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Impact of biofilm formation by vaginal *Candida* isolates on antifungal treatments

Ariana Cecibel Cedeño Pinargote

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DE TRABAJO DE FIN DE CARRERA

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Ariana Cecibel Cedeño Pinargote

Nombre del profesor, Título académico **Antonio Machado, PhD. en Ingeniería Biomédica**

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Nombres y apellidos: Ariana Cecibel Cedeño Pinargote

Código: 00206267

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RESUMEN

Candida albicans y *Candida glabrata* son patógenos emergentes que tienen una alta tasa de contagio debido a su resistencia a los antifúngicos y su capacidad para formar biopelículas. La formación de biopelículas tiene importantes repercusiones en la salud. Sin embargo, se han realizado pocas investigaciones en Ecuador sobre la capacidad de los aislados vaginales de *Candida* para formar biopelículas. El presente estudio se centró en aislar especies de *Candid*a a partir de muestras vaginales, para determinar la tasa de formación de biopelículas de *C. albicans* y *C. glabrata* durante (24 y 48 h) mediante la formación de biomasa utilizando ensayos de densidad óptica con tinción de cristal violeta (CV) y tampón fosfato salino (PBS) y su clasificación por índice de formación de biopelícula (BFI). Además, este estudio evaluó la resistencia antifúngica en células planctónicas de *Cándida* de nuestro grupo y comparó sus perfiles antifúngicos entre diferentes tipos de microbiota vaginal (microbiota sana, intermedia, candidiasis e infección/disbiosis vaginal mixta). Nuestros resultados mostraron una mayor capacidad para formar biopelículas utilizando el método de tinción CV a las 24 h, en el que el 76,19 % (16/21) de las muestras eran alta formadora HBF (del inglés *high biofilm formers*) y el 23,81 % (5/21) era intermedia formadora IBF (del inglés *intermediate biofilm formers*). Mediante pruebas de susceptibilidad en células planctónicas, los resultados mostraron que los antifúngicos anfotericina B y flucitosina no son adecuados para tratamientos de candidiasis demostrando un rango de resistencia de 90.5 y 100%, respectivamente. Por su parte, los antifúngicos de la familia equinocandinas fueron los que mejores resultados evidenciaron con un rango de resistencia de 33.3 – 42.9% contra células planctónicas de *Candida*. Por lo tanto, el presente estudio demostró cual es el mejor método de formación de biopelículas de muestras vaginales e indicó el tratamiento óptimo para la candidiasis.

Palabras claves: Biopelículas, Resistencia antimicrobiana, Tratamientos antifúngicos, *Candida albicans*, *Candida glabrata.*

ABSTRACT

Candida albicans and *Candida glabrata* are emerging pathogens that have a high contagion rate due to their resistance to antifungals and their ability to form biofilms. The formation of biofilms has important repercussions in terms of health issues. However, little research has been done in Ecuador on the ability of vaginal *Candida* isolates to form biofilms. The present study focused on isolating *Candida* species from vaginal samples, to determine the formation rate of biofilms of *Candida albicans* and *Candida glabrata* during (24 and 48 h) through biomass formation by optical density assays with crystal violet (CV) staining and phosphate-buffered saline (PBS) biomass assays, and their classification by biofilm formation index (BFI). In addition, this study evaluated the antifungal resistance in *Candida* planktonic cells of our group set and compared their antifungal profiles between different vaginal microbiota types (normal or healthy microbiota, intermediate microbiota, candidiasis, and mixed vaginal infection/dysbiosis). Our results showed a greater ability to form biofilms using the CV staining method at 24 h, in which 76.19% (16/21) samples were high biofilm formers (HBF) and 23.81% (5/21) as intermediate biofilm formers (IBF). Through susceptibility tests in planktonic cells, the results showed that the antifungals amphotericin B and flucytosine are not suitable for candidiasis treatments demonstrating a resistance range of 90.5 and 100%, respectively. Meanwhile, the antifungals of the Echinocandins family were the ones that evidenced the best results with a resistance range of 33.3 – 42.9% against planktonic *Candida* cells. Therefore, the present study proved the best method of biofilm formation of vaginal samples and the most optimal treatment for candidiasis.

Keywords: Biofilms, Antimicrobial resistance, Antifungal treatments, *Candida albicans*, *Candida glabrata*.

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INTRODUCTION

Invasive infection caused by *Candida* species (candidiasis) is a systemic mycosis associated with health care being recognized as one of the infections that most affects women of reproductive age. *Candida* is a yeast found in different microbial communities such as the vagina, mouth, skin, gastrointestinal tract, nose, and urethra. (Kıvanç & Er, 2020). In addition to having the ability to adapt to a variety of different environments, they are normally found as monastics but are also opportunistic pathogens (Atiencia-Carrera, Cabezas-Mera, Tejera, et al., 2022; Cangui-Panchi et al., 2022; Silva et al., 2009).

In recent years the number of infections caused by various species of *Candida* has increased progressively (Atiencia-Carrera, Cabezas-Mera, Tejera, et al., 2022). Approximately 15 *Candida* species are known to cause disease in humans. The most frequently isolated yeast is *Candida albicans*, however, in recent years there has been an increase in the incidence of candidiasis caused by other species like *Candida glabrata* (Munusamy et al., 2018; Rybak, Barker, et al., 2022).

One of the most important virulence factors of *Candida* species involves the formation of biofilms (Atiencia-Carrera, Cabezas-Mera, Vizuete, et al., 2022; Tobudic et al., 2012). Biofilms are defined as structured microbial communities that are attached to a surface and encased in a matrix of exopolymeric material (ECM), forming a complex three-dimensional architecture on biotic and abiotic surfaces (Cavalheiro & Teixeira, 2018; de Barros et al., 2020). In the last decades, research in the field of biofilms has increased because it is known to be the normal growth state for most microorganisms (Cangui-Panchi et al., 2022).

