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Abstract

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Keywords	Onychomycosis; Candida parapsilosis; Adhesion ability; Biofilm formation; Kodamaea ohmeri

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Full title: "Virulence properties and sensitivity profile of *Candida parapsilosis* complex species and *Kodamaea ohmeri* isolates from onychomycosis of HIV/AIDS patients"

Short title: "Virulence of *Candida parapsilosis* complex and *Kodamaea ohmeri*"

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Abstract

Cutaneous fungal infections include onychomycosis, an infection of the nail that affects both healthy and immunocompromised patients. This study investigated the *in vitro* adhesion and biofilm formation capacity of *Candida parapsilosis* and *Kodamaea ohmeri* isolates from onychomycoses of HIV/AIDS patients and also established the antifungal sensitivity profiles of these isolates. Onychomycosis in HIV/AIDS patients showed a high prevalence of emerging yeasts, among which *C. parapsilosis* complex species and *K. ohmeri* were the most frequent. Three *C. parapsilosis sensu stricto* isolates and two *C. orthopsilosis* isolates were resistant to amphotericin B and 83% were resistant to terbinafine. All isolates adhered to stainless steel and siliconized latex surfaces, and carbohydrates intensified adhesion of all isolates. Isolates adhered to keratinous nail and 50% formed biofilms with strong intensity. In multispecies or polymicrobial biofilms, *C. albicans* and *Staphylococcus aureus* regulated the formation of biofilms, decreasing the number of non-*albicans* species and of *Candida* spp. cells, respectively. Nonetheless, the presence of *C. parapsilosis sensu stricto* or *K. ohmeri* in mixed biofilms increased the number of viable *C. albicans* cells. The isolation of emerging yeast species from onychomycosis with high adhesion capacity to medical devices, with many being good biofilm producers, is a result that should be considered relevant in clinical practice. In addition, half of the isolates resistant to at least one of the tested antifungals or were susceptible in a dose-dependent manner, which corroborates the infectious capacity and viability of these isolates.

Key-words: Onychomycosis; *Candida parapsilosis*; Adhesion ability; Biofilm formation; *Kodamaea ohmeri*

1. INTRODUCTION

Cutaneous infections include onychomycosis, an infection of the nail caused by dermatophytes, yeasts, and other moulds, leading to discoloration, thickening and separation of the nail bed [1,2]. Affecting both healthy and immunocompromised patients, onychomycoses are among the most difficult fungal infections to treat[3]. Prevalence ranges from 15% to 40% in patients with the human immunodeficiency virus (HIV) [4,5].

Laboratory confirmation of infection is crutial for clinical diagnosis of onychomycosis. Cutaneous mycosis creates discomfort and several types of long-term treatment may be needed to effect a supposed cure. Even so, a complete cure, defined as clinical cure (involving whitening) and mycological cure (both microscopic and dermatophytic culture negatives), are often unachievable [1].

The yeast *Candida parapsilosis* has long been recognized as the most common etiological agent of onychomycosis [6,7,2]. In Brazil, *C. parapsilosis* is the first or second most common cause of onychomycosis lesions [8,9,10]. This species is also a significant problem in infants, patients receiving parenteral nutrition and transplant recipients [6,11]. In addition, it is the second most frequently isolated *Candida* species in blood cultures [12,13], the most commonly isolated from human hands[14] and the second most frequently isolated from body sites of hospitalized patients. *C. parapsilosis* can cause candidiasis that range from relatively mild skin mycoses to life-threatening systemic or cadidemia [15].

In addition to yeasts of the genus *Candida*, others, such as *Trichosporon*, *Rhodotorula*, *Cryptococcus* non-*neoformans*, *Geotrichum* and *Kodamaea* have been correlated with onychomycoses in immunocompromised patients [16]. *Kodamaea* (Pichia) *ohmeri* is the teleomorphic form of *C. guilliermondii var. membranaefaciens* and is an emerging pathogen and fungemia agent in the following conditions: immunocompromised patients, intravenous drug use, patients on chemotherapy with or without neutropenia, premature birth, diabetes, prolonged hospitalization and abdominal surgery [17,18].

