

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

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**Exploring Environmental *Candida* sp. Diversity and Antifungal Resistance
Compared to Clinical Strains**

**Tesis en torno a una hipótesis o problema de investigación y su
contrastación**

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Compared to Clinical Strains**

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DEDICATION

Dedicated to my parents, Carlos and Narita, and my sister Viviana for their unconditional support and life example. Because of them, this has been possible.

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RESUMEN

La diversidad y abundancia de los hongos se altera cuando las condiciones cambian abruptamente debido a su rol clave en procesos biogeoquímicos. Por esta razón, su monitoreo, al igual que coliformes totales, resulta esencial para determinar la contaminación de un nicho ecológico. Adicionalmente, el estudio de resistencia antifúngica en levaduras oportunistas que provienen de esas fuentes permite dilucidar el grado de amenaza que representan para la salud pública. Este estudio monitoreó los cambios de abundancia y diversidad de mohos, levaduras, y *Candida* spp. de muestras de agua y biofilm colectadas en tres puntos de los ríos Machángara (M0, M1 y M2) y San Pedro (SP0, SP1 y SP2) durante dos épocas lluviosa y una época seca. La cuantificación se realizó mediante ensayos de unidades formadoras de colonia por cada 100 mililitros agua (UFC/100mL), y por peso húmedo de biopelícula (UFC/g) para muestras de agua y biofilm, respectivamente. La diversidad de levaduras oportunistas se analizó mediante identificación por CHROMagar *Candida*, PCR convencional multiplex, API 20C AUX y MALDI-TOF MS. El estudio exploratorio de resistencia antifúngica comprendió la evaluación de la concentración mínima inhibitoria (CMI) al 90%, en planctónico, de las especies *Candida albicans*, *Candida tropicalis* y *Nakaseomyces glabratus* mediante el método de dilución en caldo usando los agentes antifúngicos fluconazol, flucitosina, anfotericina B y micafungina, y como tratamiento alternativo iones y nanopartículas de plata. Mediante esta investigación se revelaron altas variaciones de los hongos en agua y biofilm que reflejaban un aumento en la concentración en los puntos 1 y 2, en comparación con el punto 0 durante las tres épocas en los dos ríos. En las muestras de agua de los ríos Machángara y San Pedro, los mohos tuvieron la mayor densidad en el punto 1 y 2 de las épocas lluviosa 2 y seca (2.3×10^5 y 1.7×10^5 , respectivamente); las levaduras, en el punto 2 de la época seca (4.2×10^7 y 8.8×10^6 , respectivamente); y *Candida* spp., en el punto 2 de la época seca (5.1×10^5 y 2.5×10^5 ,

respectivamente). En las muestras de biofilm de ambos ríos, con una menor densidad que las muestras de agua, los mohos tuvieron la concentración más alta en el punto 2 de las épocas seca y lluviosa 2 (3.7×10^2 y 1.8×10^2 , respectivamente); las levaduras, en el punto 2 de la época lluviosa 2 y seca (1.1×10^4 y 3.3×10^3 , respectivamente); y *Candida* spp., en el punto 2 de la época lluviosa 2 (2.0×10^3 y 2.5×10^2 , respectivamente). Con respecto a la diversidad, se identificaron las especies de levaduras oportunistas de mayor relevancia clínica de los géneros *Candida*, *Nakaseomyces*, *Meyerozyma*, *Pichia*, *Kluyveromyces* y *Wickerhamiella*. También se encontró *Saccharomyces cerevisiae* y *Lachancea fermentati*. La sensibilidad antifúngica de *C. albicans* y *C. tropicalis*, determinada en base al CLSI y EUCAST, fue alta en su mayoría para anfotericina B (MIC₉₀: 0.006 y 0.008 µg/mL), flucitosina (MIC₉₀: 2-8 µg/mL) y micafungina (MIC₉₀: 0.13-0.25 µg/mL), pero baja para fluconazol (MIC₉₀: 4-32 µg/mL). *N. glabratus* presentó sensibilidad alta para anfotericina B y flucitosina (0.006 y 4 µg/mL, respectivamente), pero tuvo sensibilidad intermedia y resistencia para micafungina (0.13 y 0.25 µg/mL) y fluconazol (32 µg/mL). Finalmente, los iones y nanopartículas de plata lograron inhibir a las tres especies de levadura con valores de MIC₉₀ entre 0.13 y 1 mM. Los hallazgos de esta investigación corroboran la importancia de los hongos, especialmente levaduras oportunistas, como indicadores complementarios de contaminación ambiental propuesta en estudios previos. Además, otorga un primer vistazo al posible panorama de resistencia antifúngica de las levaduras oportunistas de mayor relevancia clínica en Ecuador provenientes de ambientes hospitalarios y nichos ecológicos contaminados.

Palabras clave: Hongos ambientales, fuentes hídricas naturales contaminadas, indicadores de contaminación ambiental, levaduras oportunistas, resistencia antifúngica, concentración mínima inhibitoria, tratamientos alternativos.

ABSTRACT

The diversity and abundance of fungi are altered when conditions change abruptly due to their key role in biogeochemical processes. For this reason, their monitoring, like total coliforms, is essential to determine the pollution of an ecological niche. Additionally, the study of antifungal resistance in opportunistic yeasts from these sources allows us to elucidate the degree of threat they pose to public health. This study monitored changes in the abundance and diversity of molds, yeasts, and *Candida* spp. from water and biofilm samples collected at three points in the Machángara (M0, M1, and M2) and San Pedro (SP0, SP1, and SP2) rivers during two rainy seasons and one dry season. Quantification was performed by testing colony-forming units per 100 milliliters of water (CFU/100mL), and per humid weight of biofilm (CFU/g) for water and biofilm samples, respectively. Opportunistic yeast diversity was analyzed by identification through CHROMagar *Candida*, conventional multiplex PCR, API 20C AUX, and MALDI-TOF MS. The exploratory study of antifungal resistance included the evaluation of the minimal inhibitory concentration (MIC) at 90%, in planktonic, of species *Candida albicans*, *Candida tropicalis*, and *Nakaseomyces glabratus* through broth dilution method using the antifungal agents: fluconazole, flucytosine, amphotericin B and micafungin, as well as silver ions and silver nanoparticles as alternative treatments. This study revealed high variations of fungi in water and biofilm, showing an increase in concentration at points 1 and 2, compared to point 0 during three seasons in both rivers. In water samples from the Machángara and San Pedro Rivers, molds had the highest density at points 1 and 2 of the rainy 2 and dry seasons (2.3×10^5 and 1.7×10^5 , respectively); yeasts at point 2 of the dry season (4.2×10^7 and 8.8×10^6 , respectively); and *Candida* spp., at point 2 of the dry season (5.1×10^5 and 2.5×10^5 , respectively). In biofilm samples of both rivers, having a lower density than water samples, molds had the highest concentration at point 2 of the dry and rainy 2 seasons (3.7×10^2 y

1.8×10^2 , respectively); yeasts, at point 2 of the rainy 2 and dry seasons (1.1×10^4 and 3.3×10^3 , respectively); and *Candida* spp., at point 2 of the rainy season 2 (2.0×10^3 and 2.5×10^2 , respectively). Regarding diversity, the most clinically relevant opportunistic yeast species identified belong to the genera *Candida*, *Nakaseomyces*, *Meyerozyma*, *Pichia*, *Kluyveromyces*, and *Wickerhamiella*. Antifungal susceptibility of *C. albicans* and *C. tropicalis*, determined following CLSI and EUCAST guides, was mostly high for amphotericin B (MIC₉₀: 0.006 and 0.008 µg/mL), flucytosine (MIC₉₀: 2-8 µg/mL) and micafungin (MIC₉₀: 0.13-0.25 µg/mL), but low for fluconazole (MIC₉₀: 4-32 µg/mL). *N. glabratus* presented high susceptibility for amphotericin B and flucytosine (0.006 and 4 µg/mL, respectively) but intermediate susceptibility and resistance for micafungin (0.13 and 0.25 µg/mL) and fluconazole (32 µg/mL). Finally, silver ions and silver nanoparticles could inhibit all three yeast species with MIC₉₀ values between 0.13 and 1 mM. The findings of this research corroborate the relevance of fungi, especially opportunistic yeasts, as complementary indicators of environmental pollution proposed in previous studies. Furthermore, it provides a first glimpse of the possible landscape of antifungal resistance of Ecuador's most clinically important opportunistic yeasts from hospitals and polluted ecological niches.

Keywords: Environmental fungi, contaminated natural water sources, indicators of environmental pollution, opportunistic yeasts, antifungal resistance, minimum inhibitory concentration, alternative treatments.

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PART 1: LITERATURE REVIEW

Introduction

Freshwater ecosystems harbor a diverse array of fungi, including yeasts, which play pivotal roles in nutrient cycling, organic matter decomposition, and overall ecosystem health (Hagler, 2006; Nagahama, 2006). Despite their ecological importance, the diversity and dynamics of aquatic yeasts remain underexplored, particularly when compared to the more extensively studied terrestrial fungi or aquatic bacteria. Genera such as *Candida*, *Cryptococcus*, and *Rhodotorula* are frequently detected in water bodies; however, research on these organisms has predominantly focused on polluted or urban aquatic systems (Baker et al., 2024; Ruosta et al., 2019). These yeasts constitute an integral part of the natural microbiota but are also subject to anthropogenic influences, including agricultural runoff and urbanization, which introduce contaminants that significantly shape yeast community composition (Monapathi et al., 2020).

Yeast in freshwater environments is important from ecological and public health perspectives. Opportunistic pathogenic species, including *Candida albicans* and *Cryptococcus neoformans*, pose significant infection risks, particularly to immunocompromised individuals (Arvanitidou et al., 2002; Monapathi et al., 2017). Studies have reported the widespread presence of these species in diverse aquatic settings, such as rivers, lakes, and reservoirs. Their sensitivity to environmental changes could make them effective bioindicators of organic pollution (Baker et al., 2024; Hagler, 2006). However, exposure to subtherapeutic levels of antifungal agents in contaminated waters has been associated with the development of antifungal resistance, complicating treatment strategies and amplifying public health challenges (Monapathi et al., 2020).

This short review highlights the diversity of fungi within freshwater ecosystems and examines their potential as bioindicators of water quality. It also explores the prevalence of opportunistic yeasts in aquatic environments and their associated public health risks, focusing on emerging antifungal resistance trends.

Fungi diversity in freshwater environments

Freshwater fungi constitute a diverse and ecologically significant group of organisms that inhabit various aquatic environments, including ponds, lakes, rivers, wetlands, peat swamps, streams, and artificial reservoirs. These fungi may complete their entire life cycle or part of it in such habitats or colonize submerged plant material within these ecosystems (Calabon et al., 2023; Mirabile et al., 2023). Approximately 3,000–4,000 species have been classified as aquatic fungi. However, with global fungal diversity estimated to range between 0.5 and 10 million species, knowledge of this group remains unexplored. Numerous taxonomic groups are poorly studied, and many aquatic habitats have yet to be discovered, underscoring the need for further research (Blackwell, 2011; Grossart & Rojas-Jimenez, 2016).

Freshwater yeast communities in tropical rivers, lakes, and lagoons usually include genera such as *Candida*, *Clavispora*, *Cyberlindnera*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Meyerozyma*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Torulaspora*, *Trichosporon*, and *Yarrowia* (Libkind et al., 2017). Similarly, groundwater, a key source of drinking water, exhibits a yeast diversity comparable to that of surface waters. Notable genera in groundwater include *Candida*, *Clavispora*, *Cryptococcus*, *Geotrichum*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Trichosporon*, and *Yarrowia*. These findings highlight yeasts' ecological importance and taxonomic richness in freshwater

environments, warranting further investigation (Brandão et al., 2010; Libkind et al., 2017; Pereira et al., 2009).

Opportunistic yeasts in water

Tropical ecosystems, such as rivers bordered by forests and situated near urban areas, host a diverse array of yeast species influenced by terrestrial inputs, including soil and anthropogenic activities (Hagler, 2006; Libkind et al., 2017). Among these, opportunistic pathogens such as *Candida*, *Cryptococcus*, *Kluyveromyces*, *Meyerozyma*, *Pichia*, and *Rhodotorula* have been frequently identified in freshwater systems (Monapathi et al., 2017; Ruosta et al., 2019).

Potentially pathogenic yeasts in water resources pose significant public health risks due to their potential to transmit infectious diseases through contaminated water. Yeast density and diversity have been shown to vary depending on water type and quality (Hagler, 2006).

Studies have provided quantitative insights into yeast abundance in polluted waters. Hagler and Ahearn, for example, reported average yeast counts of 5 colony-forming units (CFU)/100 mL in seawater, 1×10^1 CFU/100 mL in lakes, 5×10^1 CFU/100 mL in rivers, and as high as 2.8×10^2 CFU/100 mL in urban estuaries (Weber, 1989). In Illinois, Woollett and Hedrick observed significantly higher counts, averaging 2.7×10^4 CFU/100 mL (Woollett & Hedrick, 1970). In South Africa, yeast levels reached up to 8.7×10^2 CFU/100 mL in river and lake samples (Van Wyk et al., 2012), while studies in Lago Rico, Brazil, recorded counts of 7.2×10^1 CFU/100 mL (Brandão et al., 2017). Additionally, Maciel and colleagues reported counts of 1.7×10^2 CFU/100 mL at Brazilian beaches (Maciel et al., 2019).

Research on opportunistic yeast diversity in aquatic environments has revealed notable findings. For instance, studies in Brazilian rivers and lakes identified *Candida* spp.,

Meyerozyma guilliermondii (formerly known as *Candida guilliermondii*), and *Pichia kudriavzevii* (formerly known as *Candida krusei*) as prevalent species (Medeiros et al., 2012). In Nigeria, *Candida tropicalis* emerged as the predominant yeast in polluted water from streams and other sources (Ayanbimpe et al., 2013). Similarly, *C. tropicalis* was found to be the dominant opportunistic yeast species in surface waters in South Africa (Monapathi et al., 2021). These findings highlight the ecological significance and potential health implications of yeasts in aquatic ecosystems.

Antifungal resistance in environmental opportunistic yeasts

Efforts to monitor and understand the development of antifungal resistance in clinical yeast species have been extensive. This resistance often results from the prolonged exposure of yeasts to antifungal agents (Perfect, 2017). In polluted aquatic environments, the continuous presence of subtherapeutic levels of these agents also contributes to the emergence of antifungal-resistant and potentially pathogenic yeasts (Brandão et al., 2010; Brilhante et al., 2016).

