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Original article

## Antifungal-resistant yeasts detected in recreational coastal waters of Rio de Janeiro, Brazil

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### ABSTRACT

**Background:** Recreational coastal waters are widely used for leisure activities and require systematic microbiological monitoring to ensure the safety of bathers. However, such surveillance has traditionally focused on fecal indicator bacteria, whereas fungal communities remain largely neglected by regulatory and governmental agencies.

**Aims:** This study aimed to identify 18 yeasts isolated from three tourist beaches in Rio de Janeiro, Brazil, and to evaluate their antifungal susceptibility, biofilm-forming capacity and growth in saline conditions.

**Methods:** The molecular identification of the isolates was performed by sequencing the ITS1–5.8S–ITS2 rDNA region. Antifungal susceptibility was determined using the broth microdilution method. Biofilm formation was assessed in 96-well microtiter plates using crystal violet, safranin and XTT. Growth under different salt concentrations was also evaluated.

**Results:** Fungal isolates were identified as *Candida parapsilosis* species complex ( $n=5$ ), *Candida tropicalis* ( $n=4$ ), *Nakaseomyces glabratus* (formerly *Candida glabrata*;  $n=2$ ), *Candidozyma haemuli* species complex (formerly *Candida haemulonii* species complex;  $n=2$ ), *Wickerhamiella infanticola* ( $n=2$ ), and one isolate each of *Candida palmioleophila*, *Candida ecuadorensis* and *Pichia manshurica*. Resistance to azoles was observed in *C. tropicalis*, *N. glabratus*, *C. haemuli* and *C. palmioleophila*, with variable susceptibility to other antifungals. *C. parapsilosis* and *C. ecuadorensis* isolates were susceptible to all tested antifungals. *W. infanticola* isolates exhibited low MIC values against the antifungals tested, while *P. manshurica* exhibited elevated MICs to fluconazole and flucytosine. All yeasts formed biofilms on plastic and tolerated NaCl concentrations of up to 7.5%.

**Conclusions:** These findings demonstrate the presence of clinically relevant opportunistic yeasts in recreational seawater. The isolates exhibited antifungal resistance, salt tolerance, and the ability to form biofilms, all of which pose a potential public health concern.

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## Levaduras resistentes a los antifúngicos recuperadas de aguas costeras en zonas recreativas de Río de Janeiro, Brasil

### R E S U M E N

**Palabras clave:**  
Agua de mar  
Playas recreativas  
Especies de *Candida*  
Sensibilidad a los antifúngicos  
Biopelícula

**Antecedentes:** Las aguas costeras recreativas se utilizan ampliamente para actividades de ocio y requieren un control microbiológico sistemático para garantizar la seguridad de los bañistas. Sin embargo, esta vigilancia se ha centrado tradicionalmente en bacterias indicadoras de contaminación fecal, mientras que el control de la presencia de hongos ha sido, en gran medida, desatendido por las agencias regulatorias y gubernamentales.

**Objetivos:** Este estudio tuvo como objetivo identificar 18 levaduras aisladas de tres playas turísticas de Río de Janeiro, Brasil, y evaluar su sensibilidad a los antifúngicos, su capacidad de formación de biopelícula y su crecimiento en condiciones de salinidad.

**Métodos:** La identificación molecular de los hongos aislados se realizó mediante la secuenciación de la región ITS1–5.8S–ITS2 del rDNA. La sensibilidad antifúngica se determinó mediante el método de microdilución en caldo. La formación de biopelículas se evaluó en placas de microtitulación de 96 pozos y el uso de cristal violeta, safranina y XTT. También se investigó el crecimiento bajo diferentes concentraciones de sal.

**Resultados:** Los aislamientos fúngicos se identificaron como *Candida parapsilosis* complejo de especies ( $n = 5$ ), *Candida tropicalis* ( $n = 4$ ), *Nakaseomyces glabratus* (anteriormente *Candida glabrata*;  $n = 2$ ), *Candidozyma haemuli* complejo de especies (anteriormente *Candida haemulonii* complejo de especies;  $n = 2$ ), *Wickerhamiella infanticola* ( $n = 2$ ), y un aislamiento de cada una de las especies *Candida palmioleophila*, *Candida ecuadorensis* y *Pichia manshurica*. Se observó resistencia a los azoles en *C. tropicalis*, *N. glabratus*, *C. haemuli* y *C. palmioleophila*, con sensibilidad variable a otros antifúngicos. Los aislamientos de *C. parapsilosis* y *C. ecuadorensis* fueron sensibles a todos los antifúngicos evaluados. Los aislamientos de *W. infanticola* mostraron valores bajos de concentración mínima inhibitoria frente a los antifúngicos probados, mientras que *P. manshurica* presentó valores de concentración mínima inhibitoria elevados para el fluconazol y la flucitosina. Todas las levaduras formaron biopelículas sobre plástico y toleraron concentraciones de NaCl de hasta 7,5%.

**Conclusiones:** Estos hallazgos demuestran la presencia de levaduras oportunistas clínicamente relevantes en aguas marinas recreativas. Los aislamientos fueron resistentes a algunos antifúngicos, tuvieron tolerancia a ciertas concentraciones de sal y formaron biopelículas, cualidades que pueden suponer un potencial riesgo para la salud pública.

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Recreational coastal waters play a crucial role in promoting leisure, tourism and well-being across the world. In this context, the city of Rio de Janeiro, Brazil, stands out for its extensive coastline, featuring numerous iconic beaches that attract not only local residents, but also millions of national and international tourists throughout the year.<sup>19</sup> However, the intense and continuous use of these environments, combined with their close proximity to densely populated urban areas, subjects them to considerable anthropogenic pressure. Such impacts can compromise both the ecological balance and the microbiological quality of the sand and seawater.<sup>15</sup> Traditionally, recreational water quality has been monitored based on the detection of classical fecal indicator bacteria, such as *Escherichia coli* and Enterococci, whose presence is associated with an increased risk of acquiring waterborne diseases through contact with contaminated water.<sup>21</sup> Although this approach has proven effective for identifying bacterial contamination, it fails to account for other potentially harmful microorganisms, including opportunistic fungi, which are not currently considered in routine environmental assessments. As a result, a significant gap remains in the comprehensive evaluation of microbiological risks in these settings.<sup>21</sup>