Despite the increasing number of infections caused by emerging yeast , such as *C. parapsilosis* complex and *K. ohmeri*, few studies have been conducted to evaluate the virulence of their clinical isolates [19,20,21,22]. Although the frequency of onychomycosis due to *Candida* is increasing [23,24,25], there is little information about

the epidemiology, pathogenesis, and antifungal susceptibility of this dermatological disease causing agent. In addition, there are no reports in the literature of virulence studies about *Kodamaea*. This study aimed to provide information about the *in vitro* adhesion and biofilm formation capacity of species of the *C. parapsilosis* complexes and *Kodamaea ohmeri* isolated from onychomycoses of HIV/AIDS patients, in addition to determining the antifungal sensitivity profile of these isolates. To the best of our knowledge, this research pioneers analysis of the virulence properties of clinical *K. ohmeri* samples.

2. MATERIAL AND METHODS

2.1 Yeast samples

Yeasts were isolated from cutaneous lesions of immunocompromised patients hospitalized at a reference hospital in the city of São Luís, MA, Brazil. Reference strains *C. parapsilosis* ATCC 22019*, C. albicans* ATCC 90028 and *S. aureus* ATCC 25923 were also included and were provided by the São Paulo State University (UNESP), School of Dentistry, Araraquara, São Paulo, Brazil. The study was approved by the Ethics Committee of Ceuma University (CEP/UNICEUMA, protocol number 63522).

2.2. Isolation and identification of microorganisms

Samples were obtained from lesions by scraping them with a sterilized spatula or dental curette, after a written and informed consent was obtained, collected into steril tubes and submitted to direct mycological examination in 20% KOH (ISOFAR, Paraná, Brazil). Fungi were isolated in SGA (Sabouraud glucose agar*,* Difco, Detroit, MI, USA) and an automated Vitek-2® system (Compact, bioMérieux, Marcy-L 'Etoile, France) was used to perform yeast biochemical identification. Samples identified as *C. parapsilosis* by Vitek-2® were submitted for identification by sequencing.

2.2.1 Cycle sequencing

Pure colonies of *Candida* cultured in SGA for 48 h were used for extraction of the genomic DNA. The UltraClean Microbial DNA Isolation kit (MoBio Laboratories, Solana Beach, CA) was used according the manufacturer's recommendations to extract fungal DNA. Amplification of the divergent domains (D1 and D2) was performed using 1 pmol of the NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′ -GGTCCGTGTTTCAAGACGG-3′) primers, 0.1 µg of extracted DNA, and 25 µl of ultrapure water with PuReTaq Ready-To-Go PCR beads (GE Healthcare, Fairfield, CT). Samples were subjected to 5 min cycle at 95 ℃, for DNA denaturation, followed by 35 cycles of amplification (95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min), and

finally by a 10 min extension at 72 °C. PCR product purification was performed using a Microcon YM-100 centrifugation filter device (Millipore Corporation, Bedford, MA). The resulting sequences were assembled using MEGABACETM 1000 (Amersham Biosciences, EUA), and compared to GenBank nucleotide sequences using BLASTN (NCBI, Bethesda, MD).

2.3 Microdilution assay

Antifungal activities of Ketoconazole (KTC), Itraconazole (ITC), Fluconazole (FLC), Terbinafine (TERB) and Amphotericin B (AMB), were determined according to Clinical and Laboratory Standards Institute – CLSI-M27-A3 [26]. Antifungals were diluted in water (FLC, Pfizer, Brazil) or DMSO (KTC, ITC, AMB, TERB; Sigma-Aldrich, St. Louis, MO, USA), and then diluted in RPMI-1640 culture medium, pH 7.0 (Sigma-Aldrich, St. Louis, MO, USA), buffered with 0.165 M MOPS (morpholinepropanesulfonic acid, Sigma Chemical, St. Louis, MO) and filtered in membrane. Antifungals were used at concentrations of 0.25-64 μg ml^{-1} (FLC), 0.0625-16 μg ml^{-1} (KTC, ITC, AMB) and 0.03-16 μg ml^{-1} (TERB). All tests were performed in triplicate on two different occasions. The minimum inhibitory concentration (MIC) of each compound was defined as the lowest concentration at which no fungal growth was observed. The breakpoints used to define sensitivity, intermediate, and resistant for each species were those defined by CLSI [26].

2.4 Adhesion

2.4.1 Inoculum preparation

Isolated colonies were reactivated in a liquid medium RPMI 1640 (Sigma Aldrich Co., St. Louis, MO, USA), 37 °C for 48 h, collected by centrifugation (2318 × *g* for 5 min) and suspended in phosphate buffered saline (PBS, pH 7.0, Sigma Aldrich Co., St. Louis, MO, USA) to reach an absorbance of 0.26 at $OD_{520\ nm}$ (1×10^7 cfu ml^{-1}).