Numerous studies have examined antifungal resistance in opportunistic yeast strains from diverse ecological settings. Research conducted in three lakes in Southeastern Brazil reported resistance rates of 22% to amphotericin B, 20% to itraconazole, and 3% to fluconazole in *Candida* spp. isolates (Brandão et al., 2010). In unpolluted natural lakes in Brazil, susceptibility rates were observed at 79% for fluconazole, 13% for ketoconazole, 31% for terbinafine, and 78% for amphotericin B in several fungal species such as *Candida tropicalis*, *Candida krusei*, *Meyerozyma guilliermondii*, and *Candida parapsilosis* (Medeiros et al., 2008). Maciel et al. similarly reported resistance or susceptibility-dose dependent in 61% of *Candida*

spp. strains (Maciel et al., 2019). Additionally, a study from Catú Lake, Brazil, identified resistance in *Candida* spp. to both itraconazole and fluconazole (Brilhante et al., 2016).

These studies highlight the role of natural water sources as reservoirs for resistant microorganisms, presenting significant public health risks. This issue is particularly critical given the widespread reliance on water for domestic and agricultural activities and for direct contact uses such as recreation and religious practices (Maciel et al., 2019; Monapathi et al., 2020).

PART 2: SCIENTIFIC ARTICLE

Introduction

Water pollution is a significant global issue, driven by various factors depending on the economic context. In developing countries, insufficient wastewater treatment practices are a primary cause, while in higher-income nations, agricultural wastewater discharges play a major role (UN Water, 2021). Additionally, anthropogenic activities such as industrialization, urbanization, and hospital waste significantly contribute to water contamination on a global scale (Chaudhry & Malik, 2017).

As an essential resource for human survival, water availability is increasingly strained by growing population densities and rising demand. Over the past century, global freshwater consumption has increased sixfold and continues to grow by approximately 1% annually since 1980 (Koncagül et al., 2021). This demand has led to widespread river pollution, with an estimated 80% of untreated industrial and urban wastewater discharged directly into aquatic ecosystems (Lin et al., 2022).

The health consequences of water pollution are alarming, resulting in approximately 14,000 deaths each day due to the consumption of contaminated water (Chaudhry & Malik, 2017). Children are particularly vulnerable, with 5 million deaths annually attributed to waterborne diseases linked to unsafe water consumption (Halder & Islam, 2015). A significant source of contamination is the introduction of pathogenic microorganisms, mainly bacteria, through untreated wastewater discharges (Wolf-Rainer, 2011; Yang et al., 2020).

Polluted rivers are inhabited not only by prokaryotic microorganisms but also by eukaryotic microorganisms, including fungi such as molds and yeasts. These microorganisms play critical roles in biogeochemical processes within aquatic ecosystems; however, their

abundance and diversity are highly susceptible to changes in their environments' chemical composition and purity (Medeiros et al., 2012; Pietryczuk et al., 2018).

Despite their ecological importance and potential clinical relevance, the effects of anthropogenic activities on fungal diversity in natural aquatic systems remain insufficiently studied. Limited research exists on the taxonomic alterations and abundance of molds and yeasts in polluted environments. Nevertheless, previous studies have documented a reduction in fungal diversity in contaminated waters, accompanied by an increase in opportunistic pathogenic yeasts such as *Nakaseomyces glabratus* (formerly known as *Candida glabrata*), *Clavispora lusitaniae* (formerly known as *Candida lusitaniae*), *Meyerozyma guilliermondii*, and *Pichia kudriavzevii* (Medeiros et al., 2012; Ortiz-Vera et al., 2018; Pietryczuk et al., 2018; Steffen et al., 2023). These findings suggest that yeasts may serve as valuable bioindicators of environmental contamination (Ortiz-Vera et al., 2018; Pietryczuk et al., 2018).

Furthermore, evaluating yeasts with clinical significance in rivers remains underexplored. These microorganisms are typically found in environments with temperatures ranging from 20 to 30°C and slightly acidic pH levels. Their persistence is largely attributed to their ability to form biofilms and develop antifungal resistance, which not only enhances their survival in polluted environments but also poses significant public health concerns (Monapathi et al., 2020).

Among the fungi genera most frequently associated with human infections are *Candida*, *Trichosporon*, *Geotrichum*, *Cryptococcus*, and *Rhodotorula*. Of these, *Candida* is the primary cause of invasive fungal infections, underscoring the importance of its accurate identification (Morovati et al., 2023). Chromogenic media, such as CHROMagar *Candida*, enable species identification based on the distinct colony colors produced by yeast-specific enzymatic metabolism of substrates present in the medium (Silva et al., 2012; Tan & Peterson, 2005).

Apart from that, the API 20C AUX and polymerase chain reaction (PCR) have emerged as a rapid and effective tool for detecting potentially pathogenic yeasts (Morovati et al., 2023). Additionally, in recent years, Matrix-Assisted Laser Desorption/Ionization-time-of-flight Mass Spectrometry (MALDI-TOF MS) has gained attention for its ability to identify various potentially pathogenic and opportunistic yeast species rapidly. This technique operates by ionizing proteins, lipids, and peptides, enabling species identification through comparison with a reference database (Pote et al., 2020; Singhal et al., 2015).

In Ecuador, statistical data reveal that access to drinking water is limited, with 67.8% of urban and only 48.5% of rural populations having adequate access (Instituto Nacional de Estadística y Censos, 2019). This shortfall has forced 53.4% of the population to depend on natural water sources such as rivers, streams, creeks, and wells (Agencia de Regulación y Control del Agua, 2022). In Quito, the Machángara and San Pedro rivers, the city's primary watercourses, are heavily contaminated due to wastewater discharges originating from agricultural, urban, and industrial activities. Consequently, these rivers are among the most polluted natural water sources in the capital (Borja-Serrano et al., 2020).

Although previous research has explored microbial indicators of water quality and safety in the Machángara and San Pedro rivers (Borja-Serrano et al., 2020; Vinueza et al., 2021), studies focusing on fungal communities remain notably scarce. Given the potential clinical and environmental relevance of fungi in these ecosystems, a critical question arises if fungi could serve as supplementary indicators of environmental contamination in aquatic environments.

To address this question, the present study aims to quantify molds, yeasts, and *Candida* species and to examine their diversity in water and biofilm samples collected from the Machángara and San Pedro Rivers in Quito, Ecuador.

Materials and Methods

Sample site

Environmental samples were systematically collected from three distinct longitudinal points along the Machángara and San Pedro Rivers, as illustrated in **Figure 1**. Sampling point 0 represented a site with minimal or negligible anthropogenic influence, serving as a baseline for comparison. In contrast, points 1 and 2 were located in areas subjected to significant anthropogenic activity, encompassing urban, industrial, and agricultural impacts. Water and biofilm samples were obtained twice during three defined seasonal periods (rainy season 1, rainy season 2, and dry season) from November 2022 to July 2023, as outlined in **Table 1**. Additionally, *in situ* measurements of key physicochemical parameters, including water temperature and pH, were performed using the ProDSS Multiparameter Digital Water Quality Meter (YSI, Xylem Inc., United States), ensuring accurate and reliable data collection.

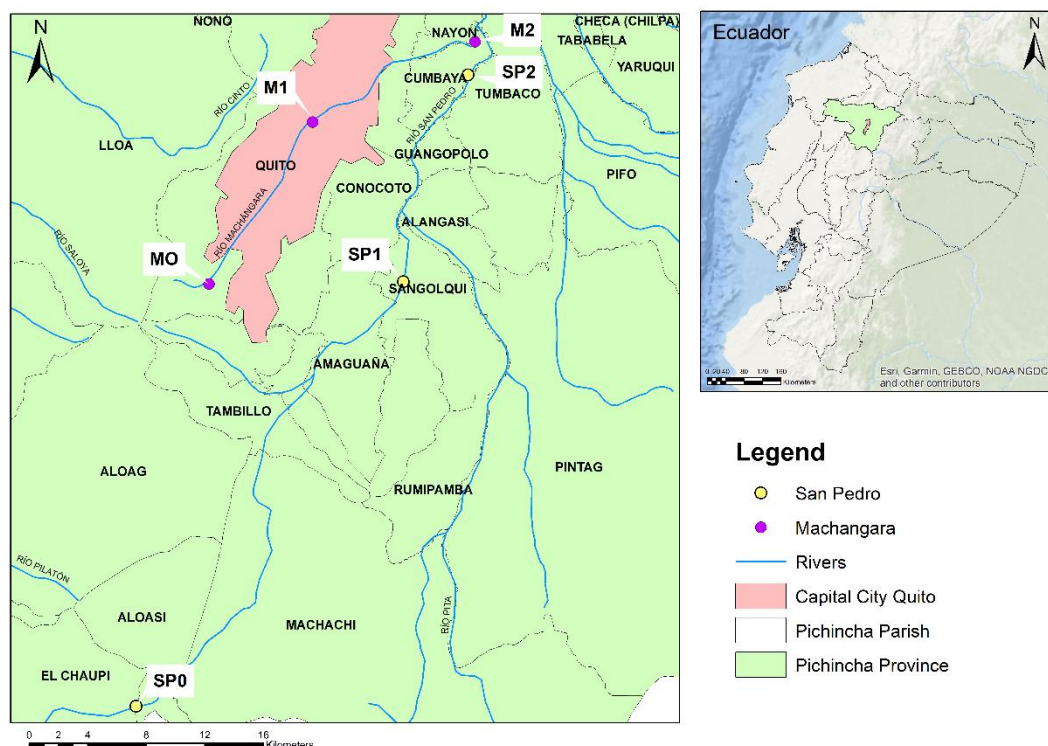


Figure 1. Geographical map illustrating the sampling locations along the Machángara and San Pedro Rivers. Sampling points on the Machángara River are marked with purple dots and include M0-Guamaní (control site), M1-Puengasí, and M2-Nayón. Sampling points on the San Pedro River are denoted by yellow dots and include SP0-Chaupi (control site), SP1-Sangolquí, and SP2-Cumbayá. The map was generated using ArcGIS Desktop software (version 10.8; accessible at <https://doc.arcgis.com/en/archive/>).

Water sample collection and preparation for microbiological analysis

Water samples were collected in duplicate using 800 mL glass jars sterilized by autoclaving at 121°C. The jars were submerged to a depth of 0.3 m in surface water, with lids opened only after full immersion to minimize the risk of contamination. Samples were maintained at 4°C during transport to the Institute of Microbiology at Universidad San Francisco de Quito (IM-USFQ) using a cooler with ice packs.

Table 1. Sample data and corresponding meteorological data by season

Sample code	River	GPS Coordinates	Parish (Province)	Region	Season	Collection date	Average water temperature (°C)	Monthly Precipitation (mm) ^a	Name of INAMHI Stations ^a	GPS Coordinates of INAMHI Stations ^a	Height of INAMHI Stations (m) ^a
M0	Machángara	0°20'8"S 78°34'58"W	Guamaní (Pichincha)	Andean	Rainy Season 1	12/11/2022 26/11/2022	7.65	111.7	M0024 Iñaquito	0°10'41.9"S 78°29'15.7"W	2789
					Rainy Season 2	12/03/2023 18/03/2023	7.95	145.9			
					Dry Season	17/06/2023 01/07/2023	8.65	38.8			
M1	Machángara	0°13'19"S 78°29'14"W	Puengasí (Pichincha)	Andean	Rainy Season 1	12/11/2022 26/11/2022	16.60	111.7	M0024 Iñaquito	0°10'41.9"S 78°29'15.7"W	2789
					Rainy Season 2	12/03/2023 18/03/2023	14.70	145.9			
					Dry Season	17/06/2023 01/07/2023	16.50	38.8			
M2	Machángara	0°11'07.0"S 78°24'54"W	Nayón (Pichincha)	Andean	Rainy Season 1	14/11/2022 28/11/2022	18.60	103.4	M0002 La Tola	0°13'54.5"S 78°22'13.4"W	2480
					Rainy Season 2	10/03/2023 17/03/2023	15.20	120.4			
					Dry Season	16/06/2023 30/06/2023	16.53	29.1			
SP0	San Pedro	0°35'44"S 78°37'26"W	Chaupi (Pichincha)	Andean	Rainy Season 1	11/11/2022 25/11/2022	9.60	149.5	M0003 Izobamba	0°21'57.0"S 78°33'18"W	3058
					Rainy Season 2	11/03/2023 19/03/2023	10.28	180.9			
					Dry Season	15/06/2023 29/06/2023	10.45	67.9			
SP1	San Pedro	0°19'48"S 78°27'35"W	Sangolquí (Pichincha)	Andean	Rainy Season 1	11/11/2022 25/11/2022	14.70	149.5	M0003 Izobamba	0°21'57.0"S 78°33'18"W	3058
					Rainy Season 2	11/03/2023 19/03/2023	13.65	180.9			
					Dry Season	15/06/2023 29/06/2023	14.30	67.9			
SP2	San Pedro	0°12'29"S 78°25'13"W	Cumbayá (Pichincha)	Andean	Rainy Season 1	14/11/2022 28/11/2022	15.95	103.4	M0002 La Tola	0°13'54.5"S 78°22'13.4"W	2480
					Rainy Season 2	10/03/2023 17/03/2023	15.23	120.4			
					Dry Season	16/06/2023 30/06/2023	15.60	29.1			

^a Data provided by the National Institute of Meteorology and Hydrology from Ecuador (INHAMI) in October 2023 (<https://www.inamhi.gob.ec/>)

For microbial analysis, filtration was performed under aseptic conditions using a vacuum pump (Chemical Duty Pump, Millipore, Merck, Burlington, MA, USA) and 0.45 μm nitrocellulose membranes (Millipore, Merck, Burlington, MA, USA). Up to two membranes per sample were used to accommodate the high particulate content at contaminated sites (M1, SP1, M2, and SP2), while a single membrane sufficed for control sites (M0 and SP0). At least 100 mL of water was filtered per sample following established protocols (Borja-Serrano et al., 2020; Vinueza et al., 2021). The membranes were then aseptically transferred to Falcon tubes containing 20 mL of sterile distilled water using sterile tweezers.

To resuspend the particles and microorganisms, the Falcon tubes were vortexed for 10 to 15 minutes at maximum speed, ensuring the integrity of the membranes. After vortexing, the membranes were removed, and the tubes were centrifuged at 7000 rpm for 15 minutes. The supernatant was discarded, and the resulting pellet was resuspended in 2 mL of sterile distilled water to prepare the sample for subsequent analysis.

Biofilm sample collection and preparation for microbiological analysis

Biofilm samples were collected following the protocol described by Rimet and colleagues (Rimet et al., 2020), with minor modifications to suit the study context. Submerged rocks in surface water were selected as sampling sites. Before each collection, a plastic tray was sanitized with 75% ethanol and rinsed with sterile distilled water to prevent contamination. Three rocks, located at depths of 20 to 50 cm below the water surface, were retrieved, briefly drained, and then placed on the prepared tray. A 100 cm^2 area of each rock was rinsed with 50 mL of sterile water and scraped using a sterile plastic spoon. The resulting material was transferred into a sterile 50 mL tube and stored at 4°C during transport to the IM-USFQ for further processing.