The microorganisms of this type of community show a lower growth rate and a higher rate of resistance to treatments, behaving very differently from planktonic cells. *Candida*

biofilms are different depending on the species, morphology, and metabolic activity. Due to these general characteristics, biofilms enhance the establishment of persistent infections in the human body (Cavalheiro & Teixeira, 2018). Additionally, it is known that biofilms are inherently resistant to antifungals, especially amphotericin B and fluconazole (Hasan et al., 2009).

Candidiasis can reappear quickly and poor medical care usually causes resistant candidiasis, so it is important to evaluate the antifungal activity of *Candida* species since there are still not many studies carried out in Ecuador. The present study was carried out to isolate *Candida* species from vaginal samples, quantify the rate of biofilm formation, compare antifungal susceptibility profiles in planktonic cells, and characterize the efficacy of antifungals at different minimum inhibitory concentrations (MICs) to prevent biofilm formation.

METHODS

Candida **Isolates and Growth Conditions** Twenty-one vaginal *Candida* isolates were selected, which eighteen *C. albicans* isolates (designated as V118, V130, V134, V161, V196, V202, V218, V251, V252, V415, V448, V449, V450, V451, V527, V535, V540, V580) and three *C. glabrata* isolates (designated as V197, V543, V601) of the Institute of Microbiology at Universidad San Francisco de Quito (IM-USFQ). All isolates were retrieved from a previous study realized (Salinas et al., 2020). *C. albicans* of the American Type Culture Collection ATCC® 10231™ was used as a reference control strain in our study set.

Evaluation of the Antifungal Resistance on *Candida* Planktonic Cells Fungal susceptibility to the drugs fluconazole, voriconazole, posaconazole, caspofungin, anidulafungin, micafungin, flucytosine, amphotericin B was performed using the SensiQuattro CANDIDA EU commercial kit (Liofilchem). Briefly, 150 µL of *Candida* suspension at 0.5 McFarland was transferred to each well of the 32-well panel containing the eight antimycotics in different concentrations, more exactly: Fluconazole, 1 to 8 µg/mL, Voriconazole, 0.06 to 0.5 µg/mL; Posaconazole and Caspofungin, 0.03 to 0.25 µg/mL; Anidulafungin, 0.015 to 0.12 µg/mL; Micafungin, 0.03 to 0.25 µg/mL; Flucytosine, 4 to 32 µg/mL; and finally, Amphotericin, 0.5 to 2 µg/mL. The plate was incubated at 34.5-35.5 °C for 24±2 hours. The results were interpreted according to EUCAST clinical breakpoints (EUCAST, n.d.) as S (Susceptible) or R (Resistant).

Biofilm formation The inoculum of $1x10^7$ CFU/mL in PBS was centrifuged at 400 rpm for 10 minutes, and the pellet was resuspended in 5 ml of sterile SDB and two different approaches were used to evaluate biofilm ability through biomass formation by optical density assays (Atiencia-Carrera, Cabezas-Mera, Vizuete, et al., 2022; Turan & Demirbilek, 2018). Briefly,

in each well of the 96-well, 200 µL of prepared *Candida* suspension was transferred. The plates were incubated at 37°C in static conditions during 24 h and a second plate was set during 48 h. Wells were then washed with 200 µL PBS three times after incubation before the biofilm quantification (see next subsection). Negative control of medium without inoculum and positive control of medium plus inoculum without the antifungal drug were also included. All assays were realized in triplicate on different days.

Quantification of Biofilm Formation To screen the strain's ability to form a biofilm, we used an optical density (OD) assay with crystal violet (CV) staining and phosphate-buffered saline (PBS) suspension (Gulati et al., 2018). Briefly, each optical density assay is described as follow. After 24 h of growth, the samples were washed three times with 200 µL of PBS. Then, the optical density values were measured at 570 nm for the 96-well plate using an ELISA Elx808 microplate spectrophotometer (Biotek). All biofilm samples and negative controls were measured and further classified (Turan & Demirbilek, 2018). Another set of two 96-well plates was set at the same growth culture conditions previously mentioned during 24 and 48 h. Both were emptied and washed three times with 200 µL of PBS. Then, wells were fixed with 200 µL of 99% methanol for 15 minutes. After this period, the wells were emptied and left to dry at room temperature. Each well was then stained with 200 µL of 2% crystal violet solution for 5 minutes. After, the wells were washed and treated with 160 µL of 33% glacial acetic acid solution, and the 96-well plate was read in the ELISA Elx808 microplate spectrophotometer at 570 nm. All biofilm samples and the negative controls were measured and further classified (Kıvanç & Er, 2020).

Biofilm classification The classification of the ability of the *Candida* isolates´ ability to form biofilm was realized through the biofilm formation index (BFI), as previously done (Atiencia-

Carrera, Cabezas-Mera, Tejera, et al., 2022). Biofilm-forming microorganisms are generally classified as non-forming (NBF), low (LBF), intermediate (IBF), and high (HBF), where BFI can be evaluated using different approaches of biofilm assays (Cangui-Panchi et al., 2022). The biofilm-forming ability was assessed using crystal violet and PBS suspension assays, and then, each isolate was classified accordingly to its biomass level. For the PBS suspension assay, non-biofilm formers (NBF) showed a biomass production less than or equal to cut-off values (OD_c) ($OD \leq OD_c$), low biofilm formers (LBF) evidenced $OD_c < OD > 2 \times OD_C$, intermediate biofilm formers (IBF) demonstrated $2 \times OD_c < OD > 4 \times OD_C$, and finally, high biofilm formers (HBF) established $OD > 4 \times OD_C$ (Turan & Demirbilek, 2018). For the crystal violet assay, *Candida isolates* were classified as non-biofilm formers (NBF) when $0 \leq OD570 \leq 0.120$, low biofilm formers (LBF) when $0.121 \leq OD570 \leq 0.240$, intermediate biofilm formers (IBF) when $0.241 \leq OD570 \leq 0.500$, and finally, high biofilm formers (HBF) when $OD570 > 0.500$ (Kıvanç & Er, 2020).