2.4.2 Adherence to siliconized latex and stainless steel

The adhesion assay was performed according to Silva et al. [22], with some modifications. A siliconized latex catheter (0.5 mm, urinary catheter, Solidor® Lamedid, Osasco, São Paulo, Brazil) and stainless-steel segments (1cm, 21g × 1-1/4" sterile disposable needles Lamedid, Osasco, São Paulo, Brazil) were washed, sterilized and added separately to 24-well microplates with 1 ml of inoculum. For negative controls, fragments were placed with 1 ml PBS. Microplates were incubated (37 ºC, 3 h), then segments were washed 3× with PBS, placed on microtubes with 1 ml of PBS and sonicated (7 W, for 30 s) to disrupt adherent cells. Crystal violet staining confirmed complete removal of adhered cells. Serial decimal dilutions (in PBS) of cell suspension

were subcultured onto SGA (aliquots of 100 µL) and incubated (48 h, 37 ºC). CFU were quantified on plates and results were expressed as Log_{10}. Three replicates of adhesion assays were carried out and assays performed on different days.

2.4.3 Carbohydrate interference of latex adhesion

Tests were conducted as described in 2.4.2 using siliconized latex catheter segments, except that after washing, fungal cells were resuspended in solutions with different carbohydrates. Cells suspended PBS were used as controls. Carbohydrate tested were glucose, sucrose, mannitol and sorbitol (50 mM) [27].

2.4.4 Adherence to keratin substrates

Human nail substrates were prepared as described previously [10], with some modifications. Nails from healthy volunteers were cut to fragments (0.5 to 1 cm) which were defatted by soaking in ethanol for 96 h. The solvent was changed once each day. Fragments were then thoroughly washed with sterilized distilled water and autoclaved for 5 min at 115 °C. A fixed volume of inoculum (1 ml) was added to the microtubes containing sterile nail fragments. Microtubes were incubated at 37 °C for 3, 6, 12 and 24 h. Fragments were then washed twice with PBS, stained with methylene blue/distilled water (1:4 v/v) and analyzed under a light microscope (Carl Zeiss Scope A1 Optical Microscope, Stockholm, Sweden).

2.5 *Candida* biofilm formation

An inoculum of each strain (200 µL of 10^7 cells ml^{-1} suspension) was added to a flat-bottom 96-well polystyrene plate for adhesion (90 min) at 37 ºC, and non-attached *Candida* cells were removed by two rounds of washing with PBS. Yeast Nitrogen Base (YNB, 200 µL, with amino acids and ammonium sulfate; Difco, Detroit, MI, USA) supplemented with glucose (100 mM) was added to the attached cells, and the microplates were incubated for 24 h at 37 ºC. Biofilms were washed 2× with PBS and 200 µL of fresh medium was added to the wells before an additional 24 h incubation. Mature biofilms (48 h) were washed in PBS, and cells were fixed with 80% ethanol for 15 min. After drying at room temperature, 200 µL of crystal violet was added to each well, and biofilms were stained for 20 min. Biofilms were washed 2× with PBS and solubilized with 200 µL of 33% glacial acetic acid. Absorbance was measured at 550 nm on a microplate reader (BioMérieux Reader 250 Version 2.0.5). Samples with optical density (OD) values lower than controls were considered as non-producers, conversely those with OD values higher than controls were considered to be producers. Among producers, those with OD less than twice that of the control were defined as weak producers, those with OD between two and four times that of the control were defined as

moderate producers, and those with OD values more than four times that of the control were defined as strong producers [28,29]. Assays were carried out in five replicates and were repeated on different days. A chi-square test of independence ($\chi 2$) was used to evaluate the variables non-producer and producer, and data were subject to logarithmic transformation and ANOVA one way and Tukey tests were done.