Culture and qualitative identification of fungi from water and biofilm samples

The molds and yeasts were quantified through serial dilutions of the samples, which were subsequently cultivated on Sabouraud Dextrose Agar (Becton, Dickinson and Company, Le Pont de Claix, France). For *Candida* spp. quantification, the samples were cultivated on Nickerson Agar (Becton, Dickinson and Company, Le Pont de Claix, France), applying the 3-drop culture technique according to previously optimized protocols (Borja-Serrano et al., 2020; Herigstad et al., 2001; Naghili et al., 2013; Vinueza et al., 2021).

Colony-forming units (CFU) were counted after 24 and 48 hours of incubation at 37°C. Yeast species were initially characterized based on colony morphology on Nickerson Agar, and subsequently, 60 out of 105 water samples and all of the 50 biofilm samples, previously collected and randomly selected, were cultured for up to 48 hours at 37°C on CHROMagar *Candida* (Becton, Dickinson and Company, Le Pont de Claix, France) for qualitative identification. All samples identified through traditional culture from the two rivers across three seasonal periods were selected for molecular identification (refer to **Supplementary Tables 1 and 2**).

DNA extraction from yeast colonies

DNA extraction was conducted following established protocols with minor adaptations (Dashti et al., 2009; Machado & Cerca, 2015; Salinas et al., 2020). Two to five colonies were suspended in 500 µL of autoclaved distilled water in a 1.5 mL sterilized tube. The samples were boiled in a water bath for 25 minutes to facilitate cell lysis, then thermally shocked at -20°C for 25 minutes. The samples were centrifuged at 13,000 rpm for 15 minutes to separate cellular debris, producing a pellet.

Subsequently, 200 μ L of the supernatant from each sample was divided between two clean, autoclaved 1.5 mL tubes. One aliquot was stored at -20°C for future analyses, while the second was used to evaluate DNA quality using a Nanodrop One Spectrophotometer (ThermoFisher, Madison, USA).

Molecular identification of yeast colonies

After extracting DNA from yeast colonies in water and biofilm samples, molecular detection was conducted using conventional multiplex polymerase chain reaction (PCR), following previously established protocols with slight modifications (Guo et al., 2010; Khan & Mustafa, 2001). The primers used for amplification, and the protocol applied for the detection are detailed in **Table 1**. Each PCR reaction, with a final volume of 10 μ L, consisted of 2 μ L of 5X GoTaq Flexi Buffer, 0.70 μ L of 25 mM MgCl_2 , 0.35 μ L of 10 μ M of each primer, 0.35 μ L of 10 mM dNTP Mix, 0.10 μ L of 5U GoTaq Flexi DNA Polymerase (all from Promega, Madison, USA), 1 μ L of template DNA, and DNA-free water to reach the final volume.

The thermocycling procedure was performed on a Bio-Rad thermocycler (Bio-Rad Laboratories, Inc., California, USA) under the following conditions: initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 5 minutes, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes.

Clinical yeast samples obtained from the microbial collection of the IM-USFQ and the National Institute for Research in Public Health (INSPI) were used as positive controls. Additionally, DNA-free water was included as a negative control to validate the results. Each sample was analyzed in duplicate or triplicate to ensure reproducibility. The PCR products were separated via electrophoresis on a 1.5% agarose gel stained with SYBR Safe and visualized after running for 30–40 minutes.

Table 2. Primers and protocol used for the detection of yeast species by conventional multiplex PCR.

Primer name	Primer Sequence (5'-3')	Size (bp)	Primer Blast Targets	Primer Cycling Parameters	Target region	Size (bp)	Reference/ GenBank accession no.	References
CTR1Fw	CAATCC TACCGC CAGAGG TTAT	356	<i>C. tropicalis</i>	3 min at 95°C; 34 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 5 min.	<i>Candida tropicalis</i> 18S ribosomal RNA gene, partial sequence; 5.8S ribosomal RNA gene, partial sequence; and 28S ribosomal RNA gene, partial sequence.	520	AF287910	(Hsu et al., 2003)
CTR2Rv	TGGCCA CTAGCA AAATAA GCGT				<i>Candida tropicalis</i> 18S ribosomal RNA gene, partial sequence; 5.8S ribosomal RNA gene, partial sequence; and 28S ribosomal RNA gene, partial sequence.	529	AF268095	
CGLFw	TTATCAC ACGACT CGACAC T	429	<i>N. glabratus</i>		<i>Nakaseomyces glabratus</i> genes for <i>ITS1</i> , 5.8S rRNA, <i>ITS2</i> , strain: IFO 0622.	793	AB032177	
CGL2Rv	CCCACA TACTGA TATGGC CTACAA				<i>Nakaseomyces glabratus</i> internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	821	AF167993	
CALB1Fw	TTTATCA ACTTGTC ACACCA GA	273	<i>C. albicans</i>		<i>Candida albicans</i> 5.8S ribosomal RNA gene, complete sequence, and 28S ribosomal RNA gene, partial sequence.	535	L47111	
CALB2Rw	ATCCCG CCTTACC ACTACC G				<i>Candida albicans</i> internal transcribed spacer 1 (<i>ITS1</i>); 5.8S ribosomal RNA; internal transcribed spacer 2 (<i>ITS2</i>).	4025	L28817	
CPAFFw	TTTGCTT TGGTAG GCCTTCT A	381	<i>C. parapsilosis</i>		<i>Candida parapsilosis</i> internal transcribed spacer 1 (<i>ITS1</i>)	520	-	(Asadzadeh et al., 2015)
CPARRv	GAGGTC GAATTT GGGAAG AAGT				<i>Candida parapsilosis</i> internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.	433	-	

Complementary identification of yeast colonies

The results of molecular identification were compared with those obtained through qualitative identification on CHROMagar *Candida*. Three water samples were selected for additional biochemical identification using the API 20C AUX system (BioMérieux, France): two samples where discrepancies were observed between the two identification methods and one sample that could not be identified through PCR (see **Supplementary Table 1**). The API 20C AUX kits were provided by the Clinical Microbiology Laboratory (LABOMIC) at Universidad San Francisco de Quito. API strips were prepared following the manufacturer's instructions and incubated at 37°C for 24 to 48 hours. Identification was considered accurate for samples with an identity score exceeding 90%.

In contrast, all biofilm samples subjected to both qualitative and molecular identification underwent complementary analysis using the MALDI-TOF MS method (see **Supplementary Table 2**). This was performed with the MALDI Biotyper mass spectrophotometer (Bruker Daltonics, Bremen, Germany) at the Hospital General IESS Quito Sur. Scores equal to or upper to 2 were considered reliable identification. Due to budgetary constraints, the API 20C AUX and MALDI-TOF MS methods could not be applied to all environmental samples in this study.

Results

Mold, yeast, and *Candida* spp. were quantified for the Machángara and San Pedro Rivers water and biofilm samples. The average and standard deviation (SD) values for water and biofilm samples are shown in **Figure 2** and **Figure 3**, respectively. Each bar in the plot represents the quantification measured in CFU/mL for water samples and CFU/g of biofilm humid weight for biofilm samples, with black bars indicating mold measurements, grey bars indicating yeast measurements, and light grey bars indicating *Candida* spp. measurements.

Detailed information can be found in **Supplementary Table 3** and **Supplementary Table 4**, respectively.

Fungi quantification in water samples

In water samples from the Machángara River, molds, yeasts, and *Candida* spp. were identified exclusively at point M0 during the first rainy season, with these microorganisms absent in subsequent seasons. The lowest mold density was recorded at point M1 during the first rainy season (8.3×10^3 CFU/100 mL), while the highest density was observed at points M1 and M2 (2.3×10^5 CFU/100 mL). Yeasts showed their lowest abundance at point M2 during the first rainy season (7.6×10^5 CFU/100 mL) and their highest abundance at the same point during the dry season (4.2×10^7 CFU/100 mL). Similarly, *Candida* spp. exhibited the lowest density at point M2 during the first rainy season (1.9×10^3 CFU/100 mL) and the highest density at point M1 during the dry season (2.5×10^6 CFU/100 mL) (**Figure 2a; Supplementary Table 3**).

In the San Pedro River, water samples revealed the presence of molds during the dry season, yeasts during the second rainy and dry seasons, and *Candida* spp. during the first rainy and dry seasons at point SP0. The lowest mold density (8.3×10^3 CFU/100 mL) was recorded at point SP1 during the two rainy seasons, whereas the highest density (1.7×10^5 CFU/100 mL) was found at point SP2. Yeasts exhibited their lowest density (6.5×10^4 CFU/100 mL) at point SP1 during the first rainy season and their highest density (8.8×10^6 CFU/100 mL) at point SP2 during the dry season. *Candida* spp. showed the lowest density (2.0×10^4 CFU/100 mL) at point SP1 during the first rainy season and the highest density (2.5×10^5 CFU/100 mL) at point SP2 during the dry season (**Figure 2b; Supplementary Table 3**).

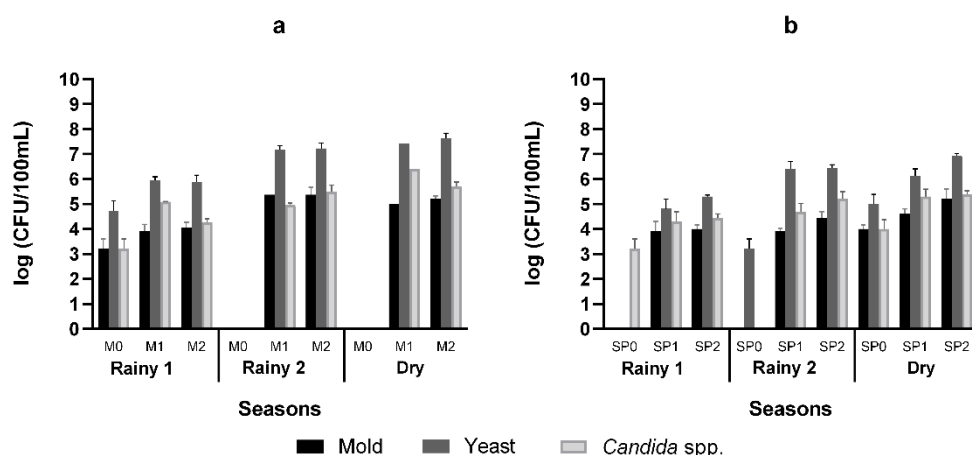


Figure 2. Average and standard deviation values of molds (black bars), yeasts (dark grey bars), and *Candida* spp. (light grey bars) from water samples. In the Machángara River (a) and San Pedro River (b) in three different sampling points during rainy season 1, rainy season 2, and dry season. Sampling collection points in the Machángara River were the following: M0 – Guamaní point; M1 – Puengasí point; and M2 – Nayón point. Sampling collection points in the San Pedro River were the following: SP0 - El Chaupi point; SP1 - San Pedro de Taboada point; and SP2 - Cumbayá point. Data represents CFU/100mL of water sample collected.

Fungi identification in water samples

Table 3 presents the distribution percentages of various *Candida* species in the Machángara and San Pedro rivers. The species *Candida albicans*, *Candida tropicalis*, *Nakaseomyces glabratus*, and *Candida parapsilosis* were identified using traditional methods and conventional multiplex PCR, while *Meyerozyma guilliermondii* was identified through the API 20C AUX test. Among the identified yeast species, *Nakaseomyces glabratus* was the most prevalent in both rivers, comprising 10.48% of the overall samples, with 12.77% in the Machángara River and 8.62% in the San Pedro River. Conversely, *Meyerozyma guilliermondii* was the least abundant, representing 1.90% of the overall samples, with 2.13% in the Machángara River and 1.72% in the San Pedro River. Other *Candida* species were *Candida*

tropicalis (5.71%, total; 8.51%, Machángara River; 3.45%, San Pedro River) and *Candida parapsilosis* (2.86%, total; 2.13%, Machángara River; 3.45%, San Pedro River). Additionally, one of the 105 samples was identified as *Cryptococcus laurentii*. Furthermore, 74.29% of the remaining isolates from samples were categorized as other yeasts (65.95%, Machángara River; 81.03%, San Pedro River) that could not be identified by any methodology applied.

Table 3. Percentage of yeast species found in water samples from Machángara and San Pedro Rivers.

Yeasts	Estimated percentage in Machángara River	Estimated percentage in San Pedro River	Estimated percentage of rivers	Presence of <i>Candida</i> spp. and other yeasts per season
<i>Candida albicans</i>	6.38% (3 of 47)	1.72% (1 of 58)	3.81% (4 of 105)	Rainy 1 and rainy 2 seasons
<i>Candida tropicalis</i>	8.51% (4 of 47)	3.45% (2 of 58)	5.71% (6 of 105)	Rainy 1, rainy 2, and dry seasons
<i>Nakaseomyces glabratus</i>	12.77% (6 of 47)	8.62% (5 of 58)	10.48% (11 of 105)	Rainy 1, rainy 2, and dry seasons
<i>Candida parapsilosis</i>	2.13% (1 of 47)	3.45% (2 of 58)	2.86% (3 of 105)	Rainy 1 and dry seasons
<i>Meyerozyma guilliermondii</i>	2.13% (1 of 47)	1.72% (1 of 58)	1.90% (2 of 105)	Dry season
<i>Cryptococcus laurentii</i>	2.13% (1 of 47)	0.00% (0 of 58)	0.95% (1 of 105)	Dry season
Other yeasts	65.96% (31 of 47)	81.03% (47 of 58)	74.29% (78 of 105)	Rainy 1, rainy 2, and dry seasons

Fungi quantification in biofilm samples

Biofilm samples from the Machángara River indicated an absence of molds, yeasts, and *Candida* spp. at point M0 during all sampling periods. The lowest mold density (1.7×10^1 CFU/g) was detected at point M1 during the first rainy season, whereas the highest density (3.7×10^2 CFU/g) occurred at point M2 during the dry season. Yeasts exhibited the lowest abundance (3.5×10^2 CFU/g) at point M1 during the first rainy season and the highest abundance (1.1×10^4 CFU/g) at points M1 and M2 during the dry and second rainy seasons,

respectively. *Candida* spp. showed the lowest density (1.7×10^1 CFU/g) at point M1 during the first rainy season and the highest density (2.4×10^3 CFU/g) at point M1 during the dry season (Figure 3a; Supplementary Table 4).

