phospholipases. However, among the M strains, using the same criterion, 4/22 strains (18.18%) have lower values than the CBS. Statistical analysis revealed...
that the difference between S and M groups is significant—$P=.039$, with the S group strains revealing higher phospholipase activity.

The analysis of the SAP activity (Figure 5) did not show marked differences between the S and M C. albicans strains.

### 3.4 | Phenotypic switching of C. albicans

C. albicans strains have the ability to undergo an epigenetic morphological switch as described by Soll et al. [27] This phenotypic switching is manifested in different colony and cell morphology and may differ with respect to various host-microbe interactions. The fungus can switch from rough opaque colonies to smooth white colonies and vice versa. The cell morphology may change from regular round-oval cells to more elongated ones.

Hence, we analysed the relative proportions of the white and opaque phenotypes among the S and M C. albicans by enumeration of each type following growth on a specific medium demonstrating these phenotypes. As shown in Figure 6 summarising this set of experiments, it can be seen that our control strain CBS 562 presents 100% white colonies. More S strains exhibited predominantly the white type colonies than the M strains. Specifically, 8/20 of the S strains exhibited only the white type (100%) and seven others had >90% of this type. Thus, 15/20 S strains, namely 80% of the cultures had the white phenotype. Among the M strains, a total of 40% (9/22) isolates revealed this phenotype. Statistically, this difference is significant, $P=.014$. Comparison of opaque colonies in the two groups also revealed a significant difference, $P=.025$. Namely, while ~18% (4/22) of the M strains cultures had at least a third of the colonies in opaque phenotype, only 5% (1/20) of S strains exhibited this phenotype.

### 3.5 | Ploidy of S and M strains measured by flow cytometry

As noted in Figure 7A, B, the DNA content of many of the S strains fell outside of the ±10% diploid DNA content region. Ten of the
S strains (50%) had higher DNA content relative to SC5314 and none of them fell below the diploid range. Among the M strains, one of the 22 isolates (M33) had higher DNA content than the control and at least one strain (M29) exhibited a lower DNA content, with two others being just below the diploid level (M37 and M39). The CBS 562 strain, which was used in other assays, had DNA content that was comparable to that of SC5314. The difference between the S and M groups is clearly demonstrated by the Scatter analysis (Figure 7B). Analysis by Student t-test showed that the S and M groups had significantly different range of ploidy levels (P value=.00018).

3.6 | CHEF analysis

CHEF analysis was performed for a subset of the strains and was compared with the flow cytometry results (Figure 8). CHEF analysis revealed generally in migration and assumed size/length of the smaller chromosomes 5, 6 and 7, with co-migration of homologues of 6 and 7 to different degrees in different isolates. Strains S8, S11 and M8, with the highest DNA content/ploidy level tested by CHEF appeared to have additional DNA in the Chr6/7 region, although it was difficult
to a sign the additional DNA to which of these two chromosomes. Strain S29, with the lowest DNA content/ploidy by flow cytometry appeared likely to be monosomic for Chr4. It is important to note that actual assignment of specific DNA identity on CHEF gels to specific bands, especially if their size is altered, requires Southern analysis, which was not performed here.

4 | DISCUSSION

Interactions of C. albicans with host cells involving both fungal determinants as well as host’s response are of complex nature and may affect the clinical outcome.12 The effect of host’s factors in determining whether the human fungal commensal C. albicans will turn into a pathogen causing cutaneous-mucosal or invasive infection was shown in numerous studies, as summarised by Romani [31], Fidel and Noverr [32], Netea and Gow [33]. This study focused on exploring specific fungal determinants, such as virulence factors and genotypic characteristics in the context of their possible role in clinical manifestations of candidiasis, with the awareness of the role of host’s factors.
Towards this end, clinical C. albicans isolates were collected from two groups of patients: (i) hospitalised patients with candidemia (strains were designated as S strains) and (ii) patients with vaginal infection treated in a community clinic (strains were designated as M strains). The C. albicans isolates were assessed in vitro as to various phenotypic characteristics considered as microbial virulence factors and analysed regarding several genetic parameters.

After determining the basic biological features, such as growth rate and doubling time of the S and M C. albicans, which did not reveal differences between the two groups of strains, we evaluated their ability to adhere to mammalian/human tissue, the rational being the significance of this mechanism in the pathogenesis of candidiasis.

Adhesion of Candida to human tissues, similar to other microbes, is a feature considered as an initial step in evolution of infection and as virulence attribute.34–37 In this study, two different test systems were used: human buccal epithelial cells (BEC) as binding cells and a human epithelial cell line—the HaCat cell line, which yielded different results. While the adhesion assays with the BEC did not show significant differences in the adhesion values of the S and M groups, with variations of individual strains within the groups, the adhesion to the cell line demonstrated a statistically significant difference between the two groups. The M strains showed higher capacity to adhere to the HaCat cell line. The dissimilarity in the results in the two different systems—BEC vs HaCat, lies probably, in the change in the binding
While the BEC consisted of a pool of cells from the same healthy donors, there still might be diversity in the binding level, whereas the HaCat cell line consists of a stable system throughout the study. Possible differences between different evaluation systems have also been indicated. The evaluation of adherence to BEC is microscopic and more subjective, whereas in the HaCat system, evaluation is more objective being based on readings of fluorescence.