The presence of fungi in recreational coastal waters has long been largely overlooked on a global scale. However, in recent years, growing attention has been directed toward the occurrence of fungi in these environments, particularly due to their potential impact on public health.<sup>2,18,31</sup> The emergence and spread of multidrug-resistant opportunistic fungal pathogens have further underscored the threat posed by fungal infections, especially in the context of medical advances that have extended the survival of individuals undergoing immunosuppressive therapies, organ

transplants or chemotherapy.<sup>7</sup> These individuals represent a growing at-risk population, highly susceptible to opportunistic fungal infections. Studies have reported the presence of opportunistic fungal pathogens in both sand and seawater samples taken from beaches in Brazil and other regions around the world.<sup>2,15,18,26,36</sup> Among these, several current and former *Candida* species have been frequently identified. They are considered fecal contaminants, due to their presence in the gastrointestinal microbiota of humans and certain animals. This fecal origin, coupled with their environmental persistence, raises concerns regarding their potential for transmission and infection in exposed individuals. Clinically relevant species such as *Candida tropicalis*, *Candida parapsilosis*, *Pichia kudriavzevii* (formerly *Candida krusei*) and *Nakaseomyces glabratus* (formerly *Candida glabrata*) have been detected in the sand and seawater of Brazilian beaches, highlighting the importance of including fungi in routine environmental monitoring programs.<sup>15,26,36</sup> Worryingly, these studies have shown that *C. tropicalis* displays resistance to at least one antifungal agent and possesses the capacity to exhibit key virulence factors, including adhesion and biofilm formation, which are crucial for the initiation of an infectious process.<sup>15,26,36</sup>

In response to growing concerns about fungal contamination in aquatic recreational environments, the Mycosands initiative was recently established by the joint working group of the International Society for Human and Animal Mycology (ISHAM) and the European Confederation for Medical Mycology (ECMM). The initiative aims to generate standardized data on the presence and diversity of fungi in beach sands, coastal waters and inland freshwater bathing sites across various European countries.<sup>2</sup> Findings from this large-scale study revealed that the most frequently isolated fungal genera in both sand and water samples included *Aspergillus*, *Candida*, *Fusar-*

ium, and *Cryptococcus*, all of which encompass species known to cause opportunistic infections in humans. The widespread detection of these fungi in recreational settings underscores the need for a broader perspective in microbial water quality assessment. Notably, the results from the Mycosands initiative contributed to the consideration of fungi as a microbial taxon of interest in the Guidelines on Recreational Water Quality, published by the World Health Organization.<sup>31</sup> This development marks an important step toward the inclusion of fungal indicators in future public health policies and monitoring protocols for recreational waters.

Considering all the aspects discussed above, the aim of the present study was to investigate the occurrence of yeast species in seawater from three recreational beaches in the city of Rio de Janeiro, Brazil. In addition, we evaluated their susceptibility to antifungal agents, their ability to grow under varying salt concentrations and their capacity to form biofilm on plastic support, which is an important virulence attribute closely associated with resistance to environmental stressors.

## Material and methods

### Microorganisms and growth conditions

In the present study, a total of 18 yeast isolates were obtained from seawater samples collected at three urban and touristic beaches in Rio de Janeiro city, each characterized by distinct levels of pollution. The sampling was conducted on June 20, 2017. The collection sites and their respective geographic coordinates (latitude, longitude) were: Botafogo (–22.944434 and –43.180056), Flamengo (–22.929164 and –43.170845) and Ipanema (–22.987266 and –43.205129). At each site, 2 L of seawater were collected 15 cm below the surface in a water column with a total depth of 100 cm. Samples were stored in sterile amber flasks, kept on ice, and processed within 8 h after collection. A volume of 500 mL from each sample was filtered using Millipore membranes (0.22- $\mu$ m pore size), which were then placed on 90-mm culture plates containing Difco™ CHROMagar™ *Candida* (Becton, Dickinson and Company, Le Pont de Claix, France) supplemented with imipenem (1  $\mu$ g/mL) to inhibit bacterial growth. The plates were incubated aerobically at 37 °C for 48 h. All colony-forming units (CFUs) were subsequently subcultured on Sabouraud dextrose agar (SDA; Sigma–Aldrich, St. Louis, MO, USA) under identical incubation conditions and further processed for molecular identification (as detailed below). For all subsequent experiments, yeast cells were cultured in Sabouraud dextrose broth (SDB; Sigma–Aldrich) at 37 °C for 48 h, and cell counts were estimated using a Neubauer chamber.

### Molecular identification

After growth in SDA, fungal DNA obtained from pure colonies was extracted with the Gentra® Puregene® Yeast and G+ Bacteria Kit (Qiagen, Germantown, MD, USA). The identification of yeasts was done by sequencing the ITS1–5.8S–ITS2 gene region of the rDNA,<sup>4</sup> using the primers ITS1 (50–TCCGTAGGTGAACCTGCGG–30) and ITS4 (50–TCTCCGCTTATTGATATGC–30). PCR amplification was carried out in a C1000 Touch thermocycler (Bio–Rad, USA) following the program: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced in both directions. Sequencing was conducted at the DNA Sequencing Platform (ABI-3730; Applied Biosystems) (PDTIS/FIOCRUZ – Rio de Janeiro, Brazil). Sequences were edited using SeqMan software, version 7.0 (DNASTAR Inc., Madison, WI,

USA) and compared by BLAST with sequences available from the NCBI/GenBank database.

### Antifungal susceptibility assay

The antifungal susceptibility assay was performed according to the broth microdilution method described in the document M27Ed4 published by the Clinical & Laboratory Standards Institute (CLSI).<sup>7</sup> The antifungal agents fluconazole (in concentrations ranging 0.125–64 mg/L), itraconazole (0.0313–16 mg/L), voriconazole (0.0313–16 mg/L), amphotericin B (0.0313–16 mg/L), caspofungin (0.015–8 mg/L), flucytosine (0.125–64 mg/L) and terbinafine (0.0313–16 mg/L) (Sigma–Aldrich) were used.<sup>7</sup> Minimal inhibitory concentration (MIC) values of yeasts with species-specific breakpoints were categorized in accordance with the document CLSI M60Ed2.<sup>8</sup> For *Candida* species and antifungals not covered by this protocol, MIC classification was based on the CLSI M27S3 protocol.<sup>6</sup> The CLSI protocol has not determined breakpoints for terbinafine and amphotericin B. However, in general, clinical isolates of *Candida* presenting MIC values greater than 1 mg/L are considered resistant to amphotericin B. The reference strains *P. kudriavzevii* (formerly *C. krusei*) ATCC 6258 and *C. parapsilosis* ATCC 22019 were used for quality control, as recommended by CLSI guidelines. For yeast species other than *Candida* for which no CLSI clinical breakpoints are available, MIC values were reported without categorical interpretation.