Biofilm samples from the San Pedro River indicated the presence of molds and yeasts at point SP0 during the dry and first rainy seasons, respectively, while *Candida* spp. was absent at this location. The lowest mold density (1.7×10^1 CFU/g) was detected at point SP2 during the first rainy season, while the highest density (1.8×10^2 CFU/g) was observed at point SP2 during the second rainy season. Yeasts exhibited their lowest abundance (2.0×10^2 CFU/g) at point SP2 during the first rainy season and their highest abundance (3.3×10^3 CFU/g) at point SP2 during the dry season. *Candida* spp. showed the lowest density (1.7×10^1 CFU/g) at point SP2 during the first rainy season and the highest density (2.5×10^2 CFU/g) at point SP2 during the second rainy season (Figure 3b; Supplementary Table 4).

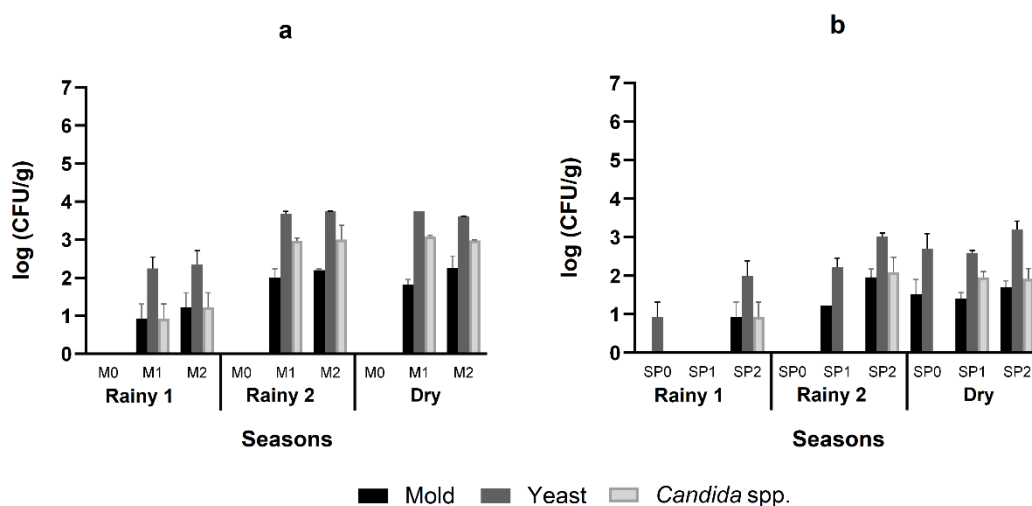


Figure 3. Average and standard deviation values of molds (black bars), yeast (grey bars), and *Candida* spp. (light grey bars) from biofilm samples in the Machángara (a) and San Pedro (b) rivers. All values are presented for the three different longitudinal sampling points during rainy season 1, rainy season 2, and dry seasons. Data represents CFU/g of biofilm humid weight.

Fungi identification in biofilm samples

The identification of *Candida* spp. and other yeast isolates was also assessed in biofilm samples by CHROMagar *Candida*, conventional multiplex PCR, and Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS). According to **Table 4**, *Nakaseomyces glabratus* constituted a significant proportion of the isolates obtained in biofilm samples in both Machángara River (40%) and San Pedro River (53.33%). *Candida albicans* was the second most prevalent yeast species, with proportions of 11.43% in the Machángara River and 13.33% in the San Pedro River. Additionally, *Candida tropicalis* (6.67%, San Pedro River), *Meyerozyma guilliermondii* (6.67%, San Pedro River), *Pichia kudriavzevii* (2.86%, Machángara River), *Nakaseomyces bracarensis* (2.86%, Machángara River), *Kluyveromyces marxianus* (6.67%, San Pedro River), *Wickerhamiella infanticola* (2.86%, Machángara River), *Saccharomyces cerevisiae* (5.71%, Machángara River) and *Lachancea fermentati* (2.86%, Machángara River) were also detected. All yeast species identified, except for *C. albicans*, *C. tropicalis*, *N. glabratus*, and *M. guilliermondii*, were determined by MALDI-TOF MS. However, this study did not detect the remaining yeast isolates (26%, total; 34.29%, Machángara River; 6.67%, San Pedro River).

Table 4. Percentage of yeast species found in Machángara and San Pedro Rivers biofilm samples.

Yeasts	Estimated percentage in Machángara River	Estimated percentage in San Pedro River	Estimated percentage of rivers	Presence of <i>Candida</i> spp. and other yeasts per season
<i>Candida albicans</i>	11.43% (4 of 35)	13.33% (2 of 15)	12.00% (6 of 50)	Rainy season 2
<i>Candida tropicalis</i>	0.00% (0 of 35)	6.67% (1 of 15)	2.00% (1 of 50)	Dry season
<i>Nakaseomyces glabratus</i>	40.00% (14 of 35)	53.33% (8 of 15)	44.00% (22 of 50)	Rainy 1, rainy 2, and dry seasons
<i>Meyerozyma guilliermondii</i>	0.00% (0 of 35)	6.67% (1 of 15)	2.00% (1 of 50)	Rainy season 1

<i>Pichia kudriavzevii</i>	2.86% (1 of 35)	0.00% (0 of 15)	2.00% (1 of 50)	Rainy season 2
<i>Nakaseomyces braccarensis</i>	2.86% (1 of 35)	0.00% (0 of 15)	2.00% (1 of 50)	Rainy season 2
<i>Kluyveromyces marxianus</i>	0.00% (0 of 35)	6.67% (1 of 15)	2.00% (1 of 50)	Dry season
<i>Wickerhamiella infanticola</i>	2.86% (1 of 35)	0.00% (0 of 15)	2.00% (1 of 50)	Dry season
<i>Saccharomyces cerevisiae</i>	5.71% (2 of 35)	0.00% (0 of 15)	4.00% (2 of 50)	Dry season
<i>Lachancea fermentati</i>	2.86% (1 of 35)	0.00% (0 of 15)	2.00% (1 of 50)	Dry season
Other yeasts	34.29% (12 of 35)	6.67% (1 of 15)	26.00% (13 of 50)	Rainy 1, rainy 2, and dry seasons

Discussion

Anthropogenic activities have significantly disrupted natural ecosystems, adversely affecting environmental sustainability and posing serious threats to public health. The monitoring and regulation of such contamination, particularly in water bodies, are, therefore, critical (UN Water, 2021). Although studies on fungal communities in polluted rivers remain limited, focusing on these microorganisms could highlight their utility as complementary indicators of environmental contamination (Cudowski et al., 2022; Medeiros et al., 2012; Pietryczuk et al., 2018; Steffen et al., 2023). Additionally, fungal diversity can provide valuable insights into the occurrence and distribution of opportunistic yeasts in rivers, which may contribute to the prevalence of invasive fungal infections among the population and high costs in Public Health (Baker et al., 2024; Steffen et al., 2023).

Fungi in water and biofilm samples

The findings of this study suggest that water and biofilm samples provide insights into the effects of anthropogenic activities on water sources. However, further studies are required

to substantiate this hypothesis. Importantly, this study represents a novel contribution as the first to quantify fungi in biofilm samples from environmental sources.

Globally, there is a notable lack of standards or regulations addressing fungal counts in natural water bodies receiving wastewater. Traditionally, fungi have not been considered indicators of environmental contamination alongside total coliforms, although interest in their potential as complementary indicators has grown in recent years (Medeiros et al., 2012; Monapathi et al., 2020). Currently, Sweden is the only country with established regulations, setting a permissible limit of 100 CFU/100 mL for molds and yeasts detected via culture-based methods (Babič et al., 2017). In comparison, fungal counts at points M1 and M2 in the Machángara and San Pedro rivers exceeded this threshold, highlighting the need for regulatory frameworks in other regions.

Points M0 and SP0, located in agricultural areas with minimal human activity, demonstrated lower fungal densities. In contrast, points M1 and M2 near urban and industrial zones showed significantly higher fungal counts. This pattern is consistent with findings from studies conducted in Central Europe (Pietryczuk et al., 2018) and South Africa (Steffen et al., 2023), which reported a direct correlation between fungal abundance and contamination levels in water sources. The increased discharge of untreated wastewater in densely populated areas likely exacerbates fungal proliferation, as chemical alterations in the aquatic environment favor their growth and diversity (Ortiz-Vera et al., 2018; Pietryczuk et al., 2018; Steffen et al., 2023).

This study was conducted from late 2022 to mid-2023, an unusual period characterized by increased precipitation, as reported by the National Institute of Meteorology and Hydrology from Ecuador (INHAMI; see **Table 1**). The study area, located in the high-altitude Andes mountain range, also experiences considerable temporal climatic variability (Portilla Farfán, 2018; Zambrano-Barragán et al., 2011). These conditions may have influenced fungal counts,

aligning with the hypothesis that yeast variability is associated with atypical rainfall patterns in certain regions (Medeiros et al., 2012). However, additional research is necessary to confirm this relationship.

Despite limited prior research on fungi in contaminated environmental water sources, earlier studies provide valuable context. Prior studies in rivers in Greece (Arvanitidou et al., 2005), lakes in Brazil (Brandão et al., 2010), and estuaries in Turkey (De Almeida, 2005) reported augmentation of yeast counts in conjunction with elevated fecal coliform levels, indicative of sewage contamination. Similarly, studies on *E. coli* and coliforms in water and biofilm samples from the Machángara and San Pedro rivers revealed higher fecal and total coliform counts at points M1 and M2 (Borja, 2024; Cabrera, 2023). Comparing these findings with the fungal data from this study suggests that elevated yeast densities correlate with high coliform levels, reinforcing their potential role as complementary pollution indicators. To assess this potential risk, a Quantitative Microbial Risk Assessment (QMRA) should be performed, as demonstrated in prior research on the Eersterivier River in South Africa (Steffen et al., 2023).

The trend in fungal counts observed in water samples closely mirrored those in biofilm samples (Figures 1 and 2). To our knowledge, no previous studies have specifically analyzed the abundance of molds and yeasts in biofilms from contaminated rivers. The patterns observed in this study may be attributed to the direct relationship between contamination levels, organic matter availability, and the proportion of opportunistic yeasts, as documented in prior research (Medeiros et al., 2012; Pietryczuk et al., 2018; Steffen et al., 2023). Additionally, the high capacity for biofilm formation reported in opportunistic yeasts, particularly *Candida* spp., could further explain this trend (Atiencia-Carrera, Cabezas-Mera, Vizuete, et al., 2022; D'Enfert & Janbon, 2016; Malinová et al., 2023).

Yeast diversity in water and biofilm samples

The findings of this study align with prior research conducted in contaminated water sources, such as the Mooi and Spruit Rivers in South Africa (Monapathi et al., 2021), groundwater and lakes in Brazil (Cupozak-Pinheiro et al., 2022; Medeiros et al., 2012), mangrove ecosystems in Hong Kong (Hau et al., 2024), and the Songhua River in Northeast Asia (Liu et al., 2015). According to these studies, the reported species, particularly those of the *Candida* genus, consistently dominated water samples, with additional contributions from genera such as *Meyerozyma*, *Pichia*, and *Nakaseomyces*. The detection of *Wickerhamiella infanticola* in this study agrees with the fungal analyses conducted in seawater off the coast of Taiwan (Chang et al., 2016).

The proportions of yeast species identified in various studies show considerable variability, with findings that differ from each other and the results of this study. For instance, Steffen et al. reported *M. guilliermondii* as the most prevalent species (55%), followed by *P. kudriavzevii* (16%), *N. glabratus* (5%), *S. cerevisiae* (3%), *C. tropicalis* (2%), and *C. albicans* (1%) (Steffen et al., 2023). In contrast, a study conducted in Brazil identified *P. kudriavzevii* as the dominant species (37%), with *M. guilliermondii* (14%), *C. tropicalis* (9%), *S. cerevisiae* (4%), *N. glabratus* (3%), and *C. parapsilosis* (2%) making up smaller proportions (Brandão et al., 2010). Similarly, a study from Hong Kong reported *C. parapsilosis* as the most abundant species (33%), with *C. tropicalis* accounting for only 2% of isolates (Hau et al., 2024). In another study, Monapathi et al. found *C. tropicalis* to be overwhelmingly dominant, representing over 90% of the yeast species identified (Monapathi et al., 2021).

Previous investigations suggest that the presence of these yeast species is a common environmental phenomenon. However, their density appears to fluctuate in response to

variations in substrate availability and levels of water contamination (Chang et al., 2016; Monapathi et al., 2021).

Although the environmental presence of these yeasts is typical, it is essential to note their potential implications for public health. In rural areas where natural water sources are frequently utilized for daily activities, opportunistic yeast species, such as those identified, may pose health risks (Hau et al., 2024; Steffen et al., 2023). To assess this potential risk, a QMRA should be performed, as demonstrated in prior research on the Eersterivier River in South Africa (Steffen et al., 2023).

The identification of molds and yeasts remains challenging due to reliance on morphological, molecular, and biochemical methods. Identification was based on the recent taxonomic reclassification of *Candida* (Kidd et al., 2023; Takashima & Sugita, 2022). While CHROMagar *Candida* proved useful for identifying *C. albicans*, *C. tropicalis*, and related species, its precision was limited, with 86% of strains misidentified due to overlapping phenotypes. Similar challenges were reported in studies from Northern Morocco, highlighting the need for complementary molecular techniques like PCR to improve accuracy (Ahaik et al., 2024). Despite budget constraints, this study successfully identified diverse yeast species, aligning with the findings of Yücesoy and colleagues (Yücesoy & Marol, 2003).

MALDI-TOF MS demonstrated superior identification capabilities compared to multiplex PCR and API 20C AUX, which were hindered by cost and limited application. MALDI-TOF MS failed in only 11.82% of strains, while multiplex PCR exhibited higher failure rates (69.09%), consistent with earlier studies from the Netherlands (Aarstehfar et al., 2019). However, molecular identification challenges may stem from suboptimal DNA extraction protocols, as effective PCR requires refined methodologies. These results underscore the necessity of integrating molecular, biochemical, and mass spectrometry techniques to

enhance yeast species identification, particularly in resource-constrained settings. The study reaffirms the critical need for refined laboratory protocols to address existing limitations, as supported by previous studies (Aarstehfar et al., 2019; Ahaik et al., 2024; Daef et al., 2014)

Conclusions and limitations

This study is the first to quantify and analyze fungal diversity in contaminated rivers in Ecuador. The findings indicate that fungal abundance and diversity are significantly influenced by anthropogenic environmental changes. Continuous monitoring of these microorganisms is recommended as supplementary indicators of environmental pollution.

One limitation of the study is the reliance on historical rainfall data to select sampling seasons. While seasonal patterns typically include rainy periods in September and March and dry months in June, the study coincided with an atypical year with unexpected precipitation patterns. Data from INHAMI showed higher rainfall during one rainy season sampling month compared to another, reflecting the challenges of defining distinct seasons in the Andean highlands due to subtle climatic variations.