2.5.3 Multi-species and polymicrobial biofilm formation

Although cells within mixed biofilms are intrinsically adherent to each other and there is probably no perfect method for determining the total number of cells of each species, we decided to investigate how some of these species behave together in biofilms. For multi-species and polymicrobial biofilm assays, good *C. parapsilosis* and *K. ohmeri* biofilm producers were chosen from clinical isolates (DM 29A1 and DM 25, respectively; see Table 5 for values). *K. ohmeri* and *C. parapsilosis sensu stricto* were chosen because they were isolated from the same sample. Reference strains *S. aureus* ATCC 25923, *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were used as controls and in the composition of multi-species and polymicrobial communities. *S. aureus* was used in experiments because it is part of the human microbiome frequently found on the skin and in the nasal fossa and has been related to onychomycosis [30]. Fungi and bacteria inocula were prepared in PBS and optically standardized to a density of 10^7 cells ml^{-1} (OD = 0.26 at 520 nm) and 10^8 cells ml^{-1} (OD = 0.01 at 520 nm), respectively. Biofilm assays were conducted in polystyrene, 96-well microtiter plates (KASVI, São José dos Pinhais, Paraná, Brazil). In multi-species biofilm tests, 100 µL of the standardized inoculum of *C. albicans* ATCC 90028 and 100 µL of either *C. parapsilosis sensu stricto* ATCC 22019, *C. parapsilosis sensu stricto* clinical isolate, or *K. ohmeri* clinical isolate were added to each well. In polymicrobial assays, 100 µL of the standardized inoculum of *S. aureus* ATCC 25923 and 100 µL of either *C. parapsilosis sensu stricto* ATCC 22019, *C. parapsilosis sensu stricto* isolate, or *K. ohmeri* isolate were transferred to each well (Table 1). After incubation for 90 min at 37 ºC for adhesion, microtiter plate wells were washed 2× with PBS pH 7.0 (Sigma Aldrich Co., St. Louis, MO, USA), before 200 µL of either YNB (multispecies biofilms) or brain–heart infusion (BHI) (polymicrobial biofilms) broth was added to the wells and the plates were incubated again at 37 º C for 48 h. 100 µL of each standardized inoculum plus medium were incubated separately as controls.

After 48 h, biofilms were washed 2× with PBS, resuspended in 100 µL PBS and transferred to microtubes with 900 µL of PBS (10× dilution). Samples were vortexed and

serial dilutions were made for each combination and controls. Aliquots of 10^{-3} and 10^{-4} dilutions of multispecies biofilms were spread on CHROmagar Candida® (*BioMerieux*, França) plates for cfu enumeration.

Bacteria samples (10^{-7} and 10^{-8} dilutions) from polymicrobial biofilms were seeded on BHI supplemented with FLC (64 μg ml^{-1}). Yeast dilutions (10^{-3} and 10^{-4}) were spread on BHI with chloramphenicol (25 μg ml^{-1}). Dilutions were seeded using the duplicate droplet technique. Cells were enumerated as cfu ml^{-1} according to the formula: cfu ml^{-1} = number of colonies × 10 n/q, where ***n*** is the absolute value of the dilution chosen and ***q*** is the quantity, in ml, seeded from each dilution in the plates (0.010 ml = 10 μL).

2.6 Statistical analysis

Data were analyzed by the *NCSS 11* statistical program (2016). Initially, the Shapiro Wilk normality test was performed with the numeric variables "adhesion", "carbohydrate interference", "48h biofilm", "mixed biofilm" and "polymicrobial biofilms". As the variables did not present normality ($p < 0.05$), they were subjected to logarithmic transformation. Two-way ANOVA was applied (for species, material and their interactions) to evaluate the logarithmic adhesion variable. One-way ANOVA was applied (for species) to evaluate the logarithmic biofilm variable. Significance for all comparisons between species was evaluated using the Tukey post hoc. To evaluate the association of the classification variables of the biofilm intensity the $\chi 2$ test of independence was used. All tests were considered significant at $p < 0.05$.

3. RESULTS

3.1 Isolation and identification of microorganisms

Samples were collected from 44 HIV/AIDS patients with clinical diagnoses of cutaneous fungal infections. The most prevalent cutaneous lesion was onychomycosis (57·4%). Among the patients there were 11 females and 33 males in the age range of 19–61 years. The patients presented CD4C cell counts ranging from 10 to 552 cells mm^{-3} and viral loads of 2567 to 256 860 copies ml^{-1}. Twenty-one (21) filamentous fungi and 25 yeasts were isolated and identified, and 13 of the isolated yeasts belonged to the genus *Candida*. Among the identified *Candida* species, a higher frequency (61.5%) of *C. parapsilosis* complex was observed (8) (Table 2). Other less prevalent species were *K. ohmeri* (12%), *C. haemulanii* (8%), *Cryptococcus laurentii* (8%), *Trichosporon asahii* (12%), *T. mucoides* (12%), *C. lusitaniae* (4%), *C. albicans* (4%), *C. tropicalis* (4%) and *Stephanoascus ciferrii* (4%). The most frequently observed isolates, i.e. *C. parapsilosis*

complex and *K. ohmeri*, first identified by VITEK as *C. parapsilosis*, both from onychomycosis were chosen for virulence and sensitivity profile tests.