Statistical Analysis Data were analyzed using SPSS (version 28.0) (IBM, 2021). Hierarchical clustering was performed with the nearest neighbor method and using Euclidean distance to compare the resistance profile between the different classes of antifungals across all samples. The dendrogram was constructed in SPSS and the chemical drawing was performed using the online MolView website (https://molview.org). Finally, we used the Chi-square tests in the biofilm formation capacity data to determine the non-random association between the categorical variables.

RESULTS

Diagnosis of samples in the study

In the present study, all *Candida* isolates were previously retrieved from a previous epidemiological study realized by our research group (Salinas et al., 2020). As shown in Table 1, the *Candida* isolates were obtained from an initial sample set of 414 volunteer women, of which the majority of women were between 21-30 years old with 61.8% (256/414) within this range being all the samples with candidiasis. All women who presented candidiasis were single (7/7), 71.4% (5/7) of them did not have a sexual partner at the time, and 28.6% (2/7) had a sexual partner. Additionally, it is observed that 71.4% (5/7) used a contraceptive method, which could be a condom, hormonal control, or among others.

As shown in Table 2, the 21 *Candida* isolates were obtained from women between 19 and 33 years old. 47.62% (10/21) indicated that they had a sexual partner (where 6/9 of the women had healthy microbiota, 3/7 of the women had candidiasis, and 1/3 of the women had a mixed infection). Meanwhile, 33.33% (7/21) of the women used condoms as a contraceptive method (6/9 healthy microbiota; 1/3 with mixed infection), 33.33% (7/21) of the women used hormonal birth control such as pills or injections (2/9 healthy microbiota; 4/7 candidiasis; 1/3 mixed infection), and finally, 28.57% (6/21) of women did not use any method (2/2 intermediate microbiota; 3/7 candidiasis; 1/3 mixed infection). Only one woman did not answer the question (1/9 healthy microbiota).

In our study set, 42.85% (9/21) of the vaginal samples were diagnosed with normal or healthy microbiota, 9.52% (2/21) as intermediate microbiota, 33.33% (7/21) as samples with candidiasis, and 14.30% (3/21) as samples with mixed infection (presence of candidiasis plus another infection/dysbiosis such as aerobic vaginitis or bacterial vaginosis) as shown in Figure

1. The 30% (3/10) of the vulvovaginal candidiasis (VC) cases showed coinfections, where two coinfections combined VC and aerobic vaginitis (AV), and the last one evidenced VC and bacterial vaginosis (BV). Figure 1 shows microscopic images of each type of vaginal microbiota in the present study. In the healthy microbiota samples (V130, V197, and V451), the cell morphology does not show any alteration, and dominance of lactobacilli is observed, evidencing well-formed epithelial cells protected by these well-known probiotic bacteria against vaginal disorders and future infections (Chen et al., 2021). Intermediate microbiota samples (V118 and V543) illustrated a different scenario characterized by a substantial reduction of *Lactobacillus* species. Finally, candidiasis and VC-related mixed infection samples demonstrated epithelial cell disruption with low levels or absence of lactobacilli.

Evaluation of the Antifungal Resistance on *Candida* Planktonic Cells

An illustration of the eight antifungal agents used to evaluate the antifungal resistance in our group set is shown in Figure 2, showing an evaluation by clustering the resistance profile of each antifungal agent against the *Candida* isolates, and a correlation was observed to their chemical structures. The antifungals fluconazole and posaconazole showed a similar resistance profile being grouped in the same cluster together with voriconazole as part of the triazole family. Likewise, anidulafungin, micafungin, and caspofungin were grouped in the same cluster demonstrating the same resistance profile against our study set. These three antifungal agents belong to the echinocandins family. Finally, flucytosine and amphotericin B belong to the fluorinated pyrimidine analog family and polyene family, respectively. Although they are structurally not related, they were grouped in the same cluster since both have a higher rate of resistance.

As shown in Figure 3, antifungal agents of the triazole family showed a range of 5 to 9 resistant isolates. The echinocandins family had a greater discrepancy among *Candida* isolates, varying between 2 and 9 resistant isolates. Caspofungin showed a higher number of resistant isolates (5-9/21), followed by anidulafungin (3-7/21), and micafungin (2-7/21). Flucytosine evidenced a range of 17 to 19 resistant isolates and all *Candida* isolates were resistant to Amphotericin B.

Biofilm formation

Due to the diversity of methodologies in the literature, the present study evaluated the ability of biofilm formation of *Candida* isolates through the two most used methodologies during the most analyzed time points (24 and 48 h). As shown in Table 4, all *Candida* isolates were classified through the biofilm formation index (BFI) according to Turan & Demirbilek (2018) and Kıvanç & Er (2020) approaches. The BFI criterium classified microorganisms into the following categories: non-biofilm-forming isolates/strains (NBF); low biofilm-forming isolates/strains (LBF); intermediate biofilm-forming isolates/strains (IBF); high biofilm-forming isolates/strains (HBF). Regarding Turan & Demirbilek's (2018) approach (Figure 4), 57.14% (12/21) of the isolates were classified as HBF (6/9 healthy microbiota; 2/2 intermediate microbiota; 2/7 candidiasis; 2/3 mixed infection), followed by 33.33% (7/21) of isolates as IBF (3/9 healthy microbiota; 3/7 candidiasis; 1/3 mixed infection), and 4.76% (1/21) of the isolates were classified as LBF (1/7 candidiasis) and NBF (1/7 candidiasis). Meanwhile, concerning Kıvanç & Er's (2020) approach at 24 h, all *Candida* isolates were classified into merely two categories (Figure 4), more exactly, 76.19% (16/21) as HBF (6/9 healthy microbiota; 2/2 intermediate microbiota; 6/7 candidiasis; 2/3 mixed infection) and 23.81% (5/21) as IBF (3/9 healthy microbiota; 1/7 candidiasis; 1/3 mixed infection). When evaluating at 48 h of biofilm

growth culture, the approach demonstrated 38.1% (8/21) of isolates as HBF (4/9 healthy microbiota; 1/2 intermediate microbiota; 2/7 candidiasis; 1/3 mixed infection), followed by 33.33% (7/21) of isolates as IBF (5/9 healthy microbiota; 1/2 intermediate microbiota; 1/3 mixed infection), and 28.57% (6/21) of isolates as LBF (5/7 candidiasis; 1/3 mixed infection).