### Biofilm formation assay

Fungal cell suspensions (200  $\mu$ L of SDB containing  $1 \times 10^6$  yeasts) were added to the wells of a flat-bottom 96-well polystyrene microtiter plate and incubated without agitation at 37 °C for 48 h. Control wells with only medium were also included in parallel. Afterwards, supernatant fluids were gently aspirated, and wells were washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove non-adhered cells. Subsequently, biofilm parameters, including biomass, metabolic activity, and extracellular matrix content were quantified. Briefly, for biomass quantification, biofilms were fixed with methanol, stained with crystal violet solution (0.4%; Sigma–Aldrich), washed once with PBS to remove excess stain, decolorized with acetic acid (33%) and the absorbance was read at 590 nm (SpectraMax M3; Molecular Devices, Sunnyvale, CA, USA).<sup>22,24</sup> The biofilm metabolic activity was assessed by the metabolic reduction of 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma–Aldrich) to a brown formazan product soluble in water.<sup>22,24</sup> In this assay, a solution of XTT (2 mg in 10 mL of preheated PBS) and menadione (100  $\mu$ L of 0.4 mM menadione solution in acetone) was added to the biofilms and incubated at 37 °C for 3 h in the dark. The colorimetric changes were read at 492 nm using a microplate reader (SpectraMax M3; Molecular Devices, San Jose, CA, USA).<sup>22,24</sup> To quantify the extracellular matrix (ECM) produced by biofilm-forming cells, unfixed biofilms were stained with safranin (0.1%; Sigma–Aldrich), the wells were washed with PBS once to remove excess stain, the ECM was decolorized with acetic acid (30%) and the absorbance was read at 530 nm (SpectraMax M3; Molecular Devices).<sup>5,24</sup>

### Osmotic stress resistance assay

Fungal suspensions (200  $\mu$ L of SDB containing  $1 \times 10^4$  yeasts) were added to 96-well polystyrene microtiter plates containing NaCl at concentrations ranging from 0.46 to 30%; plates were incubated at 37 °C for 48 h. The control of fungal growth was determined

**Table 1**

Collection sites, CHROMagar™ *Candida* colony characteristics and ITS region-based molecular identification of fungal isolates recovered from recreational beaches in Rio de Janeiro city, Brazil.

Fungal isolate identification codes	Sequencing of ITS gene			
	Local beaches	Colony color on CHROMagar™ <i>Candida</i>	Fungal identification	GenBank accession number
FL 01	Flamengo	Lilac	<i>Candida parapsilosis</i>	PV638871
FL 02	Flamengo	Blue	<i>Candida tropicalis</i>	PV638872
FL 03	Flamengo	Blue	<i>Candida tropicalis</i>	PV638873
FL 06	Flamengo	Cream/Lilac	<i>Candida parapsilosis</i>	PV638875
BT 01	Botafogo	Cream	<i>Candida palmioleophila</i>	PV638876
BT 02	Botafogo	Cream	<i>Candidozyma haemuli</i> var. <i>vulneris</i>	PV638877
BT 03	Botafogo	Lilac	<i>Candida metapsilosis</i>	PV638878
BT 04	Botafogo	Lilac	<i>Candida parapsilosis</i>	PV638879
BT 06	Botafogo	Cream	<i>Candidozyma haemuli sensu stricto</i>	PV638880
BT 07	Botafogo	Purple	<i>Wickerhamiella infanticola</i>	PV638881
BT 08	Botafogo	Cream	<i>Nakaseomyces glabratus</i>	PV638882
BT 09	Botafogo	Cream	<i>Nakaseomyces glabratus</i>	PV638883
BT 10	Botafogo	Lilac	<i>Candida orthopsilosis</i>	PV638884
IP 01	Ipanema	Cream	<i>Candida ecuadorensis</i>	PV638885
IP 03	Ipanema	Blue	<i>Candida tropicalis</i>	PV638886
IP 05	Ipanema	Blue	<i>Candida tropicalis</i>	PV638887
IP 06	Ipanema	Lilac	<i>Pichia manshurica</i>	PV638888
IP 07	Ipanema	Purple	<i>Wickerhamiella infanticola</i>	PV638889

in the same conditions without the addition of NaCl. Fungal growth was determined at 530 nm (SpectraMax M3; Molecular Devices).<sup>26</sup>

### Statistics

The experiments were conducted in triplicate across three independent experimental sets. Statistical analysis was performed using One-Way Analysis of Variance (ANOVA) for comparisons involving three or more groups. Correlations were assessed using the Pearson correlation coefficient ( $r$ ). All analyses were carried out using GraphPad Prism 9 software.  $P$  values  $\leq 0.05$  were considered statistically significant in all tests.