Another challenge was the precise identification of yeast species. Budget constraints limited the use of advanced techniques, such as API 20C AUX and MALDI-TOF MS, to a subset of water and biofilm samples, emphasizing the need for complementary assessments to understand better their potential public health impact, particularly regarding invasive fungal infections.

PART 3: SHORT COMMUNICATION

Introduction

Fungi, a diverse and historically understudied group, have gained increasing scientific attention in recent years, particularly due to their complex interactions with humans (Caetano et al., 2023). Among these, yeasts are found in many ecological niches, including the human body, constituting the human mycobiome, and residing in specific anatomical locations (Caetano et al., 2023; Segal-Kischinevsky et al., 2022). In humans, yeasts are integral components of certain regions such as the skin, oral cavity, respiratory tract, gastrointestinal tract, and genitourinary tract. Notably, the genera *Candida* and *Cryptococcus* are the most prevalent, with *Candida* being the leading cause of invasive opportunistic infections, thereby representing a significant clinical challenge (Caetano et al., 2023; Nenciarini et al., 2024; Vázquez-González et al., 2013).

The genus *Candida* accounts for the majority of fungal infections in humans (Morovati et al., 2023; Vázquez-González et al., 2013) and has been extensively studied. Recent advancements in taxonomy have resulted in reclassifying several species formerly categorized under *Candida* spp.. For instance, *Candida glabrata*, *Candida krusei*, and *Candida guilliermondii* are now recognized as *Nakaseomyces glabratus*, *Pichia kudriavzevii*, and *Meyerozyma guilliermondii* (Kidd et al., 2023; Takashima & Sugita, 2022), respectively. This study adopts the term "clinically important yeasts" to collectively describe *Candida* species and those that have undergone taxonomic updates.

Among these clinically important yeasts, *Candida albicans*, *Candida tropicalis*, and *N. glabratus* are well-documented as commensal organisms within the human mycobiome. However, under certain conditions, such as immunosuppression or external factors influencing fungal dynamics, these species can activate virulence mechanisms, resulting in invasive fungal

infections (Nenciarini et al., 2024; Tamo, 2020; Turner & Butler, 2014). These infections, broadly termed candidiasis, predominantly involve *Candida* species, including those recently reclassified (Kidd et al., 2023; Rigopoulos, 2023). Candidiasis can affect various anatomical sites, manifesting as oral candidiasis, otic candidiasis, or vaginal candidiasis (Atiencia-Carrera, Cabezas-Mera, Tejera, et al., 2022; Cangui-Panchi et al., 2022, 2023). These infections may disseminate into the bloodstream in severe cases, leading to systemic conditions such as sepsis (Rigopoulos, 2023; Tamo, 2020; Turner & Butler, 2014).

C. albicans, *N. glabratus*, and *C. tropicalis* are among the most frequently identified pathogens in cases of candidiasis (Pote et al., 2020; Turner & Butler, 2014). Together with *Candida parapsilosis* and *Pichia kudriavzevii*, these species account for approximately 92% of global candidiasis cases. *C. albicans* remains the most prevalent, with an incidence of 65.3%, followed by *N. glabratus* at 11.3% and *C. tropicalis* at 7.2% (Turner & Butler, 2014). In recent years, the incidence of non-*Candida albicans* (NAC) species, particularly *N. glabratus*, has increased, a trend attributed to advancements in diagnostic methods and the growing prevalence of antifungal resistance (Atiencia-Carrera, Tejera, & Machado, 2022; Tamo, 2020; Turner & Butler, 2014).

Antifungal agents are broadly categorized into four groups: polyenes (e.g., amphotericin B), pyrimidine analogs (e.g., 5-flucytosine), azoles (e.g., fluconazole, voriconazole, and posaconazole), and echinocandins (e.g., caspofungin, anidulafungin, and micafungin). These agents target distinct cellular pathways: polyenes compromise plasma membrane integrity, pyrimidine analogs inhibit cell division, azoles block a key enzyme in ergosterol biosynthesis, and echinocandins inhibit a membrane enzyme complex (Chen & Sorrell, 2007; Costa-de-Oliveira & Rodrigues, 2020). Despite their effectiveness, resistance to antifungal agents, particularly fluconazole, has escalated. This phenomenon is primarily linked to fluconazole's

widespread use due to its cost-effectiveness and efficacy, as well as the inherent ability of *N. glabratus* to develop resistance (Costa-de-Oliveira & Rodrigues, 2020; Rigopoulos, 2023; Tamo, 2020; Turner & Butler, 2014).

The growing challenge of antifungal resistance, coupled with the difficulty of developing new antifungal agents due to the structural and functional similarities between yeast and human cells as eukaryotes (Costa-de-Oliveira & Rodrigues, 2020; Lara et al., 2015), highlights the necessity for innovative therapeutic approaches. Silver nanoparticles (AgNPs) and silver ions (Ag^+) are promising alternatives. Previous studies have demonstrated their strong antifungal activity against various clinically significant yeast species, particularly *C. albicans*. They can disrupt membrane potential, cause DNA damage, and induce apoptosis in fungal cells (Bahey et al., 2024; Jebali et al., 2014; Lara et al., 2015; Panáček et al., 2009). Moreover, the low concentrations required for effective inhibition render silver nanoparticles a safe and promising option for the treatment of invasive fungal infections (Lara et al., 2015).

The persistent use of antifungal agents and the introduction of new treatments underscore the importance of ongoing surveillance and evaluation of antifungal resistance. The broth dilution method, endorsed by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), is among the most widely recognized techniques for this purpose. This method provides a standardized framework for determining the minimum inhibitory concentration (MIC), the lowest concentration of a drug required to inhibit the growth of a pathogen. Renowned for its reproducibility, accuracy, and ability to generate quantitative data, the broth dilution method enables direct comparison of antifungal efficacy across different agents. Its adaptability to varied clinical and research settings and its standardized protocol reduce inter-laboratory variability. These characteristics enhance its reliability, making it a cornerstone in antifungal

resistance analysis and establishing consistent, globally applicable methodologies (CLSI, 2008; EUCAST, 2023).

Although yeasts are typically opportunistic pathogens, they can be transmitted through direct contact with infected individuals, such as during sexual interactions, or via exposure to contaminated surfaces (Silva et al., 2012). While research on additional transmission pathways remains scarce, some studies suggest that clinically significant yeasts may circulate in contaminated natural resources, such as rivers (Medeiros et al., 2012; Ortiz-Vera et al., 2018; Pietryczuk et al., 2018). Exploring these transmission routes could offer valuable insights into antifungal resistance in potentially pathogenic yeasts, particularly in water resources utilized by human populations, such as irrigation water for crops, even when these resources are polluted.

Several epidemiological studies have reported on the prevalence of *Candida* species and other clinically significant yeasts in Ecuador. For example, data from three hospitals indicate an incidence rate of 0.9 cases of candidiasis per 1,000 hospital admissions, while vaginal candidiasis affects approximately 308,000 women annually (Zurita et al., 2017). A study conducted in a tertiary hospital in Guayaquil identified a significant prevalence of *C. albicans* (38%) and *C. tropicalis* (37%) among hospitalized patients, with *N. glabratus* (14%) more frequently associated with bloodstream and cerebrospinal fluid (CSF) infections (Acosta-Mosquera et al., 2024). Despite these findings, there is still a lack of studies evaluating antifungal resistance in clinically significant yeasts or even environmental yeasts circulating within Ecuador.

This gap in research underscores the need for a deeper understanding of antifungal resistance in opportunistic yeasts, particularly given their notable prevalence in hospital environments. Little is known about the resistance profiles of yeasts present in contaminated

water resources, which could constitute a significant public health concern. There is no information on the efficacy of alternative treatments such as AgNPs. To address this, the current study evaluated whether *Candida* species and other opportunistic yeasts from disturbed ecological niches exhibit greater antifungal resistance than those from hospital environments. A preliminary comparative analysis was conducted on the resistance profiles of *Candida* species and other yeast strains isolated from both clinical sources and environmental samples, specifically from the Machángara and San Pedro Rivers. This study aimed to provide a preliminary valuable insight into the public health implications of antifungal resistance in yeasts from diverse ecological and clinical contexts.

Materials and Methods

Selection of environmental and clinical samples

This study analyzed *Candida albicans*, *Candida tropicalis*, and *Nakaseomyces glabratus* from environmental and clinical samples. Environmental samples were collected from previously described sites in the Machángara and San Pedro Rivers, specifically at sampling points 1 and 2, representing contaminated locations. For each species, one sample was randomly selected from each river, yielding six environmental samples designated as: RM1B and RSP2B (*C. albicans*), RM2T and RSP2V (*C. tropicalis*), and RM2H and RSP2G (*N. glabratus*).

Clinical samples were obtained from the microbiological collection of the Institute of Microbiology at the Universidad San Francisco de Quito (IM-USFQ), the National Institute of Public Health Research (INSPI), and vaginal secretion samples collected during an epidemiological study conducted in Quito, Ecuador, between 2016 and 2017 (Salinas et al.,

2018). The clinical strains analyzed included *C. albicans* ATCC 10231 and INSPI 27, *C. tropicalis* IM-USFQ 2606 and INSPI 24, and *N. glabratus* PSV 197A.

Selection of antifungals and alternative treatments

The selection of antifungal agents in this study was informed by previous findings, which identified fluconazole, amphotericin B, flucytosine, and micafungin as exhibiting the highest inhibitory effects (Cedeño, 2022). These antifungals were, therefore, incorporated into the analysis. The concentration ranges employed were as follows: amphotericin B (0.00098 to 0.015 µg/mL), fluconazole (1 to 32 µg/mL), flucytosine (0.5 to 32 µg/mL), and micafungin (0.03 to 0.5 µg/mL). These ranges were based on the guidelines provided by EUCAST, which establishes protocols for minimum inhibitory concentration (MIC) determination (EUCAST, 2023). The final concentrations were further optimized through preliminary testing conducted during this study.

AgNPs were selected as an alternative therapeutic strategy due to their demonstrated efficacy in a previous study on foodborne bacterial pathogens (Cabascango, 2023). Following the methodology employed in the previous study, their effectiveness was evaluated using Ag⁺ as a control. Building upon the prior research findings and subsequent experimental evaluations, the MIC was examined within a range of 0.25 to 2 mM for AgNPs and 0.13 to 1 mM for Ag⁺.

Antifungal agents and alternative treatments were protected from light exposure and stored following the manufacturer's recommended conditions to ensure stability and efficacy.

Microdilution broth

The broth dilution method was performed following the CLSI M27-A3 guidelines, with modifications based on previous studies (Berkow et al., 2020; Cabezas-Mera et al., 2023; CLSI, 2008; Fernandez-Soto et al., 2023) to determine the MIC values. All strains stored at -20°C were subcultured on Sabouraud Dextrose Agar (Becton, Dickinson and Company, Le Pont de Claix, France) 24 hours before each assay to reduce the risk of mutations. After overnight incubation at 37°C, colonies from each strain were used to prepare a McFarland 0.5 standard in saline. Subsequently, 100 µL of this suspension was added to 10 mL of Sabouraud Dextrose Broth (SBD; Dipco Cía. Ltda., Quito, Ecuador) to obtain a final concentration of 1×10^5 colony-forming units (CFU)/mL.

MIC assays were conducted in duplicate or triplicate using 96-well plates. Positive control wells contained 190 µL of SBD broth mixed with the yeast inoculum and 10 µL of autoclaved distilled water, while negative control wells contained 190 µL of SBD broth with 10 µL of autoclaved distilled water (also known as sterility controls). Test wells were prepared by adding 190 µL of SBD broth, the yeast inoculum, and 10 µL of the treatment at serially increasing concentrations.

After the incubation period, optical density (OD) measurements were performed on the 96-well plates using an ELISA Elx808 Microplate Spectrophotometer (Biotek, Winooski, USA) at wavelengths of 630 nm (Atiencia-Carrera, Cabezas-Mera, Vizuite, et al., 2022; Cedeño, 2022). Subsequently, the MIC₉₀ was established as the minimum treatment concentration required to inhibit 90% of microorganism growth.

Statistical analysis

The OD values obtained were analyzed and compared to the negative control readings, as measured by the spectrophotometer. Based on this analysis, inhibition percentages were calculated. The mean and standard deviation of these values were determined using Microsoft Excel 2024 for exploratory data analysis. Additionally, a nonparametric Kruskal-Wallis's test was carried out for inhibition data using GraphPad Prism version 8.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

Results and Discussion

The results and discussion of this study focus on the MIC₉₀ values determined to evaluate the antifungal susceptibility of the analyzed samples. While inhibition percentages and their standard deviations were documented as part of this preliminary investigation, they were excluded from the analysis. This decision aligns with the guidelines set forth by the CLSI and the EUCAST, which advocate for MIC values as the standardized metric for assessing the antifungal susceptibility of opportunistic yeasts (CLSI, 2020a; EUCAST, 2023).

Antifungal resistance of environmental and clinical samples

The MIC₉₀ values for fluconazole varied among *Candida* species, with distinct differences observed between environmental and clinical strains of *C. albicans* (**Figure 4a**). Environmental strains RM1B and RSP2B exhibited MIC₉₀ values of 16 µg/mL and 8 µg/mL, respectively, whereas clinical strains ATCC 10231 and INSPI 27 displayed MIC₉₀ values of 4 µg/mL and 2 µg/mL, respectively. For *C. tropicalis*, the environmental strains RM2T and RSP2V showed MIC₉₀ values of 8 µg/mL and 16 µg/mL, respectively, while clinical strains IM-USFQ2606 and INSPI 24 had MIC₉₀ values of 4 µg/mL and 16 µg/mL, respectively.

All analyzed strains of *N. glabratus*, including environmental strains RM2H and RSP2G and clinical strain PSV 197A, exhibited a uniform MIC₉₀ of 32 µg/mL. According to CLSI guidelines, fluconazole susceptibility thresholds are defined as ≤ 2 µg/mL for sensitive strains, 4 µg/mL for susceptible-dose dependent (SDD), and ≥ 8 µg/mL for resistant strains (CLSI, 2020a). All environmental strains of *C. albicans*, *C. tropicalis*, and *N. glabratus* were categorized as resistant based on these criteria. Among the clinical strains, resistance was observed in *C. tropicalis* INSPI 24 and *N. glabratus* PSV 197A, while *C. albicans* ATCC 10231 and *C. tropicalis* IM-USFQ2606 were classified as SDD. Clinical *C. albicans* INSPI 27 was determined to be sensitive to fluconazole.