There is huge ambiguity in the results between 24 and 48 h by CV staining, contrasting also from the results obtained by the PBS suspension approach. Therefore, Chi-square tests were performed between approaches to evaluate biofilm-forming ability, using the PBS suspension approach as a reference. A p-value of 0.481 was obtained between PBS suspension and CV staining at 48 h, demonstrating a coincidence of 57.14% between the results and thus a lack of relationship between these two approaches. However, when comparing PBS suspension and CV staining at 24 h, a coincidence of 90.48% coincidence was obtained justifying the application of the 24 h PBS method.

DISCUSSION

Candida species constitute yeasts that can act as an opportunistic pathogen once there is a disruption of the host's defense. The increase in the misuse of antifungals and the number of immunocompromised patients or invasive treatments has caused candidiasis to become an alarming opportunistic infection (Mohandasy & Ballal, 2020). This study evidenced the presence of *Candida* species in every type of vaginal microbiota. As expected, the most predominant *Candida* species in our study set was *C. albicans* (85.71%), followed by *C. glabrata* (14.29%). Our finding is similar to the data shown by other studies (Marak & Dhanashree, 2018; Tortelli et al., 2020), which reported a prevalence of 45.5% of *C. albicans* and 30% of *C. glabrata* in their study set. In agreement, this study showed *C. albicans* as the dominant *Candida* species in the vaginal microbiome. However, the prevalence of non-*albicans Candida* (NAC) species in the vaginal microbiome varies among women, ranging from ~10–30% (Tortelli et al., 2020). Moreover, vaginal *Candida* colonization could lead to the development of candidiasis in women, as an opportunistic infection characterized by an overgrowth of *Candida* species and the diminution of the probiotic lactobacilli, leading to the destruction of vaginal epithelial cells and thus an aggressive immune response in the host.

In the present study, it is found that vulvovaginal candidiasis (VC) frequently occurs in different age groups when compared with other vaginal infections, more exactly aerobic vaginitis (AV) in an age group between 20-23 years and a single case of bacterial vaginosis (BV) at age 21. According to the surveys carried out by Nasir et al. (Abdullahi Nasir et al., 2015), the presence of VC was found in an age group between 21-40 years agreeing with our study, which reported VC in women between 21-30 years old. Taking into consideration single women as the group with the greatest susceptibility for VC development, our results match the

previous study by Saijan et al., which reported that the greatest recovery of *Candida* isolates came from single women through the vaginal swab technique (Sajjan et al., 2014).

The majority of BV cases were obtained from single women who did not use a safe contraceptive method, where three of them had a sexual partner and manifested symptoms (discomfort), suggesting that behavior has a direct effect on the risk of acquiring candidiasis as postulated by many authors (Hellberg et al., 1995; Puri et al., 2003; Quindós et al., 2018). It is estimated that condom use at the time of sexual intercourse is an important factor in terms of the health of the vaginal microbiota (Kobayashi et al., 2020; Salinas et al., 2020). In our group set, six of the nine women with a healthy microbiota used condoms and no symptoms were reported, contrasting women with the presence of mixed infection or dysbiosis who despite the use of condoms they already reported symptoms of both infections (1/21) and previous clinical treatments (7/21). Therefore, the success of clinical treatments is vital for these cases and it is important to monitor the antifungal resistance among *Candida* isolates, allowing optimal treatments with higher efficiency and a low rate of reinfections among patients. In our group set, all 21 *Candida* isolates were resistant to the different concentrations of amphotericin B. Similar results were previously shown by many studies (Galia et al., 2022; Maphanga et al., 2021; Rybak, Barker, et al., 2022), demonstrating that this drug is no longer effective for treating *Candida*-related infections, including *C. auris* (Wu et al., 2019).

Moreover, 42.9% of *Candida* isolates were resistant to triazoles in our study, more exactly, fluconazole, voriconazole, and posaconazole. Although azole antifungals have long provided effective treatment (Fisher et al., 2021; Shu et al., 2022), current studies showed the intrinsic resistance to azoles in various *Candida* species (Espinel-Ingroff et al., 2021; Rybak, Cuomo, et al., 2022; Whaley et al., 2017). Even though the majority of our group set was *C.*

albicans, the three *C. glabrata* isolates also showed resistance against triazoles, which agrees with the findings of Fothergill and colleagues (Fothergill et al., 2014). These authors already reported an increase in the resistance rate previously established by the Clinical and Laboratory Standards Institute (CLSI), evidencing a resistance increase from 6.1% to 18.4% for voriconazole.

The echinocandins family showed a resistance range between 33.3% and 42.9% of *Candida* isolates among our study set, demonstrating the largest number of resistant strains with caspofungin in its highest concentration in planktonic cells. Studies carried out in Europe showed that resistance to echinocandins still seems insignificant with a resistance rate between 0.5% and 10% (Martínez-Herrera et al., 2021; Mesquida et al., 2021), so our results demonstrated an alarming augmentation of the resistance against caspofungin since almost half of the *Candida* isolates were not inhibited. In addition, Galia and colleagues evidenced lower resistance rates in their group set of 30 women, more exactly, 1.4% of resistant strains for caspofungin, 2.9% for anidulafungin, and 1.3% for micafungin (Galia et al., 2022), contrasting with our results. This study revealed a higher percentage of resistance to caspofungin among Ecuadorian women.