## Results

### Yeasts identification

The yeast isolates recovered from recreational waters in Rio de Janeiro city were identified using PCR followed by sequencing of the ITS1–5.8S–ITS2 region, which is considered the gold standard for fungal species identification. In this sense, among the 18 yeasts recovered from seawater samples, clinically relevant opportunistic *Candida* species were identified, such as *C. tropicalis* ( $n = 4$ , 22.23%), *C. parapsilosis* species complex (*C. parapsilosis sensu stricto* [ $n = 3$ , 16.70%]; *Candida metapsilosis* [ $n = 1$ , 5.55%] and *Candida orthopsilosis* [ $n = 1$ , 5.55%]), *N. glabratus* ( $n = 2$ , 11.11%), *Candidozyma haemuli* species complex (*Candidozyma haemulid sensu stricto* [formerly *Candida haemulonii sensu stricto*;  $n = 1$ , 5.55%] and *Candidozyma haemuli* var. *vulneris* [formerly *Candida haemulonii* var. *vulnera*;  $n = 1$ , 5.55%]) and *Candida palmioleophila* ( $n = 1$ , 5.55%). Three other environmental yeast species were also detected, namely *Candida ecuadorensis* ( $n = 1$ , 5.55%), *Wickerhamiella infanticola* ( $n = 2$ , 11.11%) and *Pichia manshurica* ( $n = 1$ , 5.55%). The sequences were deposited in GenBank database under the accession numbers described in Table 1. Half of the fungal isolates were recovered from Botafogo beach ( $n = 9$ ; 50.0%), followed by Ipanema ( $n = 5$ ; 27.8%) and Flamengo ( $n = 4$ ; 22.2%) beaches.

### Antifungal susceptibility profile

The antifungal susceptibility profile of the fungal isolates was assessed using the broth microdilution method, following the CLSI M27-Ed4 guidelines.<sup>7</sup> The interpretation of the results was based on the breakpoints published in CLSI documents M60-Ed2<sup>8</sup> and

M27-S3.<sup>6</sup> All *C. tropicalis* isolates were resistant to fluconazole, itraconazole, voriconazole and caspofungin, and showed elevated MIC values for terbinafine (MIC >16 mg/L). However, these *C. tropicalis* isolates remained susceptible to amphotericin B and flucytosine. Among the *N. glabratus* isolates, one was resistant to fluconazole and itraconazole, while the other showed dose-dependent susceptibility to these azoles. Additionally, both *N. glabratus* isolates were resistant to caspofungin and exhibited high MICs to terbinafine (16 and >16 mg/L, respectively), but were susceptible to voriconazole, amphotericin B and flucytosine. The two isolates of the *C. haemuli* species complex, *C. haemuli sensu stricto* and *C. haemuli* var. *vulneris*, were resistant to the three azoles tested. *C. haemuli sensu stricto* was also resistant to amphotericin B, whereas *C. haemuli* var. *vulneris* was susceptible to this polyene antifungal. Moreover, both *C. haemuli* isolates were susceptible to caspofungin and flucytosine and exhibited high MICs to terbinafine (MIC >16 mg/L). The *C. palmioleophila* isolate was resistant to fluconazole, showed dose-dependent susceptibility to itraconazole, and was fully susceptible to voriconazole, amphotericin B, caspofungin and flucytosine. All isolates of the *C. parapsilosis* species complex were susceptible to the antifungal agents tested. Similarly, the *C. ecuadorensis* isolate was susceptible to all antifungals. The same pattern was observed in one *W. infanticola* isolate, with the other isolate exhibiting higher MIC to itraconazole in comparison with MIC breakpoints available for *Candida* species (1 mg/L). *P. manshurica* showed high MIC values for fluconazole (>64 mg/L), flucytosine (16 mg/L), and terbinafine (>16 mg/L), suggesting reduced susceptibility to these agents. Lower MIC values were observed for itraconazole, voriconazole, amphotericin B, and caspofungin (Table 2).

### Assessment of biofilm formation

Biofilm formation by the yeast species was assessed on a plastic (polystyrene) surface by analyzing three parameters: (i) total biomass, as determined by crystal violet staining of methanol-fixed cells; (ii) ECM production, evaluated via safranin staining of non-fixed cells; and (iii) metabolic activity, measured by the conversion of XTT to formazan in non-fixed biofilm-forming cells. All fungal isolates recovered from recreational coastal waters demonstrated the ability to form biofilms, albeit to varying extents. Biofilm biomass absorbance values ranged from 0.189 to 1.277 (overall mean  $0.525 \pm 0.311$ ), ECM production ranged from 0.172 to 2.036 (mean  $0.719 \pm 0.523$ ) and metabolic activity ranged from 0.288 to 1.506 (mean  $0.912 \pm 0.390$ ) (Fig. 1). Although no statistically signifi-

**Table 2**  
Antifungal susceptibility profile of fungal isolates recovered from recreational beaches in Rio de Janeiro city, Brazil.

Fungal species (isolates)	MIC (mg/L) <sup>a</sup>						
	FLC	ITC	VRC	AMB	CSF	5-FC	TEB
<i>C. tropicalis</i>							
FL 02	>64 (R)	>16 (R)	>16 (R)	1 (S)	>8 (R)	0.5 (S)	>16
FL 03	>64 (R)	>16 (R)	>16 (R)	0.5 (S)	>8 (R)	0.5 (S)	>16
IP 03	64 (R)	2 (R)	4 (R)	0.5 (S)	4 (R)	<0.12 (S)	>16
IP 05	>64 (R)	>16 (R)	>16 (R)	0.5 (S)	>8 (R)	1 (S)	>16
<i>C. parapsilosis</i>							
FL 01	1 (S)	0.12 (S)	<0.03 (S)	0.5 (S)	2 (S)	0.5 (S)	4
FL 06	2 (S)	<0.03 (S)	<0.03 (S)	0.5 (S)	2 (S)	0.5 (S)	2
BT 04	2 (S)	<0.03 (S)	0.06 (S)	0.12 (S)	2 (S)	0.25 (S)	2
<i>C. metapsilosis</i>							
BT 03	2 (S)	0.06 (S)	0.06 (S)	0.5 (S)	1 (S)	1 (S)	0.5
<i>C. orthopsilosis</i>							
BT 10	8 (S)	0.5 (SDD)	0.25 (S)	0.5 (S)	2 (S)	2 (S)	4
<i>N. glabratus</i>							
BT 08	64 (R)	>16 (R)	1 (S)	0.5 (S)	1 (R)	0.12 (S)	>16
BT 09	32 (SDD)	0.25 (SDD)	0.5 (S)	<0.03 (S)	1 (R)	1 (S)	16
<i>C. haemulid</i>							
BT 06	>64 (R)	>16 (R)	>16 (R)	2 (R)	0.25 (S)	0.5 (S)	>16
<i>C. haemuli</i> var. <i>vulneris</i>							
BT 02	64 (R)	4 (R)	8 (R)	1 (S)	0.12 (S)	0.5 (S)	>16
<i>C. palmiophila</i>							
BT 01	64 (R)	0.5 (SDD)	0.5 (S)	0.12 (S)	1 (S)	2 (S)	8
<i>C. ecuadorensis</i>							
IP 01	4 (S)	0.12 (S)	0.5 (S)	0.25 (S)	1 (S)	<0.12 (S)	1
<i>W. infanticola</i>							
BT 07	8	1	0.12	0.12	1	0.5	2
IP 07	4	0.12	0.06	<0.03	2	<0.12	0.25
<i>P. manshurica</i>							
IP 06	>64	0.5	0.5	0.12	0.25	16	>16