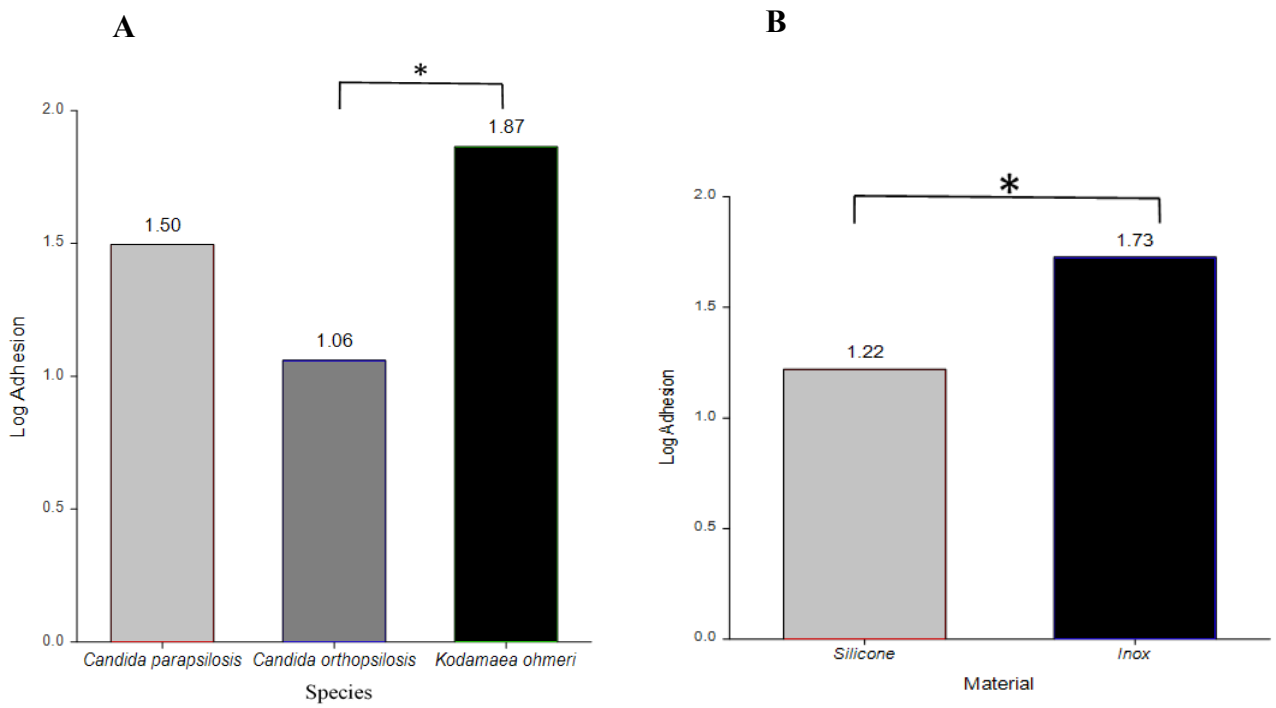
3.2 Minimum inhibitory concentration (MIC)

Most of the isolates were sensitive to FLC and ITC (67%). Almost half of the isolates showed resistance to AMB, 83% showed resistance to TERB and all were sensitive to KTC. All isolates of *C. orthopsilosis* were resistant to AMB. Some isolates of *C. parapsilosis sensu stricto* and *C. orthopsilosis* (33%) were susceptible-dose dependent (SDD) to FLU and ITC. *K. ohmeri* isolates were sensitive to all antifungal agents. MIC values for KTC were < 0.05 μg ml^{-1}. MIC for FLC ranged from 1–16 μg ml^{-1}, whereas for ITC the MIC ranged from 0.062–0.5 μg ml^{-1}. For AMB, MIC varied from 0.5 to 2 μg ml^{-1} and for TERB MIC ranged from 0.5–8 μg ml^{-1} (Table 3).