Regarding flucytosine, little has been studied about its resistance rate in microbiological studies. However, in studies realized by Charlier et al. and Jacobs et al., both studies discovered that *Candida* isolates from patients became resistant to flucytosine after the treatment was finished, from 6 days until 6 months. Their resistance rate was greater than 90%, which agrees with the 90.5% of resistant strains obtained in the present study. However, it is necessary to carry out more studies regarding this antifungal to make better comparisons with our preliminary analysis.

Finally, the ability to establish biofilms is essential for the pathogenicity and virulence of *Candida* species during vulvovaginal candidiasis (Mohandasy & Ballal, 2020; Munusamy et al., 2018). As expected, all vaginal isolates and both *Candida* species demonstrated their ability to form biofilms. The present study proved that biofilm production was 100% among all species using the 24 h CV staining assays, although the remaining applied methodologies (48 h CV staining and 24 h PBS suspension assays) showed a lower rate. In recent studies that applied similar methodologies (Atiencia-Carrera, Cabezas-Mera, Tejera, et al., 2022; Kıvanç & Er, 2020), it is reported that the application of this biomass assay (in particular, 24 h CV staining) in biofilms shows more accuracy in the data analysis, reporting a formation rate of 78%-80% among their clinical isolates. When comparing *Candida* species, both *C. albicans* and *C. glabrata* demonstrated a good ability to form biofilms. It is important to mention that only 3 of 21 vaginal isolates were *C. glabrata* and no trustful conclusion is possible to achieve, however, these vaginal isolates evidenced a high-intermediate biofilm-forming classification and further evaluation on this *Candida* species should be realized in future studies.

In this study, it was only possible to analyze antifungal resistance in planktonic cells, and therefore further evaluation of the antifungal resistance in biofilms of the present group set must also be realized constituting a limitation in the present work. The characterization of *C. albicans* and *C. glabrata* biofilms is currently an important field of research due to the large increase in persistent and severe vaginal infections among women of reproductive age (McKloud et al., 2021; Salinas et al., 2020). However, the present study has additional shortcomings such as the lack of molecular and classical analysis between the biofilms of *Candida albicans* and *Candida glabrata*, and the small number of samples used in the study does not allow to generalize the results obtained from antifungal resistance in planktonic cells to the Ecuadorian women.

CONCLUSIONS

The present study proved the ability of *Candida albicans* and *Candida glabrata* to produce a strong biofilm using different methodologies. Amphotericin B and flucytosine are not suitable for the treatment of *Candida*-related infections, neither in planktonic cells nor in biofilms, among Ecuadorian women. Anidulafungin and micafungin (echinocandins) appeared to be the most efficient fungicidal agents with 33.3 – 42.9% resistance range against planktonic *Candida* cells. The prevalence of *Candida* isolates with biofilm formation ability was 100% by biofilm index formation in 24 h CV staining assays, showing 76.19% of high biofilm formers and 23.81% of intermediate biofilm formers. To the best of the authors' knowledge, this is the first study carried out in Ecuador to analyze the antifungal activity of vaginal *Candida* isolates in planktonic cells and their ability for biofilm formation. Further studies are needed to evaluate *Candida*-related biofilms and their antifungal resistance through molecular and classical analysis.

TABLES

Table 1. General information was extracted from the data of women in the study with healthy microbiota, intermediate microbiota, candidiasis, and other vaginal infections or dysbiosis, as previously reported by our research group (Salinas et al., 2020).

		Healthy microbiota N (%)	Intermediate microbiota N (%)	Candidiasis N (%)	Other infections N (%)	Total N (%)
Total incidence		276 (66.7)	43 (10.4)	7 (1.7)	88 (21.3)	414 (100.0)
Age	Under 20	57 (20.7)	9 (20.9)	2 (28.6)	21 (23.9)	89 (21.5)
	21-30	175 (63.4)	27 (62.8)	5 (71.4)	49 (55.7)	256 (61.8)
	31-40	27 (9.8)	3 (7.0)	0 (0.0)	10 (11.4)	40 (9.7)
	41-50	13 (4.7)	2 (4.7)	0 (0.0)	3 (3.4)	18 (4.3)
	Over 50	4 (1.4)	2 (4.7)	0 (0.0)	5 (5.7)	11 (2.7)
Civil status	Single	229 (83.0)	36 (83.7)	7 (100.0)	71 (80.7)	343 (82.9)
	Free union*	4 (1.4)	1 (2.3)	0 (0.0)	4 (4.5)	9 (2.2)
	Married	39 (14.1)	5 (11.6)	0 (0.0)	9 (10.2)	53 (12.8)
	Divorced	4 (1.4)	1 (2.3)	0 (0.0)	4 (4.5)	9 (2.2)
Sexual partner	Not having	101 (36.6)	25 (58.1)	5 (71.4)	37 (42.0)	168 (40.6)
	Having	175 (63.4)	18 (41.9)	2 (28.6)	51 (58.0)	246 (59.4)
Contraceptive use	No	101 (36.6)	26 (60.5)	2 (28.6)	33 (37.5)	162 (39.1)
	Yes	175 (63.4)	17 (39.5)	5 (71.4)	55 (62.5)	252 (60.9)
Birth control methods	Condom	82 (29.7)	7 (16.3)	4 (57.1)	32 (36.4)	125 (30.2)
	Hormonal contraception	47 (17.0)	2 (4.7)	1 (14.3)	11 (12.5)	61 (14.7)
	Combined	38 (13.8)	6 (14.0)	0 (0.0)	9 (10.2)	53 (12.8)
	Others	8 (2.9)	2 (4.7)	0 (0.0)	3 (3.4)	13 (3.1)
	None or don't answer	101 (36.6)	26 (60.5)	2 (28.6)	33 (37.5)	162 (39.1)

*Free Union: Free Union couples living together for at least 3 years without being married. Epidemiological and behavioral variables among women in the study by (Salinas et al., 2020) with healthy microbiota, intermediate microbiota, candidiasis and other infections. N number of women who answered the survey within each category; % percentage assigned for each classification.