<sup>a</sup> MIC, minimal inhibitory concentration; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B; CSF, caspofungin; 5-FC, flucytosine; TEB, terbinafine; R, resistant; SDD, susceptible-dose dependent; S, susceptible. CLSI protocols have not established breakpoints for terbinafine and amphotericin B; in general clinical isolates of *Candida* with MIC values >1 mg/L are considered resistant to amphotericin B.

317 cant differences were observed among the species for the evaluated  
318 parameters, isolates of *C. tropicalis* and *C. parapsilosis* exhibited  
319 higher values across all three biofilm-related measures. On the  
320 other hand, *C. orthopsilosis* and one isolate of *N. glabratus* displayed  
321 high levels of biofilm-associated metabolic activity. Remarkably,  
322 *P. manshurica* showed prominent ECM production and metabolic  
323 activity, while the metabolic activity of *C. ecuadorensis* was also  
324 comparatively elevated. Notably, a significant positive correlation  
325 was observed between biofilm biomass and ECM ( $p=0.0008$ ;  
326  $r=0.7197$ ). However, no correlations were observed between  
327 biofilm biomass and metabolic activity ( $p=0.6129$ ;  $r=0.1279$ ), nor  
328 between ECM and metabolic activity ( $p=0.0618$ ;  $r=0.4487$ ). Fig. 2  
329 shows representative images of the biofilms formed, illustrating  
330 the variability in the extent and resistance of the biofilms among  
331 the different species.

### 332 Resistance to osmotic stress

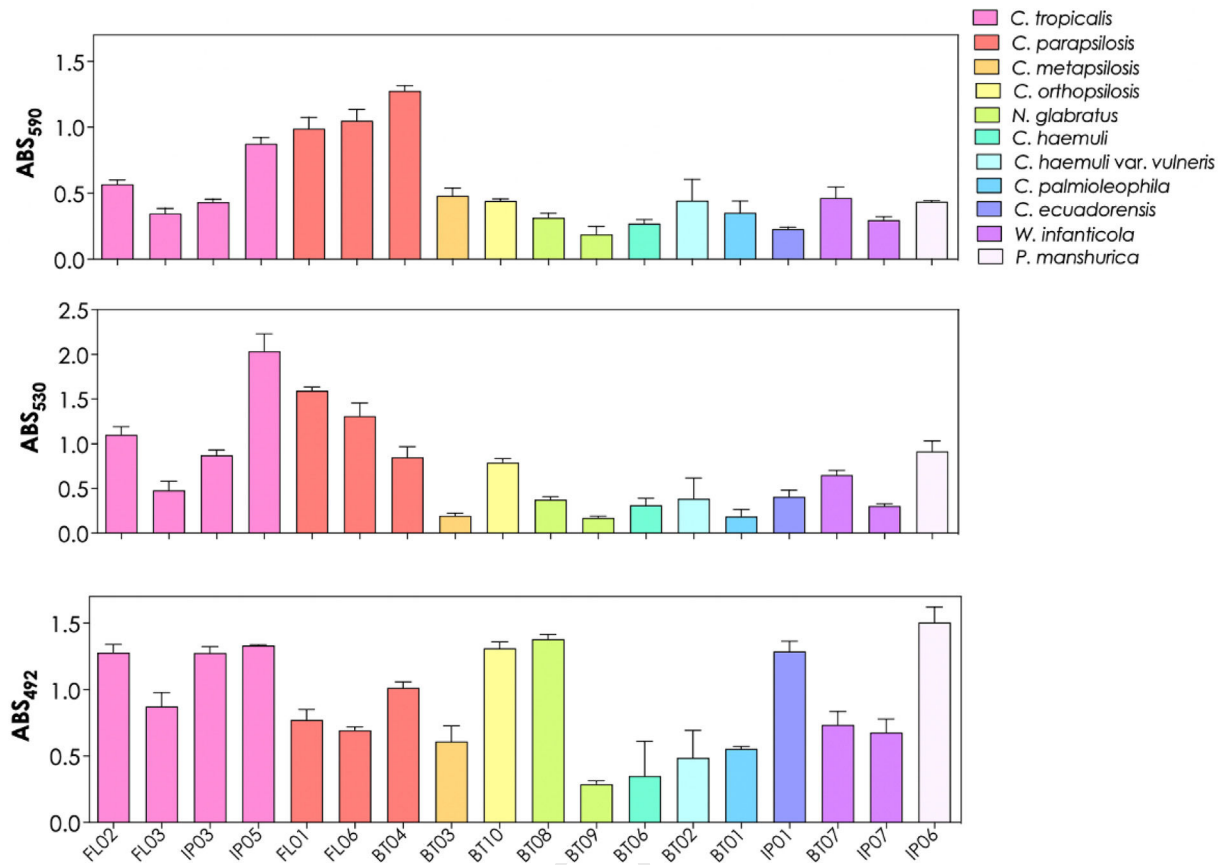
333 Since the yeast isolates used herein were recovered from marine  
334 waters, we investigated their resistance to osmotic stress using  
335 NaCl. In this sense, all yeast isolates were able to grow in concen-  
336 trations up to 7.5% NaCl, except for the isolate of *C. ecuadorensis*  
337 that grew up to 3.75% (Fig. 3).