3.3 Adherence to siliconized latex and stainless steel

As silicone latex and stainless steel are common constituents of medical devices used in hospitals that are potential sources of infection, we evaluated the ability of isolates to adhere to these materials. All isolates were able to adhere to silicone and stainless steel. *C. parapsilosis* and *K. ohmeri* isolates had higher adhesion capacity compared to *C. orthopsilosis* isolates ($p < 0.05$, Figure 1). In addition, ANOVA test found a significant difference ($p < 0.05$) between the mean yeast adhesion to stainless steel (1.73 ± 0.1) and silicone (1.22 ± 0.1). Thus, the ability of the isolates to adhere to stainless steel was significantly higher than to silicone (Figure 1B). Multiple comparison tests of all pairwise differences between the means of the variables "inert material" and "species" showed significant difference between *K. ohmeri* and *C. orthopsilosis* in relation to the adhesion on material and that all clinical isolates adhered more strongly to inox ($p < 0.05$, ANOVA and Tukey Multiple-Comparison Test, Figure 1B).

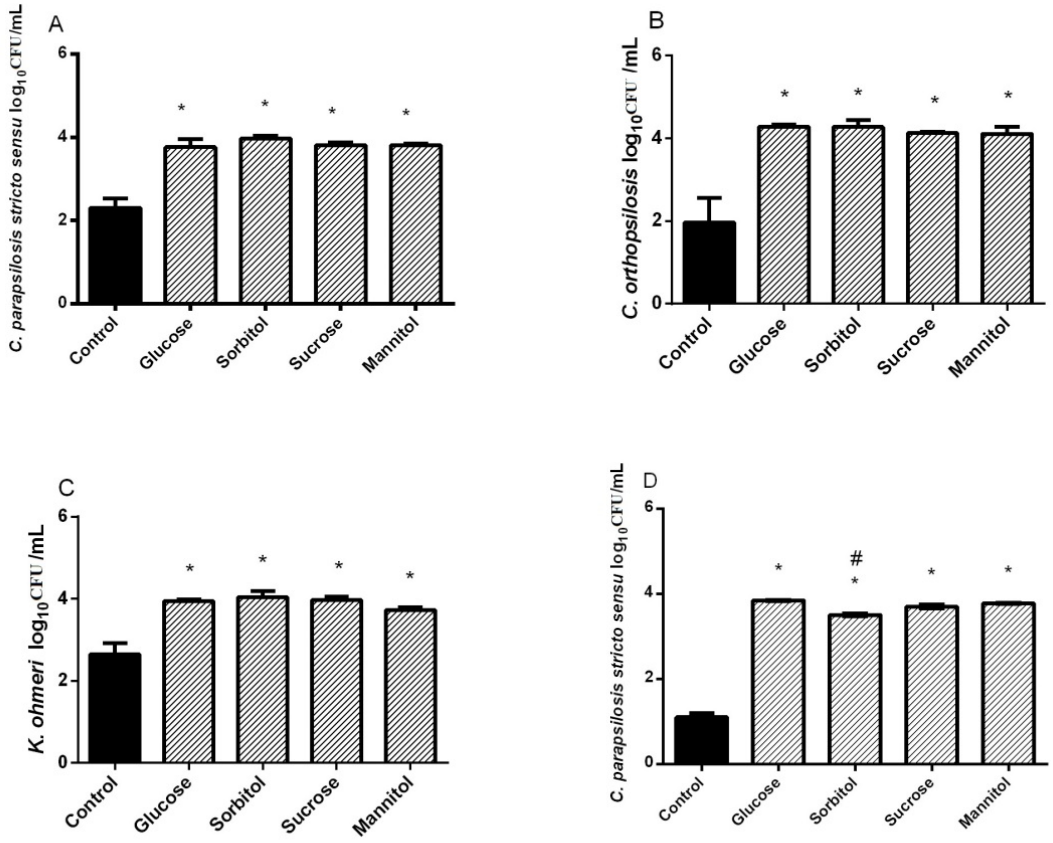
Figure 1. (A). Mean of *Candida* onychomycosis isolates (*Candida parapsilosis*, *Candida orthopsilosis* and *Kodamaea ohmeri*) capability of adhesion to medical devices (silicone latex and stainless steel) *K. ohmeri* isolates presented higher mean adhesion capacity when compared to *C. orthopsilosis* ones. * p <0.05. **(B).** Mean ability of *Candida* onychomycosis isolates to adhere to silicone latex and stainless steel, constituents of medical devices. A significant difference between the mean yeast adhesion to stainless steel and silicone was found (p <0.05), showing that isolates showed greater ability to adhere to stainless steel. *p <0.05.