Table 2 . General information was extracted from the initial data with healthy microbiota, intermediate microbiota, candidiasis, and mixed vaginal infections or dysbiosis..

Isolates	Sample	Age	Behavior										
			Sexual partner	Contraceptive use	Number of sexual partners in the last year	Vaginal discharge	Strong odor of vaginal discharge	Number of treatments	Discomfort due to vaginal secretion	Have Children	Medical consultation for infection	Number of treatments during life	Coinfections
HEALTHY MICROBIOTA													
C. albicans	V130	33	Yes	HC	1	Yes	Yes	1	Yes	No	No	3	2
C. albicans	V134	25	Yes	Condom	1	No	No	1	No	No	Yes	1	1
C. albicans	V196	22	Yes	Condom	1	Yes	Yes	1	No	No	Yes	NA	1
C. glabrata	V197	21	Yes	HC	1	No	No	0	No	No	No	NA	1
C. albicans	V202	19	No	NA	NA	Yes	No	NA	NA	NA	NA	NA	NA
C. albicans	V251	20	Yes	Condom	1	Yes	No	1	No	No	Yes	1	1
C. albicans	V448	24	No	Condom	2	Yes	No	1	Yes	No	No	NA	1
C. albicans	V451	22	No	Condom	1	Yes	No	NA	No	No	No	NA	1
C. albicans	V580	24	Yes	Condom	NA	NA	NA	NA	NA	No	No	NA	1
INTERMEDIATE MICROBIOTA													
C. albicans-E. coli	V118	22	No	None	NA	Yes	NA	NA	NA	NA	NA	NA	1
C. glabrata-Gram positive coccus	V543	20	NA	None	NA	Yes	No	1	No	No	NA	NA	1

CANDIDIASIS													
C. albicans	V161	30	Yes	None	1	No	No	2	No	Yes	No	NA	1
C. albicans	V218	23	No	None	NA	Yes	No	1	No	No	No	NA	1
C. albicans	V252	21	NA	HC	NA	Yes	No	NA	No	No	No	NA	1
C. albicans	V449	24	Yes	HC	1	Yes	NA	NA	Yes	Yes	No	NA	1
C. albicans	V450	23	Yes	HC	1	NA	NA	NA	No	No	Yes	1	1
C. albicans	V535	24	No	None	NA	No	No	NA	No	No	No	NA	1
C. albicans	V540	19	No	HC	NA	No	No	1	No	No	No	NA	1
MIXED INFECTION													
C. albicans Candidiasis- Aerobic vaginitis	V415	20	No	HC	NA	Yes	Yes	1	Yes	No	Yes	3	1
C. albicans Candidiasis- Aerobic Vaginitis	V527	23	Yes	Condom	>4	Yes	Yes	1	No	No	No	NA	3
C. glabrata Candidiasis- Bacterial vaginosis	V601	21	No	None	NA	Yes	No	NA	No	No	No	NA	1

HC: hormonal birth control. NA: Not answer. Behavior variables of the 21 samples of women in the study with healthy microbiota, intermediate microbiota, candidiasis, and with the presence of mixed infection.

Table 3. Biofilm classification of *Candida* isolates according to Turan & Demirbilek (2018) and Kıvanç & Er (2020).

#	Isolates	Sample	Biofilm formation capacity According to (Turan & Demirbilek, 2018)		Biofilm formation capacity According to (Kıvanç & Er, 2020)		
			PBS OD value 24h (± standard deviation)	Biofilm formation categories	CV OD value 24h (± standard deviation)	CV OD value 48h (± standard deviation)	Biofilm formation categories
HEALTHY MICROBIOTA							
1	*C. albicans*	V130	0.69 (± 0.21)	HBF	1.10(± 0.29)	0.23 (± 0.33)	HBF/IBF
2	*C. albicans*	V134	0.71 (± 0.21)	HBF	0.49(± 0.29)	0.40(± 0.33)	IBF
3	*C. albicans*	V196	0.64 (± 0.21)	HBF	0.75(± 0.29)	0.49(± 0.33)	HBF/IBF
4	*C. glabrata*	V197	0.53 (± 0.21)	IBF	0.50(± 0.29)	1.48(± 0.33)	HBF
5	*C. albicans*	V202	0.63 (± 0.21)	HBF	0.88(± 0.29)	0.53(± 0.33)	HBF
6	*C. albicans*	V251	0.35 (± 0.21)	IBF	0.57(± 0.29)	0.55(± 0.33)	HBF
7	*C. albicans*	V448	0.68 (± 0.21)	HBF	0.36(± 0.29)	0.38(± 0.33)	IBF
8	*C. albicans*	V451	0.33 (± 0.21)	IBF	0.24(± 0.29)	0.29(± 0.33)	IBF
9	*C. albicans*	V580	1.09 (± 0.21)	HBF	1.12 (± 0.29)	0.59 (± 0.33)	HBF
INTERMEDIATE MICROBIOTA							
10	*C. albicans*-*E. coli*	V118	0.99 (± 0.21)	HBF	0.95 (± 0.29)	0.68 (± 0.33)	HBF
11	*C. glabrata*-Gram positive coccus	V543	1.18 (± 0.21)	HBF	1.09 (± 0.29)	0.39 (± 0.33)	HBF/IBF
CANDIDIASIS							
12	*C. albicans*	V161	0.14 (± 0.21)	NBF	0.50(± 0.29)	0.69 (± 0.33)	HBF
13	*C. albicans*	V218	0.28 (± 0.21)	LBF	0.68(± 0.29)	0.11 (± 0.33)	HBF/LBF
14	*C. albicans*	V252	0.34 (± 0.21)	IBF	0.61(± 0.29)	0.22(± 0.33)	HBF/LBF
15	*C. albicans*	V449	0.59 (± 0.21)	HBF	0.56(± 0.29)	0.17(± 0.33)	HBF/LBF
16	*C. albicans*	V450	0.69 (± 0.21)	HBF	0.45(± 0.29)	0.15(± 0.33)	IBF/LBF
17	*C. albicans*	V535	0.39 (± 0.21)	IBF	0.62(± 0.29)	0.16(± 0.33)	HBF/LBF
18	*C. albicans*	V540	0.39 (± 0.21)	IBF	0.65(± 0.29)	1.09(± 0.33)	HBF
MIXED INFECTION							
19	*C. albicans* Candidiasis-Aerobic vaginitis	V415	0.73 (± 0.21)	HBF	0.48(± 0.29)	0.23(± 0.33)	IBF/LBF
20	*C. albicans* Candidiasis-Aerobic Vaginitis	V527	0.49 (± 0.21)	IBF	0.68(± 0.29)	0.82 (± 0.33)	HBF
21	*C. glabrata* Candidiasis-Bacterial vaginosis	V601	1.04 (± 0.21)	HBF	0.86 (± 0.29)	0.41(± 0.33)	HBF/IBF