### 338 Discussion

339 The use of recreational waters for leisure activities is of recog-  
nized importance worldwide, and especially in Brazil, a country

340 internationally known for the beauty of its coastal regions. These  
341 environments are of significant tourist interest, attracting both  
342 domestic and international visitors, which is a key factor in the  
343 Brazilian economy. In this sense, the city of Rio de Janeiro stands  
344 out as tourist destination due to its natural beauty, including sev-  
345 eral beaches distributed throughout its territory. The Brazilian  
346 legislation classifies the quality of seawater for primary contact  
347 based solely on the presence of fecal indicators bacteria, while  
348 the presence of other microorganisms, including fungi, remains  
349 neglected.<sup>21</sup> Recently, the World Health Organization included  
350 fungi as a taxon of interest for monitoring recreational water  
351 quality,<sup>31</sup> based on the results of the studies developed by the  
352 European working group called Mycosands.<sup>2</sup> This working group  
353 investigated the occurrence of fungi in beach sand and seawater of  
354 13 different European countries, identifying as the most frequently  
355 detected fungal genera yeasts like *Candida* and *Cryptococcus*, and  
356 molds like *Aspergillus* and *Fusarium*.<sup>2</sup> The authors found a median  
357 of site-blinded total fungal of 89 CFUs/g and suggested that this  
358 fungal concentration could be utilized as a baseline for beaches in  
359 Europe where analytical data are unavailable.<sup>2</sup>

360 In the present study, we recovered both environmental  
361 yeast species and clinically relevant opportunistic yeasts from  
362 recreational coastal waters of Rio de Janeiro city. Among the  
363 opportunistic yeasts identified, *C. tropicalis*, *C. parapsilosis* species  
364 complex and *N. glabratus* are notable etiological agents of inva-  
365 sive fungal infections, especially in critically ill patients.<sup>32</sup> Indeed,  
366 these fungal species are among the most frequently isolated *Can-*  
367 *dida* species in candidemia cases in Brazilian hospitals, second only



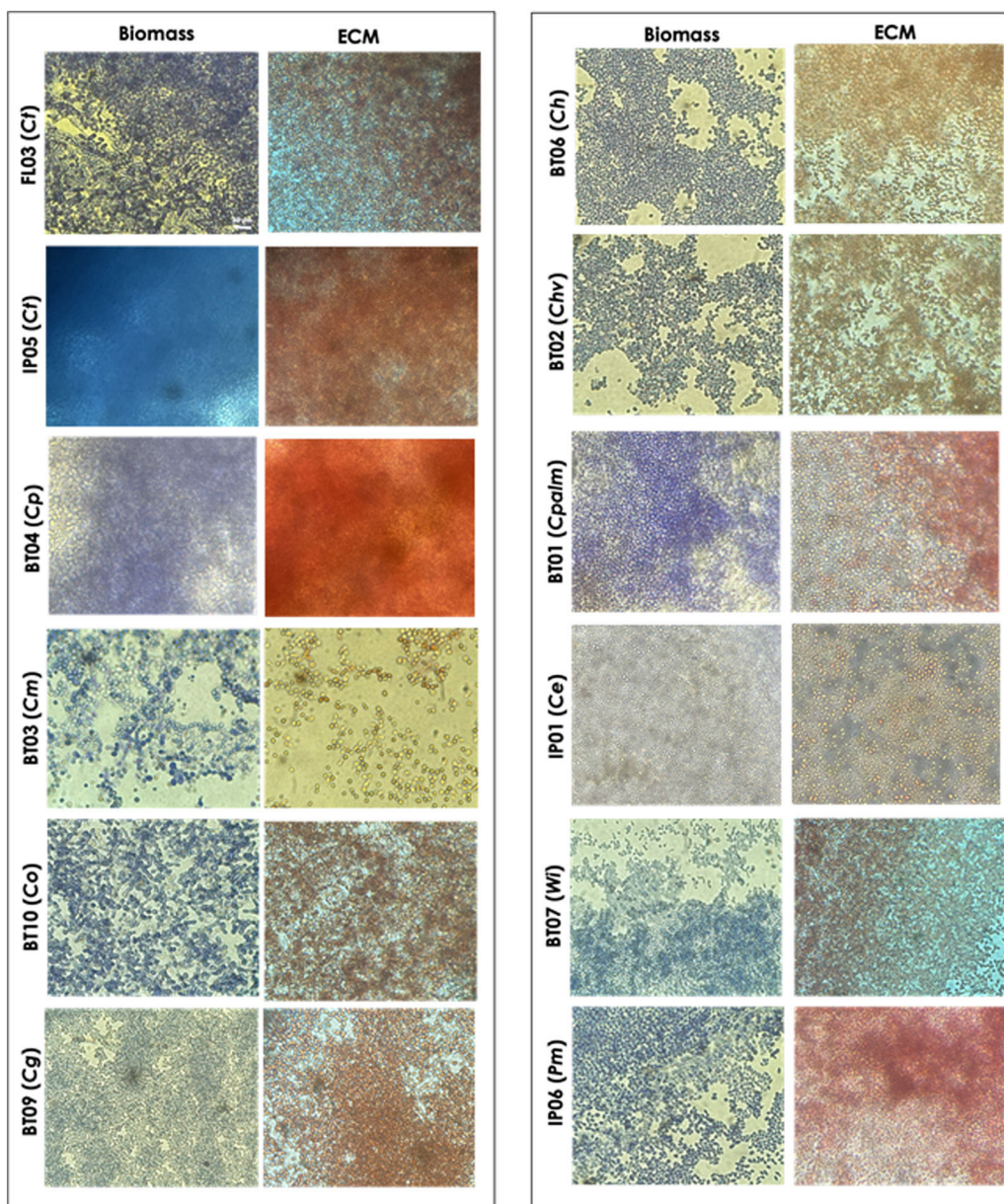
**Fig. 1.** Biofilm formation by the fungal isolates obtained from recreational coastal waters of Rio de Janeiro. Biofilms were developed on a polystyrene surface at 37 °C for 48 h and subsequently prepared for quantification of fungal biomass by measuring absorbance at 590 nm, extracellular matrix at 530 nm and metabolic activity at 492 nm. The results are expressed as absorbance values (ABS) per isolate studied. The results represent means  $\pm$  standard deviation of three independent experiments. The codes on the X-axis of the graph represent each of the 18 yeast isolates used.

