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Prevalence and Changing Virulence Factors and Biofilm Formation among Non-Albicans *Candida* Species in Suspected Vulvovaginitis Infections

Rahul Gopichand Walide[1*], Ahire Karuna R[2], Ravidas Vasave[1]

[1]Department of Microbiology, Government Medical College, Nandurbar, Maharashtra, India; [2]Department of Microbiology, ACPM Medical College, Dhule, Maharashtra, India

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***Correspondence**
Email: rahulwadile123@gmail.com
Tel: +919890315091

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ABSTRACT

Introduction: Vulvovaginal candidiasis (VVC), a common gynecological condition predominantly attributed to *Candida albicans*, frequently affects pregnant women. Non-*albicans Candida* species (NACs) are increasingly recognized as important etiological agents, potentially contributing to adverse pregnancy outcomes such as preterm birth and miscarriage. This study aimed to determine the prevalence of NACs and characterize their virulence factor profiles, including biofilm formation, in women with suspected vulvovaginitis, with the goal of informing and optimizing clinical management strategies for this condition. **Methods:** Cervicovaginal swabs were prospectively collected from pregnant women presenting with clinical signs and symptoms suggestive of vulvovaginitis. Identification of *Candida* species was performed using standard mycological techniques, encompassing microscopic examination, germ tube testing, and carbohydrate assimilation tests. The assessment of virulence factor production included biofilm formation, and the enzymatic activities of hemolysin, phospholipase, lipase, and protease (evaluated via agar diffusion assays). Statistical analyses were conducted using SPSS software (version 25.0). **Results:** Among the 370 cervicovaginal swabs collected from women presenting with suspected vulvovaginitis, *Candida* species were identified in 123 swabs (33.24%). *C. albicans* constituted 53.66% of the isolates, while NACs accounted for the remaining 46.34%. Within the NACs isolates, *Candida tropicalis* (49.12%) and *Candida glabrata* (28.07%) were the predominant species. Diabetes mellitus was the most common risk factor identified in women with *Candida* infection. Hemolysin production was the most frequently detected virulence factor among the NACs isolates, observed in 40.9% of these isolates. **Conclusion:** This study demonstrates the significant prevalence of NACs in vulvovaginal candidiasis cases, concurrent with the notable presence of diverse virulence factors. Our findings underscore the importance of routine mycological investigations for accurate species identification and suggest that virulence factor profiling may be critical for informing effective management strategies for VVC, particularly given the observed prevalence and potential pathogenic implications of the diverse array of virulence factors among NACs.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a frequent fungal infection characterized by inflammation of the vulva and vagina. The etiology of VVC is predominantly attributed to *Candida* (*C.*) species, with *C. albicans* being the most frequently isolated organism, accounting for approximately 85%–90% of cases [1, 2]. Notably, VVC exhibits a heightened prevalence among pregnant women, with global estimates suggesting that over 40% will experience this condition [3]. Furthermore, recurrent VVC affects an estimated 5-10% of pregnant women, posing a significant clinical challenge [4]. Increasingly, recent studies have reported that NAC species are implicated in 10% to 30% of VVC cases [5]. While *C. albicans* remains the predominant etiological agent, other *Candida* species, particularly NAC species, are being recognized with increasing frequency as

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significant contributors to VVC. Among NAC species, *C. glabrata* is frequently cited as the most prevalent, followed by *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* [6].

Predisposing factors for VVC encompass both host-related conditions, such as diabetes mellitus, obesity, HIV/AIDS, and immunosuppression, and pathogen-related attributes, including enhanced adherence capabilities and the production of virulence factors [7]. During pregnancy, physiological changes including hormonal fluctuations and alterations in vaginal pH and glucose concentration create a more permissive environment for *Candida* proliferation, thereby increasing the susceptibility to VVC [8]. Prolonged antifungal treatment, diabetes mellitus, and prior antifungal use have been associated with an increased prevalence of NACs [4]. VVC has been implicated in several adverse pregnancy outcomes, including low birth weight, preterm birth, miscarriage, and premature rupture of membranes. These adverse outcomes are potentially linked to the inflammatory response elicited by the systemic effects of *Candida* infection [9].