The MIC₉₀ values reported for clinical strains in this study are consistent with findings from hospital-based studies conducted in China (Lei et al., 2018), Malaysia (Amran et al., 2011), Iran (Mirshekar et al., 2021), Taiwan (Tseng et al., 2020), and Costa Rica (Mora-Lee et al., 2023). These investigations also noted a high prevalence of resistant strains, with resistance typically associated with MIC₉₀ values equal to or upper to 8 µg/mL, particularly in *C. tropicalis* and *N. glabratus*. Conversely, the results from environmental samples align with those from a study on mangrove water ecosystems in Hong Kong, where multiple species of *Candida* and other opportunistic yeasts demonstrated low sensitivity to azoles. This suggests that environmental strains may exhibit higher resistance to antifungals, particularly fluconazole, likely due to urban pollutant discharges contaminating natural water sources (Hau et al., 2024).

As expected, the environmental samples of *C. albicans* and *C. tropicalis* in this study displayed greater resistance than their clinical counterparts. In contrast, both environmental and clinical samples of *N. glabratus* were uniformly resistant to fluconazole, evidencing that environmental isolates showed the same antifungal resistance. This can be attributed to the intrinsic fluconazole resistance characteristic of *N. glabratus* (Hassan et al., 2021).

Flucytosine susceptibility assays revealed notable variability in MIC₉₀ values among *C. albicans* strains, with clear differences between environmental and clinical samples (**Figure 4b**). Environmental strains RM1B and clinical strain ATCC 10231 exhibited MIC₉₀ values of 4 µg/mL, while strain RSP2B demonstrated an MIC₉₀ of 8 µg/mL. The highest MIC₉₀ value, 32 µg/mL, was observed in clinical strain INSPI 27. For *C. tropicalis*, the environmental strain RM2T showed an MIC₉₀ of 4 µg/mL, whereas the remaining strains (RSP2V, IM-USFQ2606, and INSPI 24) exhibited MIC₉₀ values of 2 µg/mL. In the case of *N. glabratus*, all analyzed strains, including environmental strains RM2H and RSP2G and the clinical strain PSV 197A, presented an MIC₉₀ of 4 µg/mL.

The determination of precise flucytosine sensitivity was complicated by the recent removal of susceptibility thresholds for this antifungal in the CLSI M60 guidelines (CLSI, 2020a). MIC₉₀ values were compared with the ranges recommended in the CLSI M61 guidelines to address this. The MIC₉₀ values reported in this study exceeded the recommended range for *Candida parapsilosis* ATCC 22019 (0.06–0.25 µg/mL) but generally aligned with the range established for *Candida krusei* ATCC 6258 (4–16 µg/mL) (CLSI, 2020b). The exception was *C. albicans* INSPI 27, which exhibited an MIC₉₀ of 32 µg/mL.

In this study, the MIC₉₀ values for *C. albicans*, *C. tropicalis*, and *N. glabratus* were consistently higher than those reported in previous studies. For example, a flucytosine efficacy study conducted in Iowa, USA, identified MIC₉₀ values of 1 µg/mL and 0.12 µg/mL, respectively (Pfaller et al., 2002). Similarly, a hospital-based study in Atlanta, USA, documented MIC₉₀ values ranging from 0.13 to 1 µg/mL for *Candida* spp. (Lockhart et al., 2011). While a Brazilian study reported MIC₉₀ values of 0.78 µg/mL, 0.039 µg/mL, and 0.024 µg/mL for *C. albicans*, *C. tropicalis*, and *N. glabratus*, respectively (Pinto et al., 2008). For environmental samples, the MIC₉₀ values recorded in this study also exceeded the range (0.06–

0.125 µg/mL) reported in the Hong Kong environmental opportunistic yeasts study (Hau et al., 2024).

The discrepancy between the MIC₉₀ values observed in this study and those reported in prior studies could indicate a broader trend of decreasing flucytosine sensitivity in *Candida* spp., as suggested in recent reviews (Sigera & Denning, 2023). These findings highlight the low susceptibility to flucytosine in environmental and clinical samples of *C. albicans*, *C. tropicalis*, and *N. glabratus*. This tendency underscores the critical need for ongoing surveillance and assessment of antifungal resistance in both clinical and environmental settings to understand better and address emerging resistance patterns.

The MIC₉₀ values for amphotericin B in the analyzed strains are presented in **Figure 4c**. Among *C. albicans* strains, the environmental isolates RM1B and RSP2B, along with the clinical strain ATCC 10231, exhibited an MIC₉₀ of 0.006 µg/mL, while the clinical strain INSPI 27 demonstrated a slightly higher MIC₉₀ of 0.008 µg/mL. In *C. tropicalis*, all environmental and clinical strains showed consistent MIC₉₀ values of 0.006 µg/mL. For *N. glabratus*, the environmental strains RM2H and RSP2G displayed MIC₉₀ values of 0.006 µg/mL, whereas the clinical strain PSV 197A exhibited a slightly lower MIC₉₀ of 0.004 µg/mL.

When assessed against the EUCAST susceptibility threshold for amphotericin B (S, MIC ≤ 1 µg/mL) (EUCAST, 2023), none of the strains—whether environmental or clinical—showed resistance. The MIC₉₀ values identified in this study were significantly lower than those reported in studies conducted in Italy (Lovero et al., 2017), southern Iran (Badiee & Alborzi, 2011), and Turkey (Eksi et al., 2013), where MIC₉₀ values for *C. albicans* ranged from 0.125 to 0.5 µg/mL, *C. tropicalis* from 0.25 to 0.75 µg/mL, and *N. glabratus* from 0.5 to 0.75 µg/mL. However, our findings align with a multicenter study across four Latin American countries

(Argentina, Brazil, Peru, and Venezuela) (Godoy et al., 2003), where MIC₉₀ values for *Candida* species ranged between 0.03 and 0.5 µg/mL. These discrepancies likely reflect regional differences in antifungal resistance patterns and temporal shifts in susceptibility. Consistent with prior research, resistance to amphotericin B remains rare (Badiee & Alborzi, 2011; Eksi et al., 2013).

However, it is important to mention that the formulation of amphotericin B is another factor potentially contributing to variability in MIC values. For example, the study by Lovero et al. used a liposomal formulation, which they hypothesized could influence MIC results, but further research is required to establish an association (Lovero et al., 2017).

For environmental isolates, only *C. albicans* and *C. tropicalis* can be compared with findings from Hau and colleagues' study, which reported MIC₉₀ values for *Candida* spp. in the range of 0.25–0.50 µg/mL, higher than those observed in the present study (Hau et al., 2024). Based on these results, there is no indication of resistance to amphotericin B in environmentally opportunistic yeasts in this preliminary evaluation, paralleling the susceptibility observed in clinical strains. These findings underscore the continued efficacy of amphotericin B in both environmental and clinical contexts, emphasizing the importance of ongoing surveillance to detect emerging resistance.

The micafungin results (**Figure 4d**) showed short variability in MIC values among environmental and clinical strains across the three analyzed opportunistic yeast species. Environmental *C. albicans* RM1B and RSP2B, along with the clinical INSPI 27, exhibited an MIC₉₀ of 0.25 µg/mL, while ATCC 10231 presented a slightly lower MIC₉₀ of 0.20 µg/mL. Environmental *C. tropicalis* RM2T and clinical strains IM-USFQ 2606 and INSPI 24 showed equal MIC₉₀ values of 0.195 µg/mL, whereas the environmental strain RSP2V exhibited a

MIC₉₀ of 0.125 µg/mL. For *N. glabratus*, the environmental strains RM2H and RSP2G and the clinical strain PSV 197A displayed a MIC₉₀ of 0.125 µg/mL.

When assessed against the established sensitivity thresholds for micafungin (CLSI, 2020a), the environmental and clinical strains of *C. albicans* and *C. tropicalis* were classified as sensitive. In contrast, strains of *N. glabratus* showed varied responses: RM2H (environmental) and PSV 197A (clinical) exhibited intermediate sensitivity, while RSP2G (environmental) was resistant. The MIC₉₀ values observed in this study are higher than the MIC₉₀ of 0.015 µg/mL reported in a resistance analysis of pediatric samples from Japan (Ikeda et al., 2009) and exceed the MIC₉₀ of 1 µg/mL identified in a six-year study of clinical isolates collected from 90 medical centers worldwide (Pfaller et al., 2008). However, the MIC₉₀ values for environmental strains align with the range of 0.016 to 2 µg/mL reported by Hau and colleagues (Hau et al., 2024).

These findings emphasize the substantial efficacy of micafungin, along with other echinocandins, against a range of opportunistic yeast species (Pfaller et al., 2008). Nevertheless, the reduced sensitivity observed in *N. glabratus* may be associated with azole resistance and amino acid substitutions in hotspot regions of Fks subunits of glucan synthase (Perlin, 2015). In this study, the high sensitivity of both environmental and clinical *C. albicans* and *C. tropicalis* to micafungin contrasted with the low sensitivity observed across all *N. glabratus* strains.

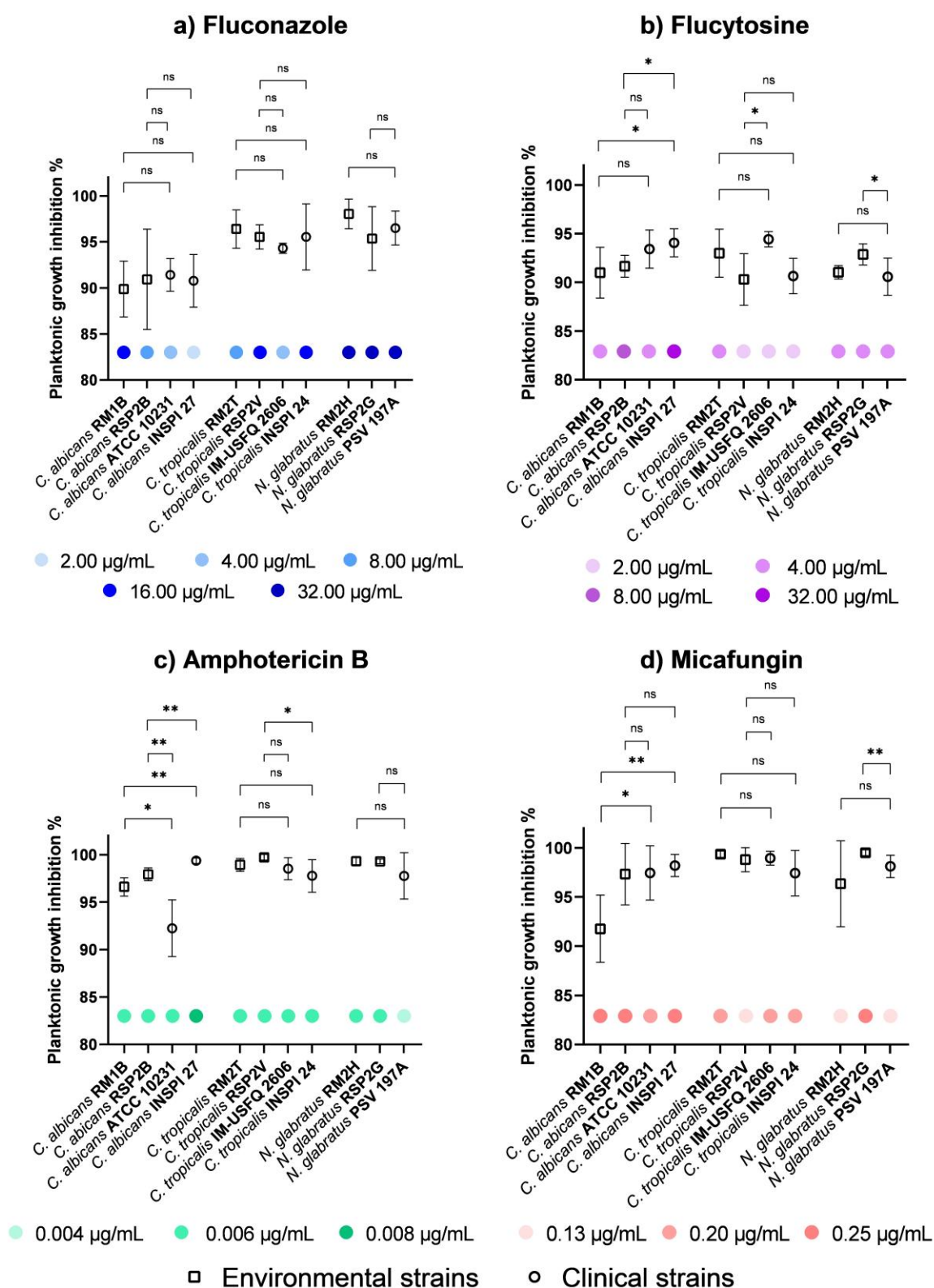


Figure 4. Percentage inhibition and MIC values of yeasts from environmental and clinical samples evaluated with fluconazole (a), flucytosine (b), amphotericin B (c), and micafungin (d). The dot plot illustrates the mean percentage inhibition and standard deviation

for each yeast species, divided into environmental strains (left) and clinical strains (right). An exploratory statistical analysis comparing environmental and clinical strains is provided above each graph, conducted using the nonparametric Kruskal-Wallis's test in GraphPad Prism 8.0. Dots are color-coded by antifungal agent: blue for fluconazole, purple for flucytosine, green for amphotericin B, and terracotta for micafungin with varying shades indicating specific MIC concentrations, detailed below the plot.

AgNPs as an alternative treatment for opportunistic yeasts

Figure 5 illustrates the MIC₉₀ values for various opportunistic yeast species treated with AgNPs (a) and Ag⁺ (b). For *Candida albicans*, the environmental strains RM1B and RSP2B exhibited an MIC₉₀ of 0.5 mM with AgNPs, whereas the clinical strains ATCC 10231 and INSPI 27 demonstrated a higher MIC₉₀ of 1 mM. In contrast, the MIC₉₀ for Ag⁺ was 0.5 mM for RM1B, ATCC 10231, and INSPI 27, while RSP2B showed an MIC₉₀ of 1 mM.

For *Candida tropicalis*, AgNPs yielded MIC₉₀ values of 0.25 mM for the environmental strain RM2T and the clinical strain INSPI 24, 1 mM for the environmental strain RSP2V, and 0.5 mM for the clinical strain IM-USFQ 2606. Ag⁺ varied slightly, with RM2T, RSP2V, IM-USFQ 2606, and INSPI 24 exhibiting MIC₉₀ values of 0.25, 1, 0.5, and 0.13 mM, respectively.