3.4 Carbohydrate interference of adhesion to silicone

In addition to carbohydrates being considered important mediators of the adhesion process in many biological models, they are present as the main component in solutions for parenteral nutrition, which is administered through special intravenous catheters. Glucose, sorbitol, sucrose and mannitol increased adhesion of samples to silicone in relation to the control ($p < 0.05$) by more than 50%. Sorbitol increased the capacity of adhesion less intensely than the other carbohydrates ($p < 0.05$) (Figure 2).

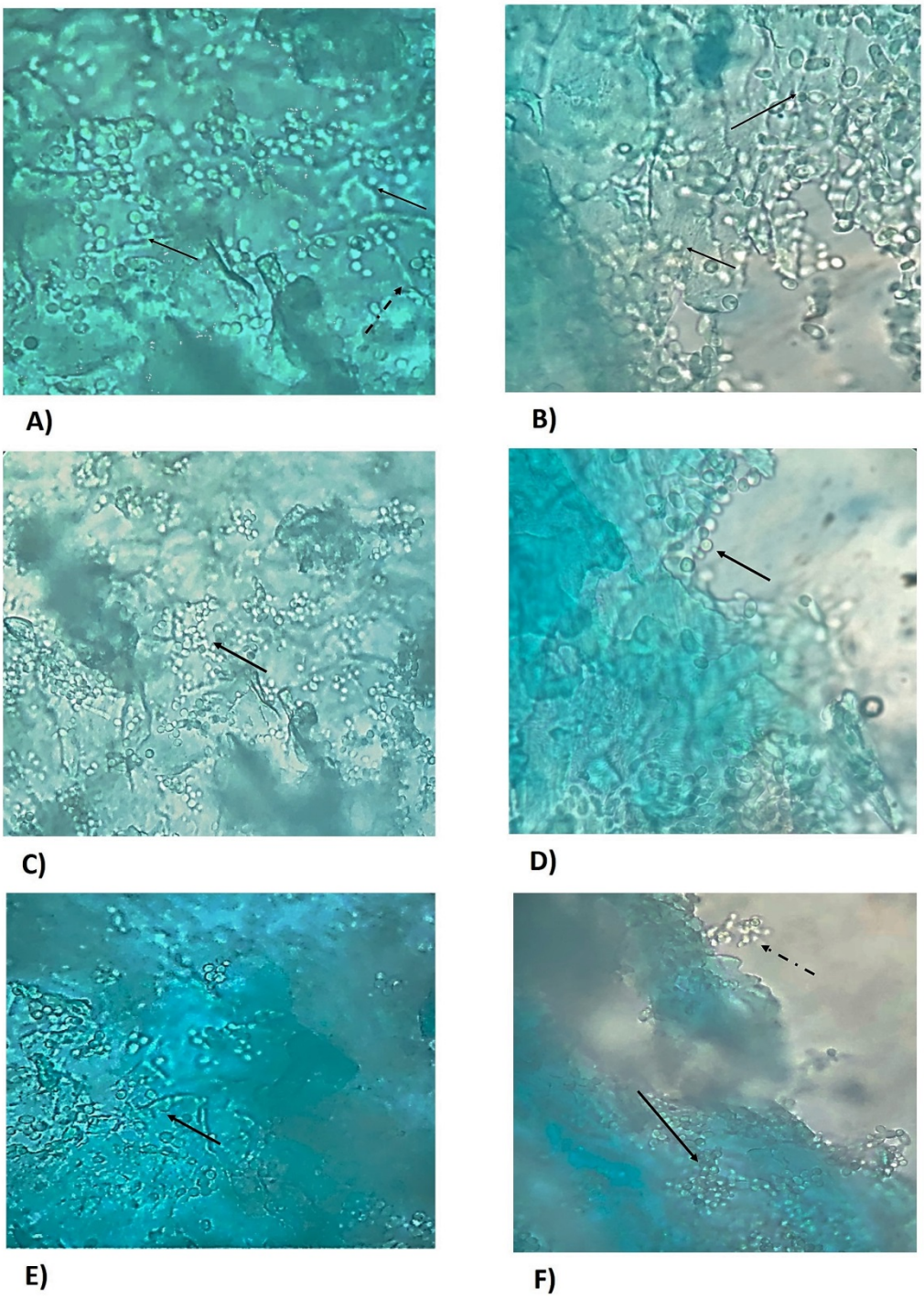
Figure 2. Carbohydrate interference on the adhesion of *C. parapsilosis sensu stricto* ATCC 22019 and the clinical isolates of the species *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *K. ohmeri* on silicone. The clinical isolates were from the onychomycosis of HIV / AIDS patients, hospitalized in a public network hospital in the city of São Luis, Brazil. **A)** Carbohydrates increased the adhesion of *C. parapsilosis sensu stricto* in relation to the control without presence of sugars. **B)** All sugars tested increased the adhesion of *C. orthopsilosis* in relation to control. **C)** The presence of carbohydrates increased the adhesion of *K, ohmeri* in relation to control. **D)** All sugars tested increased the adhesion of the reference strain. Sorbitol was the sugar that less intensified the adhesion in relation to the other carbohydrates. Note. **(*)** means statistical difference between the control group when compared to the carbohydrates sources; **(#)** means statistical diferrence between the glucose compared to the other carbohydrates sources.