Candida species possess a repertoire of virulence factors that contribute to their pathogenicity. These include the capacity to evade host defenses, adhere to host epithelial surfaces, form biofilms, exhibit hemolytic activity, and secrete hydrolytic enzymes such as phospholipases, proteinases, and esterases [10]. It is important to note that different *Candida* species exhibit varying degrees of virulence; for instance, *C. albicans* is more frequently associated with VVC compared to certain other species [11]. The prevalence of NACs as etiological agents of VVC is demonstrably increasing [12]. Despite this rising prevalence of NACs in VVC, comprehensive data regarding their virulence factor profiles and the specific challenges present in the clinical management of VVC remains limited, particularly within the Indian population.

This study aimed to determine the prevalence of NACs in women presenting with suspected vulvovaginitis and to characterize the virulence factors, including biofilm formation, associated with NAC-related vulvovaginal candidiasis. A comprehensive understanding of the evolving epidemiology of vulvovaginal candidiasis and the potential impact of NAC species on patient outcomes is of paramount importance, and this research contributes valuable insights to this area.

MATERIAL AND METHODS

Study design and setting. This prospective observational study was conducted over a one-year period, from January 2022 to December 2022, at the Department of Micobiology, JMF's ACPM Medical College & Hospital, located in North Maharashtra, India. This setting was selected due to the anticipated high prevalence of vulvovaginitis in this population and the scarcity of epidemiological data regarding non-*albicans* *Candida* species in this geographic region.

Ethical considerations. This study received ethical approval from the Institutional Ethics Committee of JMF's A.C.P.M. Medical College, Dhule, Maharashtra, India (Approval Number: 78/IEC/ACPMMC/Dhule) on 28 March 2023.

Inclusion criteria. Pregnant women presenting with clinical signs and symptoms suggestive of vulvovaginitis, including but not limited to abnormal vaginal discharge, pruritus, burning sensation, or pain, and who provided written informed consent, were eligible for inclusion in this study.

Exclusion criteria. Individuals meeting any of the following criteria were excluded from participation: age below 18 years (due to ethical considerations regarding consent and potential developmental differences in the vaginal microbiome) and those who declined to provide written informed consent.

Sample collection and processing. Cervicovaginal swabs (two per participant) were collected aseptically from the posterior fornix of the vagina from 370 pregnant women with suspected vulvovaginal candidiasis. One swab was utilized for direct microscopic examination following preparation of a 10% potassium hydroxide (KOH) wet mount (HiMedia, India) and Gram staining. The second swab was inoculated onto Sabouraud's Dextrose Agar (SDA) (HiMedia, India) supplemented with gentamicin (to inhibit bacterial growth) and incubated at 37°C for 24–48 h to facilitate the growth and isolation of *Candida* species.

Species identification by conventional methods. *Candida* species identification commenced with the germ tube test. In brief, a suspension of yeast cells was prepared and inoculated into a small volume of human serum on a microscope slide. This preparation was then incubated at 37°C for 2 h. Microscopic examination for the presence of germ tubes was subsequently performed. The formation of elongated, hyphal-like projections emanating from the yeast cells, lacking constrictions at their point of origin, was considered as a positive result, indicative of either *C. albicans* or *Candida dubliniensis*. It is important to note that the germ tube test alone cannot differentiate between these two species, as both are capable of germ tube formation under these conditions.

Dalmau plate culture. Dalmau plate cultures, utilizing cornmeal agar supplemented with Tween 80, were employed to assess the formation of pseudohyphae, true hyphae, and chlamydospores. Briefly, a small inoculum of the test organism was transferred to a well created on the surface of a cornmeal agar plate using a sterile loop. Subsequently, the inoculum was lightly streaked across the agar surface in a manner to achieve a dilution gradient, facilitating the development of isolated colonies. A sterile coverslip was then aseptically placed

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the inoculated well and a portion of the streak lines, creating a microaerophilic environment conducive to hyphal development. The plates were incubated at 22–25°C for 48–72 h. Microscopic examination was performed directly on the plate, with the condenser lowered to enhance contrast. The area beneath the coverslip was initially examined at lower magnification of 10X to locate regions exhibiting optimal hyphal growth. Subsequently, higher magnifications of 40X were used to assess the presence and morphology of pseudohyphae, true hyphae, and chlamydospores. If these structures were not readily apparent under the coverslip, the submerged growth along the streak lines was carefully examined at higher magnification to identify these morphological features.