For *N. glabratus*, the environmental strains RM2H and RSP2G, as well as the clinical strain PSV 197A, consistently exhibited an MIC₉₀ of 0.5 mM with AgNPs. Across all tested strains, MIC₉₀ values for both AgNPs and Ag⁺ ranged between 0.25 and 1 mM, except for *C. tropicalis* INSPI 24, which displayed an MIC₉₀ of 0.13 mM for Ag⁺.

These results align with previous studies. A study conducted in the Czech Republic reported similar MIC₉₀ values for AgNPs and Ag⁺ (Panáček et al., 2009). Additionally, the MIC values obtained in this study were generally lower than the 1 mM MIC reported in an Egyptian study against *C. albicans*, *C. tropicalis*, and *N. glabratus* strains (Bahey et al., 2024). Notably,

a study in Mexico reported an MIC₉₀ of 0.25 mM for *C. tropicalis* treated with AgNPs (Guerra et al., 2020), consistent with the values observed for the environmental strain RM2T and the clinical strain INSPI 24 in this study.

Overall, these findings corroborate existing literature, demonstrating the efficacy of AgNPs in inhibiting *Candida* species in vitro (Bahey et al., 2024; Panáček et al., 2009). These results further support the potential of AgNPs as an effective antifungal agent for diverse yeast strains and species, avoiding antifungal resistance among them. Previous studies have reported promising outcomes when combining silver nanoparticles (AgNPs) with antifungal agents, demonstrating the potential to reverse antifungal resistance (Darwish et al., 2021; Santos Souza et al., 2020; Sun et al., 2016). Additionally, the synergistic effects of AgNPs in combination with plant-derived secondary metabolites have been explored, resulting in reduced minimum inhibitory concentration (MIC) values (Jadhav et al., 2020). Nonetheless, further research is necessary to assess the cytotoxicity and safety of these combinations through *in vivo* models (Jia & Sun, 2021).

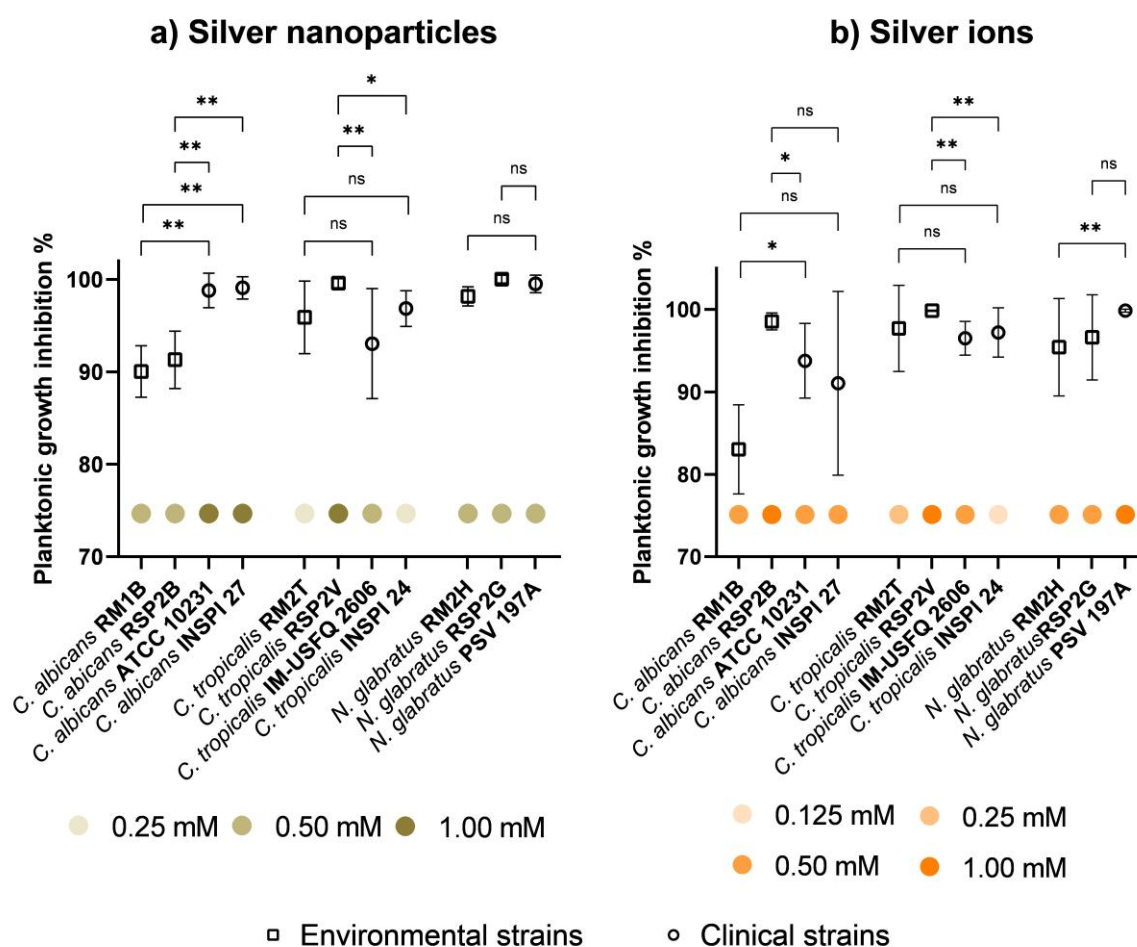


Figure 5. Percentage inhibition and MIC values of yeasts from environmental and clinical samples evaluated with silver nanoparticles (AgNPs) (a) and silver ions (Ag^+) (b). Each graph includes a statistical comparison of environmental and clinical strains performed using the nonparametric Kruskal-Wallis's test in GraphPad Prism 8.0. Data points represented as olive green dots for Ag-NPs and orange dots for Ag-I, with varying shades, indicate different MIC concentrations, as specified below the dot plot.

Conclusions and limitations

This exploratory study provides preliminary insights into Ecuador's potential landscape of antifungal resistance. Environmental samples of the three species exhibited susceptibility to amphotericin B, flucytosine, and micafungin comparable to that observed in clinical isolates. However, higher resistance to fluconazole was noted in environmental samples. Among the

species analyzed, *N. glabratus* was the only one demonstrating reduced susceptibility to micafungin.

This study demonstrated the effectiveness of silver nanoparticles (AgNPs) in inhibiting various opportunistic yeast species in their planktonic form. Notably, previous research suggests that combining AgNPs with antifungal agents can reverse resistance while adding plant-derived molecules can further reduce their minimum inhibitory concentration (MIC). However, these findings warrant further investigation, particularly through *in vitro* studies, to confirm and better understand their potential synergistic effects.

The primary limitation of this study was the small sample size of both environmental and clinical strains, which impeded robust statistical analysis to identify significant differences between the two groups or the treatments applied. As this research was a preliminary exploratory analysis, antifungal resistance was assessed solely in the planktonic state rather than the biofilm state. This limitation constrained the accuracy of the findings, as the yeast species analyzed are known to be strong biofilm formers. Comprehensive resistance evaluation in both planktonic and biofilm states is essential for a complete understanding of antifungal efficacy.

Future perspectives

Future research should quantify the biofilm formation of these yeast strains, followed by assessments of biofilm inhibition and eradication. Evaluating biofilm formation in the studied strains will allow for additional information, such as the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC). These assessments will facilitate a comparative statistical analysis of the MIC, MBIC, and MBEC for the antifungal treatments used in this study.

These additional methodologies, such as optical density measurements, colony-forming unit enumeration, and fluorescence microscopy analysis, should be added. Expanding the sample size would enable robust statistical comparisons of antifungal resistance between environmental and clinical strains. This approach could provide deeper insights into antifungal resistance in clinically significant yeasts, especially those linked to human and anthropogenically impacted environments. Additionally, alternative treatments should be considered, such as plant-based extracts and antimicrobial compounds from microalgae.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Characterization of randomly selected water samples from the Machángara and San Pedro Rivers across three periods using CHROMagar *Candida* (Qualitative identification), conventional multiplex PCR (Molecular identification), and API 20C AUX (Biochemical identification).

Season	Sample Origin	No.	Sample	BIGGY Morphology	CHROMagar <i>Candida</i> Morphology	Qualitative Identification	Molecular Identification	Identification by API 20C AUX
Rainy season 1	Machángara River samples	1	RM2O	Medium-sized colonies are smooth and shiny, with a brown color and a thin white halo.	Dark blue colonies.	<i>Candida tropicalis</i>	Not identified	Not applied for this strain
		2	RM2I	Small, smooth, and opaque colonies with a black color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		3	RM2H	Small and rough colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		4	RM2G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		5	RM1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Purple colonies.	<i>Candida tropicalis/ Nakaseomyces glabratus</i>	<i>Candida tropicalis</i>	Not applied for this strain
		6	RM1O	Medium-sized colonies are smooth and shiny, with a brown color and a thin white halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		7	RM1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	<i>Candida tropicalis</i>	Not applied for this strain
		8	RM1B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	<i>Candida albicans</i>	Not applied for this strain
		9	RM1H	Small and rough colonies with a white color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		10	RM1O	Medium-sized colonies are smooth and shiny, with a brown color and a thin white halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		11	RM0B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	<i>Candida albicans</i>	Not applied for this strain
		12	RM0F	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
	San Pedro River samples	13	RSP2A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		14	RSP2K	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Candida parapsilosis</i>	Not applied for this strain
		15	RSP1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Light blue colonies.	<i>Candida albicans</i>	Not identified	Not applied for this strain
		16	RSP2M	Large, rough, and opaque colonies with a black color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain

Rainy season 2	Machángara River samples	17	RM2K	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		18	RM2D	Small, smooth, and opaque colonies with a medium dark brown color, a thin white halo, and a protruding center.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		19	RM2F	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		20	RM2E	Small, smooth, and opaque colonies with a dark brown color and a darker protruding center.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Candida albicans</i>	Not applied for this strain
		21	RM2H	Small and rough colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		22	RM2I	Small, smooth, and opaque colonies with a black color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Candida tropicalis</i>	Not applied for this strain
		23	RM2A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		24	RM1G	Medium-sized, smooth, and shiny colonies with a white color.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		25	RM2J	Medium-sized, smooth, and opaque colonies with a black color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		26	RM2B1	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	<i>Candida albicans</i>	Not applied for this strain
		27	RM2B2	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
	San Pedro River samples	28	RSP2I	Small, smooth, and opaque colonies with a black color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		29	RSP1J	Medium-sized, smooth, and opaque colonies with a black color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		30	RSP2G	Medium-sized, smooth, and shiny colonies with a white color.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		31	RSP2P	Medium-sized, smooth, and opaque colonies with a dark brown color and a protruding center.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		32	RSP2O	Medium-sized colonies are smooth and shiny, with a brown color and a thin white halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		33	RSP2F	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		34	RSP2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	<i>Candida albicans</i>	Not applied for this strain
		35	RSP2D	Small, smooth, and opaque colonies with a medium dark brown color, a thin white halo, and a protruding center.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		36	RSP2M	Large, rough, and opaque colonies with a black color.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	<i>Candida tropicalis</i>	Not applied for this strain

Dry season	Machángara River samples	37	RM1B	Large, smooth, and shiny colonies with a dark brown or black color.	Green colonies	<i>Candida albicans</i>	<i>Candida tropicalis</i>	<i>Cryptococcus laurentii</i>
		38	RM1C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	<i>Meyerozyma guilliermondii</i>
		39	RM1G	Medium-sized, smooth, and shiny colonies with a white color.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		40	RM1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		41	RM2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	<i>Candida parapsilosis</i>	Not applied for this strain
		42	RM2N	Large, smooth, and shiny colonies with an orange color and a protruding light brown center.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		43	RM1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		44	RM1G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		45	RM2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	Not identified	Not applied for this strain
		46	RM2T	Large colonies, dark brown or black, with a protruding center and a thin halo of lighter brown, rough texture.	Colonias grandes, color azul turquesa	<i>Candida tropicalis/Candida albicans</i>	<i>Candida tropicalis</i>	Not applied for this strain
		47	RM2E	Small, smooth, and opaque colonies with a dark brown color and a darker protruding center.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		48	RM2M	Large, rough, and opaque colonies with a black color.	Blue colonies.	<i>Candida tropicalis/Candida albicans</i>	<i>Candida tropicalis</i>	Not applied for this strain
	San Pedro River samples	49	RSP1C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		50	RSP1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of turquoise green color.	<i>Candida albicans</i>	Not identified	Not applied for this strain
		51	RSP1B	Large, smooth, and shiny colonies with a dark brown or black color.	Light green colonies	<i>Candida albicans</i>	<i>Candida tropicalis</i>	<i>Meyerozyma guilliermondii</i>
		52	RSP2O	Medium-sized colonies are smooth and shiny, with a brown color and a thin white halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not applied for this strain
		53	RSP2L	Small, smooth, non-shiny colonies, dark brown in color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Candida parapsilosis</i>	Not applied for this strain
		54	RSP2C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain
		55	RSP2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	Not identified	Not applied for this strain
		56	RSP2C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	<i>Nakaseomyces glabratus</i>	Not applied for this strain

		57	RSP2Y	Colonies characterized by a large, dark brown to black appearance with a thin white halo encircling their outer margin. Within the colony, two distinct halos are observed: an outer white halo and an inner brown halo. The central structure of the colony is elevated, displaying a white protrusion with a black core.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain
		58	RSP2V	Large colonies, dark brown or black, with a rocky appearance.	Blue colonies.	<i>Candida tropicalis/Candida albicans</i>	<i>Candida tropicalis</i>	Not applied for this strain
		59	RSP2W	Large colonies, light brown, opaque, with a cottony appearance.	Blue colonies.	<i>Candida tropicalis/Candida albicans</i>	Not identified	Not applied for this strain
		60	RSP2X	Medium-sized colonies, dark brown, rough with irregular edges, and a prominent circle in the center.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	Not applied for this strain

Supplementary Table 2. Characterization of randomly selected biofilm samples from the Machángara and San Pedro Rivers across three periods using CHROMagar *Candida* (Qualitative identification), conventional multiplex PCR (Molecular identification), and MALDI-TOF MS (Mass-Spectrometry identification).