HBF: High biofilm formers; IBF: Intermediate biofilm formers; LBF: Low biofilm formers; NBF: non-biofilm formers. Classification of the biofilm formation capacity of the 21 samples analyzed in the study.

FIGURES

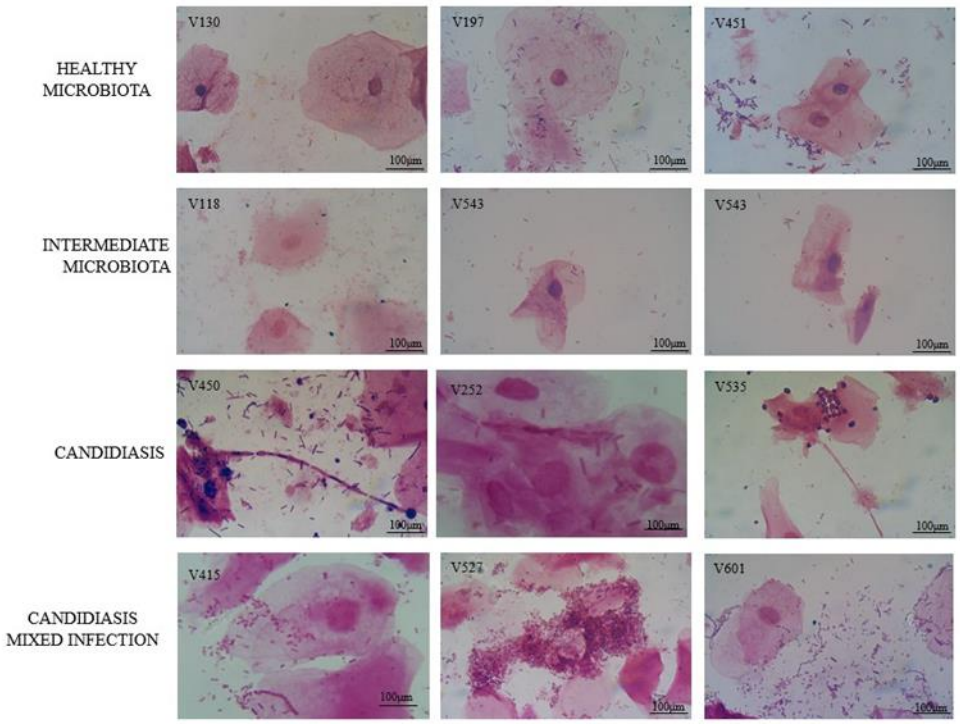

Figure 1. An illustration of the representative images of each type of vaginal microbiota is observed in the vaginal samples selected in the present study.

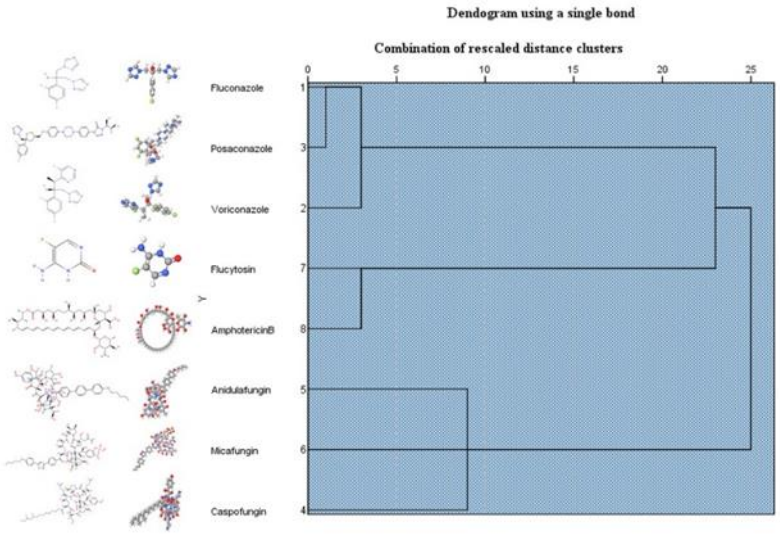

Figure 2. Cluster evaluation of the resistance profile obtained by different classes of antifungal agents against the *Candida* isolates in our study set.