CHROMagar. *Candida* isolates, obtained in pure culture from the initial SDA plates, were subcultured onto CHROMagar *Candida* (HiMedia, India), a selective and differential medium designed for the presumptive identification of common *Candida* species based on distinctive colony coloration. The inoculated CHROMagar plates were incubated at 35–37°C for 24–48 h. Presumptive species identification was then performed by observing colony color and morphology, strictly adhering to the manufacturer's instructions and the established colorimetric key for CHROMagar *Candida* [13].

Biofilm production. Biofilm formation was quantitatively assessed using a crystal violet staining assay in sterile 96-well flat-bottom polystyrene microtiter plates. Initially, *Candida* isolates were cultured on SDA plates for 24 h at 35°C. A loopful of yeast cells was then transferred to 15 mL of Sabouraud Dextrose Broth (SDB) in a sterile polypropylene tube and incubated for 24 h at 37°C with shaking (150 rpm) to prepare a standardized inoculum. After incubation, the SDB was carefully aspirated, and the tubes were gently washed twice with sterile distilled water to remove non-adherent cells. The tubes were then stained with 1% safranin for 10 min and examined for the presence of an adherent layer on the tube walls. Each isolate was tested in duplicate, and the results were independently evaluated by two different observers. Biofilm production was categorized as negative (-), weak (+), moderate (++), or strong (+++) according to the previously described protocol [14]. *Staphylococcus epidermidis* ATCC 35984 was used as a positive control.

Phospholipase activity. Phospholipase activity was assessed by placing 10 µL of yeast suspension in sterile saline onto the surface of an agar medium containing egg yolk (pH 4.3). The plates were incubated at 37°C for five days. Phospholipase activity was indicated by the formation of a precipitation zone (PZ) around the yeast colonies, resulting from the hydrolysis of lecithin in the egg yolk agar. The phospholipase activity was quantitatively determined using the following formula:

Pz value = (diameter of the colony) / (diameter of the colony + PZ)

Phospholipase activity, as determined by the Pz value, was interpreted as follows: a Pz value of 1.0 indicated no detectable phospholipase activity (-); values between 0.90 and 0.99 were considered weak (+); values between 0.80 and 0.89 were considered moderate (++); values between 0.70 and 0.79 were considered strong (+++); and values below 0.70 were considered very strong (++++) [15]. *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, and *C. tropicalis* ATCC 750 were included as reference strains for quality control purposes.

Statistical analysis. Data were entered and analyzed using SPSS software (version 25.0, IBM Corp., Armonk, NY, USA). Descriptive statistics, including frequencies and percentages for categorical variables and means with standard deviations or medians with interquartile ranges for continuous variables, were used to summarize the data.

RESULTS

Prevalence of *Candida* species and associated risk factors. Among the 370 pregnant women presenting with suspected vulvovaginitis, 123 women (33.24%) yielded positive cultures for *Candida* species. *C. albicans* was the predominant species identified (53.66%, n = 66), followed by NACs (46.34%, n = 57). Within the NACs group, *C. tropicalis* was the most frequently isolated species, succeeded by *C. glabrata* (refer to Table 1 for detailed species distribution). Diabetes mellitus was the most prevalent predisposing factor for vulvovaginal candidiasis in this study cohort, followed by prolonged antibiotic therapy and a history of sepsis (detailed information in Table 2). Considering all *Candida* isolates, hemolysin production was the most frequently observed virulence factor (51.93%, n = 64), followed by protease production (38.53%, n = 47) and biofilm formation (31.54%, n = 39) (refer to Table 3 for a comprehensive overview of virulence factor distribution).