Season	Sample Origin	No.	Sample	BIGGY Morphology	CHROMagar <i>Candida</i> Morphology	Qualitative Identification	Molecular Identification	Mass-Spectrometry Identification		
								Species	Score value (best-match)	Score value (second best-match)
Rainy season 1	Machángara River biofilm samples	01	BM1Q	Large, smooth, and opaque colony with a brown color. Internally, it is rough, with a khaki-brown color and a protruding center.	Colonies of beige or white color.	<i>Candida parapsilosis</i> / <i>Nakaseomyces glabratus</i>	Not identified	Not identified	1.32	1.28
		02	BM2G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus</i> / <i>Candida parapsilosis</i>	<i>Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	2.1	2.07
	San Pedro River biofilm samples	03	BSP2O	Medium-sized colonies are smooth and shiny, with a brown color and a thin white halo.	Purple colonies.	<i>Candida tropicalis</i> / <i>Nakaseomyces glabratus</i>	Not identified	<i>Meyerozyma guilliermondii</i>	2.3	2.24
		04	BSP2H	Small and rough colonies with a white color.	Purple colonies.	<i>Candida tropicalis</i> / <i>Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.16	2.14
Rainy season 2	Machángara River biofilm samples	05	BM1R	Medium sized, smooth, low gloss, orange colonies with a thin white halo and a whitish center	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus</i> / <i>Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.28	2.27
		06	BM1Q	Large, smooth, and opaque colony with a brown color. Internally, it is rough, with a khaki-brown color and a protruding center.	Pink colonies with a cotton-like white halo	<i>Pichia kudriavzevii</i>	Not identified	<i>Pichia kudriavzevii</i>	2.05	2
		07	BM1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of beige or white color.	<i>Candida parapsilosis</i> / <i>Nakaseomyces glabratus</i>	Not identified	Not identified	1.32	1.28
		08	BM1B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	<i>Candida albicans</i>	<i>Candida albicans</i>	2.3	2.21
		09	BM1C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of beige or white color.	<i>Candida parapsilosis</i> / <i>Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces braccarensis</i>	2	2
		10	BM1G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus</i> / <i>Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.12	2.02
		11	BM2L	Small, smooth, non-shiny colonies, dark brown in color.	Purple colonies.	<i>Candida tropicalis</i> / <i>Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.16	2.12
		12	BM2L	Small, smooth, non-shiny colonies, dark brown in color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus</i> / <i>Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.23	2.15
		13	BM2C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of beige or white color.	<i>Candida parapsilosis</i> / <i>Nakaseomyces glabratus</i>	Not identified	Not identified	1.34	1.33

Dry season	San Pedro River biofilm samples	14	BM2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.21	2.18
		15	BM1O	Medium-sized colonies are smooth and shiny, with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Candida albicans</i>	2.13	2.13
		16	BM1K	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.29	2.23
		17	BSP2A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of turquoise green color.	<i>Candida albicans</i>	<i>Candida albicans</i>	<i>Candida albicans</i>	2.11	2.11
		18	BSP2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	<i>Candida albicans</i>	<i>Candida albicans</i>	2.37	2.31
		19	BSP2F	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not identified	1.34	1.33
		20	BSP2C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.14	2.14
	Machángara River biofilm samples	21	BM1K	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not identified	1.44	1.35
		22	BM1G	Medium-sized, smooth, and shiny colonies with a white color.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	<i>Lachancea fermentati</i>	1.75	1.62
		23	BM1C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not identified	1.32	1.3
		24	BM1D	Small, smooth, and opaque colonies with a medium dark brown color, a thin white halo, and a protruding center.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not identified	1.43	1.43
		25	BM2L	Small, smooth, non-shiny colonies, dark brown in color.	<i>Colonies of beige or white color.</i>	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	Not identified	1.43	1.43
		26	BM2G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	Not identified	1.36	1.33
		27	BM2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.37	2.36
		28	BM2F	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.28	2.28
		29	BM2K	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Saccharomyces cerevisiae</i>	2.19	2.14
		30	BM2A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Purple colonies.	<i>Candida tropicalis/Nakaseomyces glabratus</i>	Not identified	<i>Wickerhamiella infanticola</i>	1.89	1.32

	31	BM2C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of turquoise green color.	<i>Candida albicans</i>	Not identified	<i>Candida albicans</i>	2.28	2.28
	32	BM2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of turquoise green color.	<i>Candida albicans</i>	Not identified	<i>Candida albicans</i>	2.29	2.24
	33	BM1A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Not identified</i>	1.29	1.28
	34	BM1H	Small and rough colonies with a white color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.25	2.16
	35	BM1C	Medium-sized, shiny colonies with a white color and a light brown center, resembling a fried egg.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.3	2.3
	36	BM1G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.32	2.26
	37	BM1F	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.27	2.25
	38	BM2K	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Not identified</i>	1.29	1.28
	39	BM2F	Small, smooth, and shiny colonies with a brown color and a thin white halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Saccharomyces cerevisiae</i>	2.16	2.09
	40	BM2U	Large opaque brown colonies with non-uniform halo with irregular edges both internally and externally. An opaque brown spot is present in the halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Not identified</i>	1.35	1.32
	41	BM2A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Not identified</i>	1.3	1.28
San Pedro River biofilm samples	42	BSP1H	Small and rough colonies with a white color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	<i>Nakaseomyces glabratus</i>	2.21	2.2
	43	BSP1B	Large, smooth, and shiny colonies with a dark brown or black color.	Light green colonies.	<i>Candida albicans</i>	<i>Candida tropicalis</i>	<i>Candida tropicalis</i>	2.22	2.2
	44	BSP1D	Small, smooth, and opaque colonies with a medium dark brown color, a thin white halo, and a protruding center.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.25	2.26

		45	BSP2B	Large, smooth, and shiny colonies with a dark brown or black color.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.32	2.31
		46	BSP2A	Large, smooth, and shiny colonies with a brown color and a thin whitish halo.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.21	2.12
		47	BSP2N	Large, smooth, and shiny colonies with an orange color and a protruding light brown center.	Colonies of beige or white color.	<i>Candida parapsilosis/Nakaseomyces glabratus</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.42	2.38
		48	BSP2G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.36	2.32
		49	BSP1N	Large, smooth, and shiny colonies with an orange color and a protruding light brown center.	Ambar colonies with a pink center	Not identified	Not identified	<i>Kluyveromyces marxianus</i>	2.24	2.19
		50	BSP2G	Medium-sized, smooth, and shiny colonies with a white color.	Colonies of pale lilac or pink color.	<i>Nakaseomyces glabratus/ Candida parapsilosis</i>	Not identified	<i>Nakaseomyces glabratus</i>	2.46	2.42

Supplementary Table 3. Molds, yeasts and *Candida* sp. quantification on traditional culture media in both Machángara and San Pedro Rivers from water samples.

Rivers	Sampling points/Seasons	Medium Sabouraud Dextrose Agar						Medium Nickerson Agar		
		Molds (CFU/100mL $\pm$ SD)			Yeasts (CFU/100mL $\pm$ SD)			<i>Candida</i> spp. (CFU/100mL $\pm$ SD)		
		Rainy season 1	Rainy season 2	Dry Season	Rainy season 1	Rainy season 2	Dry Season	Rainy season 1	Rainy season 2	Dry Season
Machángara	M0	1.7×10^3 (2.4×10^3)	0 (0)	0 (0)	5.5×10^4 (7.8×10^4)	0 (0)	0 (0)	1.7×10^3 (2.4×10^3)	0 (0)	0 (0)
	M1	8.3×10^3 (7.1×10^3)	2.3×10^5 (0)	1.0×10^5 (0)	9.2×10^5 (3.4×10^5)	1.5×10^7 (6.5×10^6)	2.7×10^7 (0)	1.8×10^4 (7.1×10^3)	2.4×10^3 (1.9×10^2)	2.5×10^6 (0)
	M2	1.2×10^4 (7.1×10^3)	2.3×10^5 (2.4×10^5)	1.7×10^5 (4.7×10^4)	7.6×10^5 (6.7×10^5)	1.7×10^7 (1.1×10^7)	4.2×10^7 (2.7×10^7)	1.9×10^3 (7.1×10^1)	1.2×10^5 (4.7×10^3)	5.1×10^5 (2.5×10^5)
San Pedro	SP0	0 (0)	0 (0)	1.0×10^4 (4.7×10^3)	0 (0)	1.2×10^3 (2.4×10^3)	1.1×10^5 (1.4×10^5)	1.7×10^3 (2.4×10^3)	0 (0)	1.0×10^4 (1.4×10^4)
	SP1	8.3×10^3 (1.2×10^4)	8.3×10^3 (2.4×10^3)	4.0×10^4 (2.4×10^4)	6.5×10^4 (9.2×10^4)	2.5×10^6 (2.6×10^6)	1.3×10^6 (1.2×10^6)	2.0×10^4 (2.8×10^4)	5.0×10^4 (5.7×10^4)	2.0×10^5 (1.9×10^5)
	SP2	1.0×10^4 (4.7×10^3)	2.8×10^4 (2.1×10^4)	1.7×10^5 (2.4×10^5)	2.0×10^5 (2.8×10^4)	2.7×10^6 (1.0×10^6)	8.8×10^6 (1.5×10^6)	2.7×10^4 (1.4×10^4)	1.6×10^5 (1.5×10^5)	2.5×10^5 (9.4×10^4)

Supplementary Table 4. Molds, yeasts and *Candida* sp. quantification on traditional culture media in both Machángara and San Pedro Rivers from biofilm samples.

Rivers	Sampling points/Seasons	Medium Sabouraud Dextrose Agar						Medium Nickerson Agar		
		Molds (CFU/g $\pm$ SD)			Yeasts (CFU/g $\pm$ SD)			<i>Candida</i> spp. (CFU/g $\pm$ SD)		
		Rainy season 1	Rainy season 2	Dry Season	Rainy season 1	Rainy season 2	Dry Season	Rainy season 1	Rainy season 2	Dry Season
Machángara	M0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	M1	1.7×10^1 (2.4×10^1)	2.0×10^2 (1.41×10^2)	1.3×10^2 (4.7×10^1)	3.5×10^2 (3.5×10^2)	9.7×10^3 (1.4×10^3)	1.1×10^4 (4.7×10^1)	1.7×10^1 (2.4×10^1)	1.9×10^3 (3.5×10^2)	2.4×10^3 (1.9×10^2)
	M2	3.3×10^1 (4.7×10^1)	3.2×10^2 (2.4×10^1)	3.7×10^2 (3.8×10^2)	4.5×10^2 (5.9×10^2)	1.1×10^4 (1.4×10^2)	8.2×10^3 (4.7×10^1)	3.3×10^1 (4.7×10^1)	2.0×10^3 (2.8×10^3)	1.9×10^3 (7.1×10^1)
San Pedro	SP0	0 (0)	0 (0)	6.7×10^1 (9.4×10^1)	1.7×10^1 (2.4×10^1)	0 (0)	1.0×10^3 (1.4×10^3)	0 (0)	0 (0)	0 (0)
	SP1	0 (0)	3.3×10^1 (0)	5.0×10^1 (2.4×10^1)	0 (0)	3.3×10^2 (2.4×10^2)	7.7×10^2 (1.4×10^2)	0 (0)	0 (0)	1.8×10^2 (7.1×10^1)
	SP2	1.7×10^1 (2.4×10^1)	1.8×10^2 (1.2×10^2)	1.0×10^2 (4.7×10^1)	2.0×10^2 (2.8×10^3)	2.1×10^3 (5.2×10^2)	3.3×10^3 (1.2×10^3)	1.7×10^1 (2.4×10^1)	2.5×10^2 (3.5×10^2)	1.7×10^2 (1.4×10^2)

Supplementary Table 5. Minimum inhibitory concentration (MIC) and percentage inhibition of clinically significant yeast species from environmental and clinical strains.

	Antifungals (µg/mL)				Alternative treatment (mM)	
	Amphotericin B	Flucytosine	Fluconazole	Micafungin	Silver nanoparticles	Silver ions
<i>Candida albicans</i> RM1B						
MIC ₉₀	0.006	4.00	16.00	0.25	0.50	0.50
Inhibition, SD (%)	96.61 ±0.97	90.99 ±2.61	89.87±3.03	91.77 ±3.42	90.05 ±2.79	83.02 ±5.39
<i>Candida albicans</i> RSP2B						
MIC ₉₀	0.006	8.00	8.00	0.25	0.50	1.00
Inhibition, SD (%)	97.92 ±0.68	91.65 ±1.12	90.92 ±5.44	97.33 ±3.13	91.31 ±3.11	98.53 ±1.02
<i>Candida albicans</i> ATCC 10231						
MIC ₉₀	0.006	4.00	4.00	0.20	1.00	0.50
Inhibition, SD (%)	92.24 ±2.97	93.41 ±1.97	91.41 ±1.77	97.44 ±2.76	98.82 ±1.87	93.76 ±4.53
<i>Candida albicans</i> INSPI 27						
MIC ₉₀	0.008	32.00	2.00	0.25	1.00	0.50
Inhibition, SD (%)	99.10±0.22	94.05 ±1.45	90.77 ±2.87	98.20 ±1.12	99.10 ±1.21	95.17 ±4.15
<i>Candida tropicalis</i> RM2T						
MIC ₉₀	0.006	4.00	8.00	0.20	0.25	0.25
Inhibition, SD (%)	98.92 ±0.67	92.98 ±2.46	96.39 ±2.08	99.36 ±0.33	95.90 ±3.93	97.68 ±5.22
<i>Candida tropicalis</i> RSP2V						
MIC ₉₀	0.006	2.00	16.00	0.13	1.00	1.00
Inhibition, SD (%)	99.72 ±0.30	90.29 ±2.65	95.54 ±1.32	98.79 ±1.22	99.60 ±0.5	99.84 ±0.08
<i>Candida tropicalis</i> IM-USFQ 2606						
MIC ₉₀	0.006	2.00	4.00	0.20	0.50	0.50
Inhibition, SD (%)	98.52 ±1.16	94.42 ±0.80	94.30 ±0.55	98.95 ±0.71	93.07 ±5.95	96.51 ±2.04
<i>Candida tropicalis</i> INSPI 24						
MIC ₉₀	0.006	2.00	16.00	0.20	0.25	0.13
Inhibition, SD (%)	97.77 ±1.72	90.64 ±1.81	95.54 ±3.59	97.43 ±2.31	96.86 ±1.94	97.21 ±3.00
<i>Nakaseomyces glabratus</i> RM2H						
MIC ₉₀	0.006	4.00	32.00	0.13	0.50	0.50
Inhibition, SD (%)	99.32 ±0.36	91.03 ±0.71	97.08 ±2.96	96.34 ±4.37	98.17 ±1.04	95.41 ±5.91
<i>Nakaseomyces glabratus</i> RSP2G						
MIC ₉₀	0.006	4.00	32.00	0.25	0.50	0.50
Inhibition, SD (%)	99.30 ±0.24	92.86 ±1.08	98.59 ±0.47	96.21 ±3.23	100.00 ±0.43	96.61 ±5.15
<i>Nakaseomyces glabratus</i> PSV 197A						
MIC ₉₀	0.004	4.00	32.00	0.13	0.50	1.00
Inhibition, SD (%)	97.76 ±2.45	90.58 ±1.91	98.70 ±0.84	98.11 ±1.13	99.52 ±0.95	99.96 ±0.30