Although it might be difficult to assign, based on in vitro assays a specific clinical relevance, interestingly, higher capacity of M strains to adhere may be compatible with findings that women with known epidemiological evidence of increased susceptibility to vaginal candidiasis demonstrate higher ability of adherence.

FIGURE 7 Flow cytometry for ploidy measurements of S and M strains. (A) DNA content of S and M strains. (B) Scatter Analysis comparing ploidy of S and M groups. The Candida was stained with Sybr Green I and analysed using a FACSCalibur. DNA content of S and M strains was compared to the control strain SC5314. The CBS 562 strain, which was used as control in other assays, had DNA content that was comparable to that of SC5314. Analysis by Student t-test showed that S strains had significantly higher range of ploidy levels than M strains (P value=.00018).
cellular actin and thereby on the cytoskeleton, as shown by Sandovsky and Segal [40], Schindler and Segal [41] and Sandovsky et al. [42].

As the evolution of infection includes formation of biofilms, which follows adhesion of the fungus, both in mucosal as well as in invasive candidiasis, it seems plausible that no major differences were noted between the S and M groups in this respect, however demonstrated variability between strains within the groups. Variability among strains was noted also by other investigators, eg Sherry et al. [45] indicated that biofilms of blood stream isolates reveal phenotypic and transcriptional heterogeneity that are associated with resistance and pathogenicity.

Production of hydrolytic enzymes is another feature associated with pathogenicity of fungi. The secreted aspartic proteinases (Saps), a family of 10 enzymes is considered as factors involved both in adhesion and invasion of C. albicans. Moreover, studies have indicated that different Saps are associated with specific clinical entities of candidiasis. Specifically, the study by Shaller et al. [47] showed a crucial role for Sap1 and Sap2 in vaginal infections caused by C. albicans. Another study of the Hube group demonstrated that C. albicans mutants lacking the genes SAP4 to SAP6 presented reduced invasiveness. In our study, we did not find differences between the M and S groups, possibly, due to the assay we used, which did not differentiate which SAP is involved.

We did find a difference between the S and M groups regarding phospholipase activity, which was higher among the S strains. The ability to break down by phospholipases, the phospholipids, a major component of the cell membrane, may contribute to the fungal potential to invade into the host and thus possibly be associated with Candida strains causing invasive disease. It is of interest that Price reported that blood isolates produced much higher levels than isolates from wounds or urine.

Figure 8: CHEF analysis of S and M strains. CHEF analysis was performed for a subset of the strains and was compared with the flow cytometry results. (A) strains S8,11,12,14; M29,33. (B) strains S4,5,6,7.
In the context of differences between mucosal and invasive Candida infections, it is of interest to mention a recent study by Luna-Tapia et al. [49], which indicated that ERG2 and ERG24 are required for pathogenicity of C. albicans in experimental mouse systemic infection but not vaginal. Another recent study by Moyes et al. [50] described a toxin, candidalysin, which is critical for mucosal infection and elucidates mucosal pathogenesis.

Analysis of genetic characteristics such as the epigenetic morphological switch—the phenotypic switching and ploidy analysis by flow cytometry have also shown differences between the S and M strains. The phenotypic switching assay demonstrated that among the five most populous clades, our MLST study showed significant differences in the proportions of isolates from blood, commensal carriage and superficial infections. Moreover, our standard strain CBS 562, which was virulent in various in vivo models: murine systemic [14,52,53] and vaginal infection, [54] skin infection in guinea pigs [55] and more recently in the non-vertebrate model Galleria mellonella, [14] was composed of 100% white colonies.

Ploidy studies by flow cytometry have indicated that all S strains were diploid and a half of the studied strains in this group had higher DNA content than the diploid control strain, with a statistical difference between the S and M groups.

It is of interest that a study of Odds et al. [56] reported that a multi-locus sequence typing (MLST) analysis of European C. albicans isolates showed significant differences in the proportions of isolates from blood, commensal carriage and superficial infections among the five most populous clades. Our MLST study [13] of the S and M strains indicated a specific CC type found only among the S strains.

In summary, our present study comparing in vitro phenotypic characteristics related to virulence and genotypic characteristics of strains from two different clinical entities of candidiasis indicated that although no comprehensive differentiation between the strains of the two groups could be demonstrated, several characteristics are more specific to one of the groups than to the other. Namely, while M strains isolated from vaginitis patients have higher capacity to adhere, the S strains isolated from candidemia patients have higher activity of phospholipase, are composed primarily of white type colonies and have higher DNA content. The study is unique in view of the concurrent comparison of isolates from different clinical entities, both at the phenotypic and genotypic level, using in vitro and in vivo [14] parameters. However, as indicated above, it should be noted that the fungal features possibly affecting the clinical presentation, are only one part in the equation of microbe and host.

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