Table 1. Distribution of NACs isolated from women with suspected vulvovaginal candidiasis

Species	No. of isolates (%)
C. tropicalis	28 (49.12)
C. glabrata	16 (28.07)
C. krusei	8 (14.03)
C. parapsilosis	5 (8.77)

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Table 2. Predisposing factors for vulvovaginal candidiasis in the study population

Predisposing factors	No. of patients (%)
Diabetes Mellitus	39 (68.42)
Prolonged antibiotic use	18 (31.57)
History of sepsis	16 (28.07)
Immunosuppression	12 (21.05)
Intravascular catheter	7 (12.28)

Table 3. Distribution and frequency of virulence factors among *Candida* species isolated from women with suspected vulvovaginal candidiasis

Virulence factor	Total isolates (n = 123)	*C. albicans* (n = 66)	*C. tropicalis* (n = 28)	*C. glabrata* (n = 16)	*C. krusei* (n = 8)	*C. parapsilosis* (n = 5)
Adherence	123 (100.0%)	66 (100.0%)	28 (100.0%)	16 (100.0%)	8 (100.0%)	5 (100.0%)
Phospholipase	22 (17.89%)	10 (15.15%)	6 (21.43%)	3 (18.75%)	3 (37.50%)	0 (0.0%)
Lipase	8 (6.50%)	4 (6.06%)	4 (14.29%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Protease	52 (42.28%)	27 (40.91%)	11 (39.29%)	10 (62.50%)	4 (50.00%)	0 (0.0%)
Hemolysin	54 (43.90%)	27 (40.91%)	14 (50.00%)	5 (31.25%)	3 (37.50%)	5 (100.0%)
Biofilm	51 (41.46%)	30 (45.45%)	14 (50.00%)	4 (25.00%)	3 (37.50%)	0 (0.0%)
Coagulase	26 (21.14%)	16 (24.24%)	10 (35.71%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

DISCUSSION

VVC represents a common fungal infection among women of reproductive age, clinically manifesting with symptoms such as abnormal vaginal discharge, pruritus, and a burning sensation. The pathogenesis and recurrence of VVC are governed by a complex interplay of factors, including dynamic shifts within the vaginal microbiota, alterations in vaginal pH, hormonal fluctuations across the menstrual cycle and during pregnancy, and host-related factors encompassing immune status and sexual behaviors. These elements can disrupt the delicate homeostatic balance of the vaginal ecosystem, thereby fostering an environment permissive to the proliferation of *Candida* species and the subsequent establishment of infection. *C. albicans* remains the predominant etiological agent in VVC. The increasing incidence of NACs presents novel challenges for the accurate diagnosis and effective management of this condition. The expression of virulence factors by *Candida* species is a complex and dynamic process, modulated by a multitude of factors including the specific type of infection (*e.g.*, acute *vs*. recurrent), the host's immune response, geographical location, and the intrinsic virulence attributes of the *Candida* species involved [15-18].

In this study, we investigated the prevalence of several key virulence factors among *Candida* isolates recovered from women presenting with suspected vulvovaginal candidiasis. These virulence determinants included hemolytic activity, biofilm formation capacity, and the enzymatic activities of protease and phospholipase. While prior studies have explored these virulence factors in *Candida* isolates from India, further research is warranted to comprehensively elucidate their specific roles in the pathogenesis and diverse clinical manifestations of VVC within this particular population [19]. Our findings underscore the increasing prevalence of NACs in VVC, thereby emphasizing the clinical importance of species-level identification for appropriate management**.** Furthermore, a comprehensive understanding of the virulence factor profiles associated with these emerging species is essential for the development of targeted therapeutic strategies aimed at effectively managing VVC and preventing recurrent episodes.

Our study identified *C. albicans* in 53.66% and NACs in 46.34% of 123 women with vulvovaginitis**.** These findings are comparable to those reported by Malak *et al*. (2021) in Mumbai [20], who observed a prevalence of 49.3% for *C. albicans* and 50.7% for NACs among 150 isolates. In contrast, our results diverge from those of Chaudhary *et al*. (2020) in Jaipur [21], who documented a prevalence of 26% for *C. albicans* and 74% for NACs in their analysis of 200 isolates. These variations in prevalence rates across different geographical locations likely reflect differences in patient populations, diagnostic methodologies, and local antifungal usage patterns.