to *C. albicans*, which remains the predominant species recovered from these patients.<sup>14</sup> Other studies have also demonstrated the high occurrence of *C. tropicalis* in both sand and seawater samples of different Brazilian beaches.<sup>15,26,36</sup> Additionally, the occurrence of *C. parapsilosis* species complex and *N. glabratus* in recreational coastal waters of Brazil and other countries, such as Iran and Qatar, has already been reported.<sup>15</sup> With regard to the antifungal susceptibility of *C. tropicalis*, this species has been shown to exhibit prominent resistance to azoles. This finding is corroborated by the results of the present study, which revealed that all isolates of *C. tropicalis* were resistant to the three azole antifungals tested. Similar results were observed in *C. tropicalis* isolates obtained years prior in these same beaches.<sup>26</sup> Other studies also reported high resistance rates to azole in *C. tropicalis* isolates obtained from coastal environments.<sup>15,36</sup> All the isolates of *C. tropicalis* tested in this study exhibited resistance to caspofungin, a finding that differs from the literature on this species, which has been classified as susceptible to this echinocandin.<sup>26,36</sup>

In the present study, isolates within *C. haemuli* species complex, an emergent multidrug-resistant group of yeasts, were also recovered from the recreational coastal waters of Rio de Janeiro city. The origin of species within the *C. haemuli* species complex remains a subject of ongoing debate, with increasing speculation regarding a possible environmental reservoir. Interestingly, the species *C. haemuli* was first isolated from the gut of the fish *Haemulon scirus*, from the water of Biscayne Bay, Florida, and from the seawater of the coast of Lisbon, Portugal, being initially called *Torulopsis haemulonii*.<sup>29</sup> Members of the *C. haemuli* species complex have recently been isolated from sand and sea-

water samples collected from different Brazilian beaches, a finding that corroborates the aforementioned hypothesis.<sup>15</sup> The supposition that coastal environments may serve as ecological niches and potential sources for the emergence and dissemination of these opportunistic multidrug-resistant yeasts is thus reinforced. Species belonging to the *C. haemuli* species complex have emerged over the past decades as clinically significant multidrug-resistant pathogens in hospitals worldwide, including Brazil.<sup>23,25</sup> These yeasts have been increasingly associated with a broad spectrum of infections, ranging from superficial colonization to severe invasive disease, particularly among immunocompromised and critically ill patients.<sup>13,23,25</sup> The antifungal susceptibility profile of the species within this yeast group is a cause of concern, as its members are typically resistant to azoles and amphotericin B, yet generally susceptible to echinocandins,<sup>4,23,25</sup> as evidenced by the results of the present study. Their intrinsic reduced susceptibility to multiple classes of antifungal agents and their frequent misidentification by conventional diagnostic methods further complicate the clinical management and may contribute to unfavorable therapeutic outcomes.

The rare opportunistic species *C. palmioleophila* was also isolated in our study. This species has been associated with different marine ecosystems, including oligotrophic hypersaline coastal waters of the Arabian Gulf,<sup>11</sup> a hydrographic basin in Minas Gerais state, Brazil, especially during the dry season<sup>17</sup> and wastewaters in Niteroi city, Rio de Janeiro, Brazil, during COVID-19 pandemic.<sup>9,10</sup> *C. palmioleophila* was first isolated from soil samples,<sup>20</sup> being further associated with catheter-related fungemia.<sup>28</sup> In recent years, the isolation frequency of *C. palmioleophila* from clinical cases has



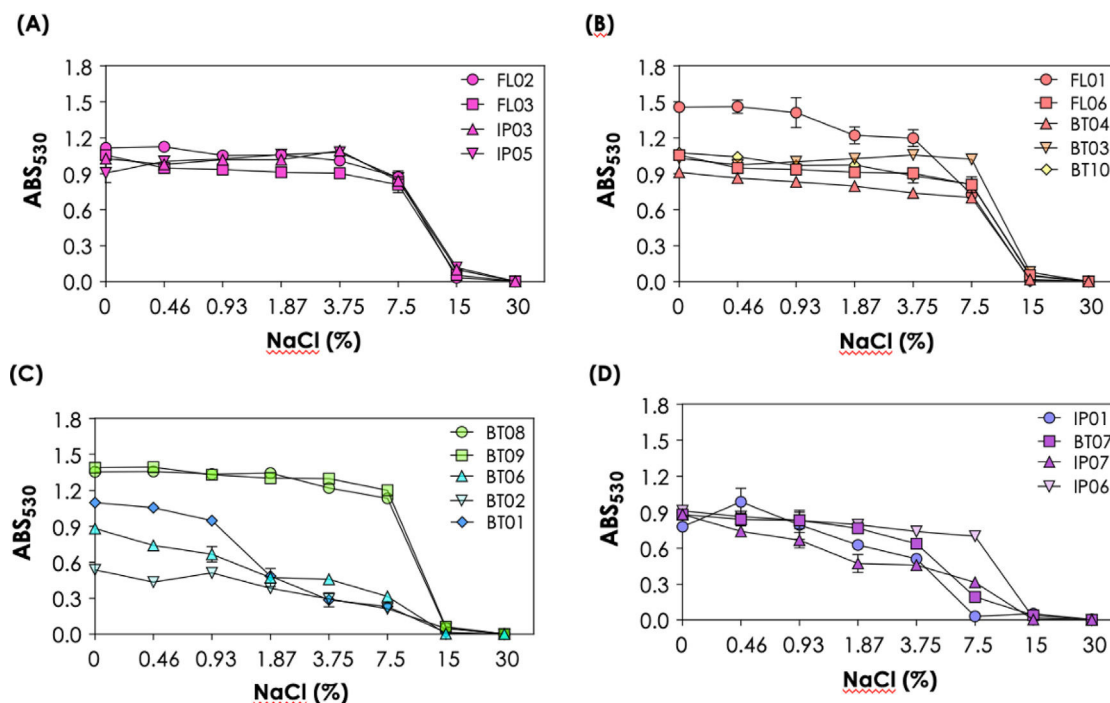
**Fig. 2.** Representative micrographs of biofilms formed by the fungal isolates obtained from recreational coastal waters of Rio de Janeiro after 48 h of incubation at 37 °C in contact with polystyrene. The biofilms were visualized by means of crystal violet staining, to highlight the biomass, and by safranin staining, to highlight the extracellular matrix (ECM). The isolates used were FL03 and IP05 of *C. tropicalis* (Ct), BT04 of *C. parapsilosis* (Cp), BT03 of *C. metapsilosis* (Cm), BT10 of *C. orthopsilosis* (Co), BT09 of *N. glabratus* (Cg), BT06 of *C. haemuli* (Ch), BT02 of *C. haemuli* var. *vulneris* (Chv), BT01 of *C. palmioleophila* (Cpalm), IP01 of *C. equadorensis* (Ce), BT07 of *W. infanticola* (Wi) and IP06 of *P. manshurica* (Pm).