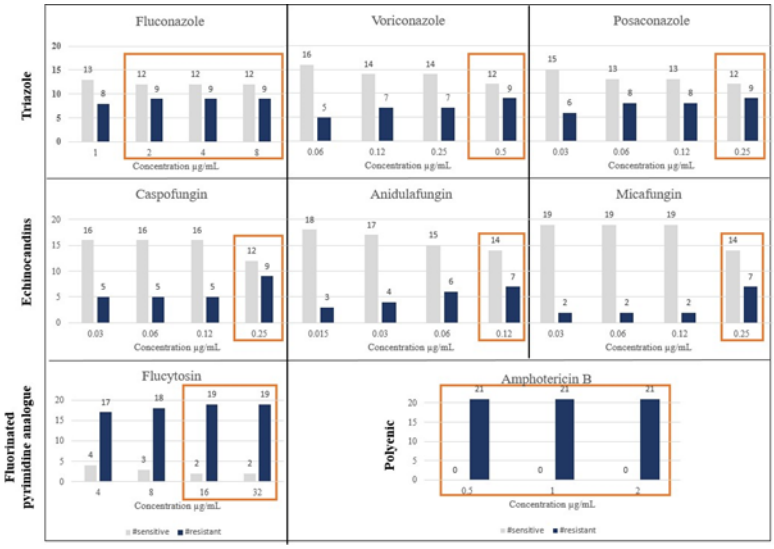

Figure 3. Illustration of the antifungal susceptibility and resistance evaluation obtained on planktonic cells of the *Candida* isolates in the present study.

Description: The orange squares indicate the concentration at which the antifungal is resistant according to the previously mentioned literature.

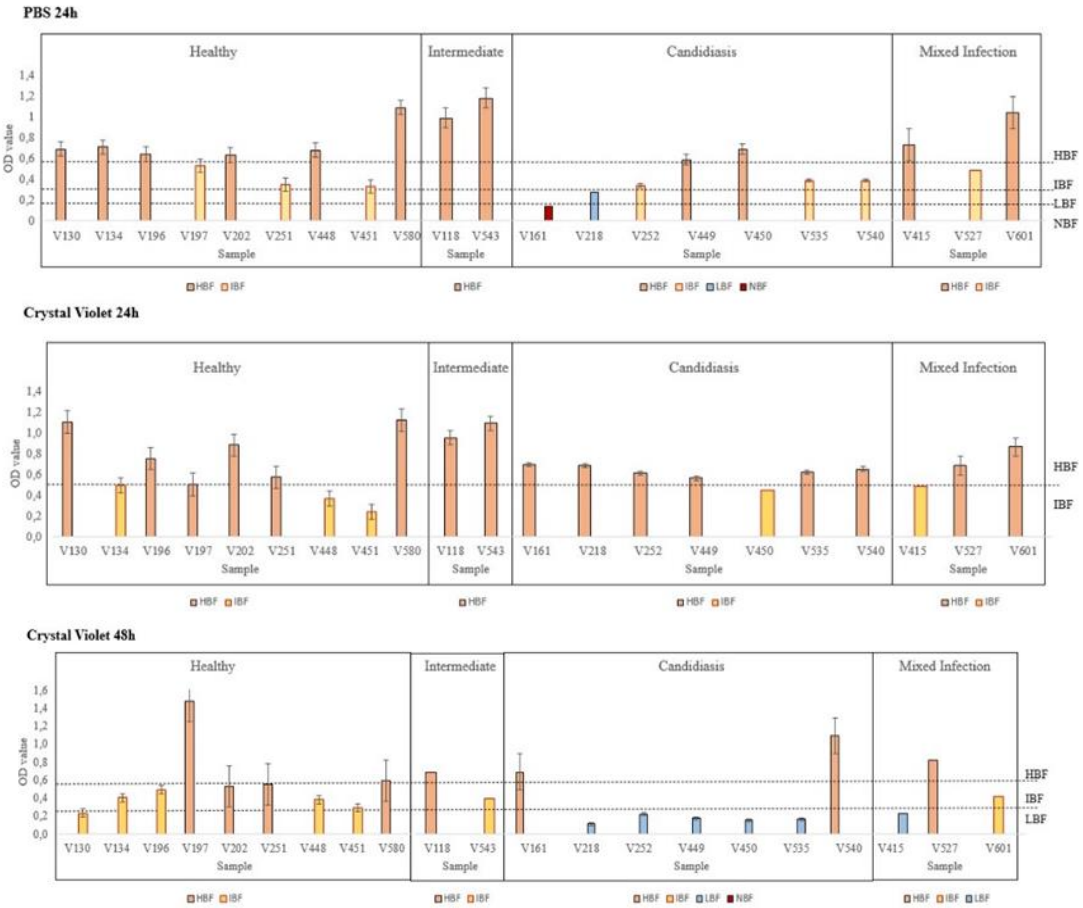

Figure 4. Illustration of the biofilm-forming ability of *Candida* isolates and their classification according to Turan & Demirbilek (2018) and Kıvanç & Er (2020).

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APPENDIX

Appendix 1. EUCAST and CLSI recommended ranges for the classification of susceptible (S) and resistant (R) isolates against different antifungal agents..

Family	Antifungal Agent	Species	S ≤ (mg/L)	R > (mg/L)	Reference
Triazoles	Fluconazole	*C. albicans*	2	4	(EUCAST, n.d.)
		C. glabrata	0.001	16	(EUCAST, n.d.)
	Voriconazole	*C. albicans*	0.06	0.25	(EUCAST, n.d.)
		C. glabrata	ND	ND	(EUCAST, n.d.)
	Posaconazole	*C. albicans*	0.06	0.06	(EUCAST, n.d.)
		C. glabrata	ND	ND	(EUCAST, n.d.)
Echinocandins	Caspofungin	*C. albicans*	0.25	1	(Alexander & CLSI, n.d.)
		C. glabrata	0.12	0.5	(Alexander & CLSI, n.d.)
	Anidulafungin	*C. albicans*	0.03	0.03	(EUCAST, n.d.)
		C. glabrata	0.06	0.06	(EUCAST, n.d.)
	Micafungin	*C. albicans*	0.016	0.016	(EUCAST, n.d.)
		C. glabrata	0.03	0.03	(EUCAST, n.d.)
Fluorinated pyrimidine analog	Flucytosine	*C. albicans*	50	150	(Inderbir Padda & Mayur Parmar, 2022)
		C. glabrata	50	150	(Inderbir Padda & Mayur Parmar, 2022)
Polyenic	Amphotericin B	*C. albicans*	1	1	(EUCAST, n.d.)
		C. glabrata	1	1	(EUCAST, n.d.)