Among the NACs isolated from patients with VVC in the present study, *C. tropicalis* was the most frequently encountered, comprising 49.12% of all non-*albicans Candida* isolates. This was followed by *C. glabrata* (28.07%), *C. krusei* (14.03%), and *C. parapsilosis* (8.77%).

A study by Deorukhkar *et al*. (2014) [22] in Maharashtra, India, examined 523 *Candida* spp. isolates from various clinical specimens and reported a higher prevalence of non-*albicans Candida* isolates compared to *C. albicans*. Among the NACs species, *C. tropicalis* (35.1%) was the predominant isolate, followed by *C. glabrata* (28.1%), findings that align with our current observations. These findings are further supported by a study conducted in Tripura, India, which reported a 23.7% prevalence of vulvovaginal candidiasis among 275 symptomatic women, with *C. tropicalis* (44.11%) being the primary causative agent, followed by *C.*

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glabrata (35.29%) and *C. krusei* (20.58%) [23]. However, a study conducted in Mumbai, India, reported a higher incidence of *C. glabrata* (19.3%) compared to *C. tropicalis* (21.4%), a finding that contrasts with the results of the present study [20]. In our study, *Candida parapsilosis* constituted 8.77% of the isolates among the 123 positive VVC cases. This prevalence is comparable to the 7.1% of *C. parapsilosis* isolates reported in a study conducted in Lucknow and is also consistent with the findings reported by Jahan *et al*. (2020) [23].

Diabetes mellitus was the most prevalent risk factor identified in our study, a finding consistent with reports by Balaraman *et al*. (2018) [24], who also found diabetes mellitus to be the most common risk factor among 181 *Candida* isolates. Notably, diabetes mellitus is a well-established predisposing factor for various *Candida* infections, including candidemia. Severe hyperglycemia, a hallmark of poorly controlled diabetes, has been statistically correlated with an increased incidence of candidemia [25].

Upon entrance into a mammalian host, fungal cells transfromation from a saprophytic to a parasitic mode of nutrition, necessitating rapid adaptation to the altered microenvironment. The successful establishment of infection is critically dependent on the fungal cell's ability to adapt to these dynamic conditions. Adhesion to host tissues represents the initial and crucial step in the infectious process, enabling the pathogen to resist clearance by mucosal secretions and the host's natural defenses. This adherence is mediated by adhesins, specialized surface molecules expressed by the pathogen, which facilitate the initial attachment to host cells. The adhesion process typically commences with non-specific interactions, including van der Waals forces, Brownian motion, and hydrophobic and ionic interactions. Subsequently, these initial, weaker interactions are reinforced and stabilized through more specific and durable receptor-ligand interactions [26].

In our study, all *Candida* isolates demonstrated robust *in vitro* adherence properties. These isolates effectively adhered to various surfaces, most notably to epithelial cells, which are the primary target cells in vulvovaginal candidiasis. While all species exhibited adherence properties, *C. albicans*, often considered to possess heightened virulence, demonstrated a notably high degree of adherence. These findings are partially consistent with previous research by Nikawa *et al*. (2003) [27], who reported 100% adherence rates for *C. tropicalis*, *C. glabrata*, and *C. krusei*. However, it is important to note that our study suggests a potentially higher degree of adherence for *C. albicans* compared to these NACs, although direct quantitative comparisons with the Nikawa *et al*. study may be limited by methodological differences.

In our study, a substantial proportion of isolates exhibited multiple virulence factors, with some isolates co-expressing up to five of the assessed factors. The present study's findings do not support the notion, prevalent in some literature, that the visual appearance of a lesion and the presence of sufficient yeast forms upon direct microscopic examination with 10% KOH are invariably sufficient to establish pathogenesis [28]. Our data suggest that the presence and interplay of multiple virulence factors likely contribute significantly to the pathogenic potential of *Candida* species in VVC. Protease synthesis, a crucial hydrolytic enzyme implicated in both adhesion and tissue invasion, was identified as another prominent virulence factor in our study. *C. albicans* (40.9%) and *C. glabrata* (62.5%) demonstrated protease synthesis. Hemolysin production and biofilm formation were the next most frequently observed virulence attributes. These findings stand in contrast to the results reported by Deorukhkar *et al*. (2014) [22], who observed proteinase production in a considerably higher proportion of *C. albicans* (82.1%) and *C. tropicalis* (80%).