increased, particularly in Europe.<sup>1,3</sup> This species is commonly resistant to fluconazole,<sup>10,34</sup> as demonstrated in this study. Indeed, a case of *C. palmioleophila* infection with a fatal outcome was reported in China.<sup>33</sup> Conversely, *C. palmioleophila* is usually susceptible to the other antifungal agents, a finding that was corroborated by the present study's results.<sup>9</sup>

Environmental yeasts such as *C. equadorensis*, *W. infanticola* and *P. manshurica* were also recovered from seawater samples in the present study. The species *W. infanticola* has also been isolated from recreational beaches of Paraná state, Brazil, being susceptible to fluconazole and amphotericin B, and susceptible dose-dependent to itraconazole.<sup>15</sup> The two isolates of *W. infanticola* of our study exhibited low MIC values for all the antifungals tested, and would be considered susceptible according to the breakpoints established for

*Candida* in CLSI document M27-S3,<sup>6</sup> except for one isolate, which would be classified as resistant to itraconazole.

Biofilm, as a syntrophic community, is widely regarded as the preferred way of life for microorganisms.<sup>27</sup> Biofilms are complex structured communities of microorganisms attached to a surface or interface, covered by an ECM produced by the microorganisms themselves, being important during infection processes and in the environment colonization.<sup>27</sup> All environmental isolates used in our study were able to form biofilm on a plastic (polystyrene) surface. In this sense, biofilm formation by clinically relevant *Candida* species, including *C. tropicalis*, *C. parapsilosis* species complex, *C. haemuli* species complex, *N. glabratus* and *C. palmioleophila*, have been described in the literature.<sup>16</sup> Indeed, catheter-related fungemia caused by *Candida* species in hospital settings represents



**Fig. 3.** Resistance to osmotic stress of the fungal isolates obtained from recreational coastal waters of Rio de Janeiro. Fungal cells in SDB ( $1 \times 10^4$  cells in 200  $\mu$ L) containing different concentrations of NaCl (0–30%) were incubated in polystyrene microtiter plates at 37 °C for 48 h. Fungal growth was read at 530 nm and the results are expressed as absorbance values (ABS) per isolate studied. The results represent mean  $\pm$  standard deviation of three independent experiments. (A) *C. tropicalis* isolates (FLO2, FLO3, IPO3 and IPO5), (B) *C. parapsilosis* species complex isolates (*C. parapsilosis sensu stricto*: FLO1, FLO6 and BT04; *C. metapsilosis*: BT03; *C. orthopsilosis*: BT10), (C) *N. glabratus* (BT08 and BT09), *C. haemuli* species complex (*C. haemuli sensu stricto*: BT06; *C. haemuli* var. *vulneris*: BT02) and *C. palmioleophila* (BT01), (D) *C. equadorensis* (IPO1), *W. infanticola* (BT07 and IPO7) and *P. manshurica* (IPO6).

a huge problem for public health worldwide, since these fungal pathogens are able to adhere to different types of catheters, forming biofilms, which facilitates the access to the bloodstream of patients. Fungal cells forming biofilms exhibit different phenotypes in comparison to their planktonic counterparts, being generally more resistant to antifungal treatments, aggravating the clinical situation of patients.<sup>16</sup> Biofilms also play a significant role in many different environmental niches, including recreational coastal waters. In this sense, biofilms can protect microorganisms from challenging conditions, such as high salinity, ultraviolet radiation, poor nutrients, extreme pH and temperatures, acting as “protective clothing”.<sup>35</sup> Additionally, microorganisms can form biofilms on microplastics and, interestingly, microplastics with biofilms exhibit greater pollutant sorption capacity than virgin counterparts, ultimately serving as vectors capable of introducing contaminants and microbial agents to aquatic habitats.<sup>30</sup> The environmental yeast species tested in the present study were able to form biofilms on polystyrene surface similarly to the opportunistic yeasts, corroborating the relevance of this virulence attribute regardless of whether the yeast is pathogenic or not.

Given that the yeasts used in this study were isolated from marine environments, we assessed their ability to grow under high NaCl concentrations. Except for *C. equadorensis* that grew up to 3.75% NaCl concentration, all the other yeast isolates were able to grow up to 7.5% NaCl. This finding is in agreement with our prior research, in which isolates of *C. tropicalis* and *C. parapsilosis* recovered from recreational coastal waters of Rio de Janeiro were also able to grow in concentrations up to 7.5% NaCl.<sup>26</sup> Additionally, several *C. tropicalis* isolates obtained from sand samples of beaches in Northeast Brazil were able to grow in higher salt levels (15% NaCl).<sup>36</sup>

Taken together, our results reveal the presence of clinically relevant opportunistic *Candida* species, as well as traditionally environmental yeasts, in seawater samples from beaches in Rio de Janeiro. Notably, except for the isolates within the *C. parapsilosis* species complex, all clinically important *Candida* species, and the environmental yeast *P. manshurica*, exhibited a concerning antifungal resistance profile. Furthermore, all isolates exhibited some virulence attributes, such as biofilm formation on plastic surface and growth under high salinity, which are associated with environmental persistence and pathogenic potential. Altogether, these findings highlight the presence of potentially pathogenic and drug-resistant yeasts in recreational coastal waters of Rio de Janeiro. To better assess the public health risk, future studies should include seasonal and long-term monitoring of fungal communities in marine environments, incorporate quantitative assessments of fungal load, and investigate the potential of yeast transmission to humans through recreational water exposure. The integration of fungal surveillance into routine water quality programs, alongside bacterial indicators, is also recommended to provide a more comprehensive understanding of microbial risks in coastal areas.

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## Conflict of interest

The authors report no conflict of interest.

513 **Q3** **Uncited reference**

514 12.

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