The present study demonstrated phospholipase activity in 17.89% of the *Candida* isolates, with *C. krusei* exhibiting the highest proportion of activity among the tested species at 37.5%. This finding contrasts with the higher overall phospholipase activity reported by Deorukhkar *et al*. (2014) [22], who observed phospholipase activity in *C. tropicalis* (81.8%), *C. glabrata* (65.5%), *C. parapsilosis* (33.3%), and *C. krusei* (22.2%). The lower overall phospholipase activity observed in our study, despite a higher proportion in *C. krusei*, may reflect variations in the distribution of *Candida* species or differences in the sensitivity of the assays used.

Hemolysin production was detected in all *Candida* species tested in our study, with *C. parapsilosis* demonstrating a 100% rate and *C. albicans* showing a 40.9% rate. All hemolysin-producing isolates exhibited alpha hemolysis. This finding contrasts sharply with the observations of Luo *et al*. (2001) [11], who reported hemolytic activity in *C. glabrata* (42.5%), *C. tropicalis* (6.25%), *C. parapsilosis* (6.25%), and *C. krusei* (5%). The significantly higher hemolysin production rate observed for *C. parapsilosis* in our study, compared to Luo *et al*.'s findings, warrants further investigation.

Plasma coagulase produced by *Candida* species functions by converting prothrombin to thrombin via proteolysis. This enzymatic process subsequently activates thrombin, which then converts fibrinogen to fibrin, a key component of blood clots. However, the precise role of coagulase in *Candida* virulence remains incompletely understood, and studies specifically investigating its contribution to pathogenesis are limited [29].

In this study, coagulase production was observed in *C. albicans* (16 isolates, 24.24%) and *C. tropicalis* (10 isolates, 35.71%). Conversely, coagulase production was not detected in *C. krusei* or *C. glabrata* isolates. These findings show partial consistency with those reported by

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Rodrigues *et al*. (2003) [30], who also observed coagulase production in *C. albicans* (43.47%) and *C. tropicalis* (25.27%). However, Rodrigues *et al*. [30] also reported coagulase production in *C. parapsilosis* (31.86%) and *C. glabrata* (27.47%), which was not observed in our study. Biofilm formation, a critical virulence mechanism, confers enhanced resistance to antifungal agents and host immune defenses. Biofilm ecosystems are demonstrably more recalcitrant to treatment and persistent than their planktonic counterparts. This increased virulence is attributed to the protective matrix of the biofilm, which impedes the penetration of antifungal drugs and shields the fungal cells from host immune factors, thereby promoting attachment and subsequent tissue invasion. This tissue invasion, in turn, further exacerbates virulence and contributes to drug resistance. Several recent studies have reported elevated levels of phospholipase, protease, and adhesion production within biofilms compared to planktonic cells [31, 32]. In our study, biofilm formation was most prevalent among *C. albicans* isolates (45.45%), while *C. tropicalis* exhibited the highest proportion of biofilm-forming isolates among the non-*albicans Candida* species (50%). These findings show some similarity to those reported by Majumdar *et al*. (2016) [19], who observed biofilm formation in a significant proportion of NACs (56.41%), with *C. tropicalis* (47%) exhibiting a high rate of biofilm production. However, direct comparisons should be made cautiously due to potential variations in biofilm assay methodologies and interpretation criteria.

This study elucidates the heterogeneous virulence factor repertoire among *Candida* species associated with vulvovaginitis. Notably, *C. albicans* isolates consistently demonstrated a greater propensity for expressing multiple virulence factors, including adhesion, protease, hemolysin, coagulase, and biofilm formation. Furthermore, isolates demonstrating biofilm formation exhibited distinct virulence profiles, characterized by increased production of hemolysin and coagulase, yet reduced protease activity, compared to their planktonic counterparts. These findings underscore the critical role of both species-specific virulence determinants and the contribution of biofilm formation in the pathogenesis of vulvovaginitis.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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