3.5 Adherence to keratin substrates

The *C. parapsilosis sensu stricto* ATCC 22019 sample, as well as clinical samples were adhered to nail fragments at intervals of 3, 6, 12 and 24 h with observed growth in the form of blastopores and pseudohyphae (Figure 3).

Figure 3. Optical micrographs showing the *in vitro* adhesion of yeast cells to human keratin substrates. Samples of nail fragments were placed on glass slides and stained with methylene blue. They were viewed in a Carl Zeiss Scope A1 Optical Microscope. Magnification of 1000× (some examples are in a large zoom such as letters B and D). A, C, E are *Candida orthopsilosis* (sample DM9B) and B, D, F are *Kodameae ohmeri* (sample DM25B). Arrows indicate some blastopores (⟶) and pseudohyphae (- · ⟶).

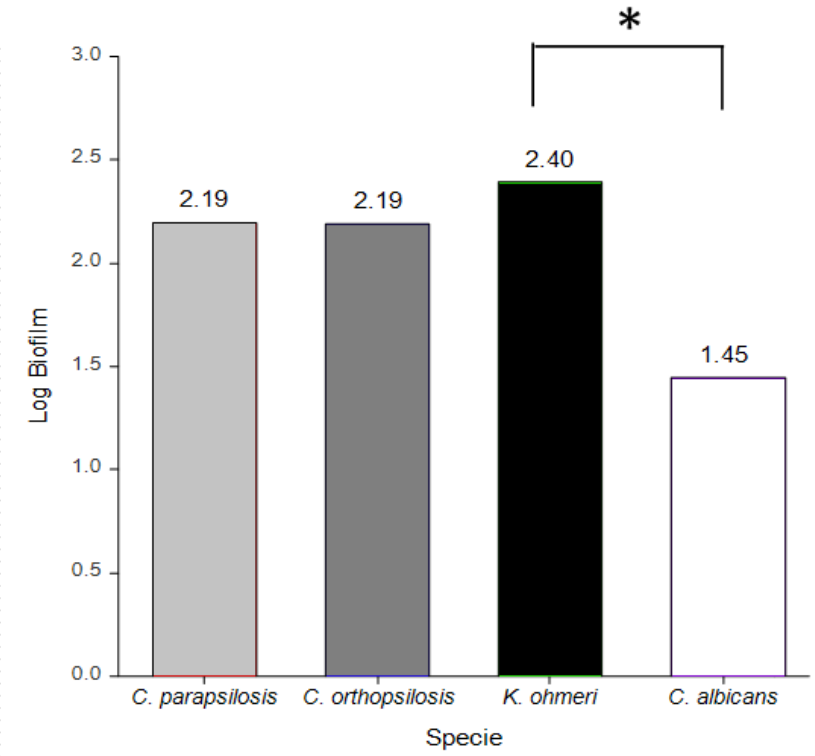


3.6 Biofilm formation

All species formed biofilms (Table 5) but there was a significant difference ($p < 0.05$) in the proportions of biofilm production by *K. ohmeri* and *C. albicans* (Figure 4). *C. parapsilosis sensu stricto* ATCC 22019 formed strong biofilms in 48 h (Tables 4 and 5). The majority (66.7%) of *K. ohmeri* and half (50%) of *C. orthopsilosis* formed strong biofilms in 48 h (Table 5). *C. parapsilosis* isolates exhibited lowest biofilm formation

capacity. *K. ohmeri* had the greatest ability to form biofilms among the different species ($p < 0.05$; Figure 4).

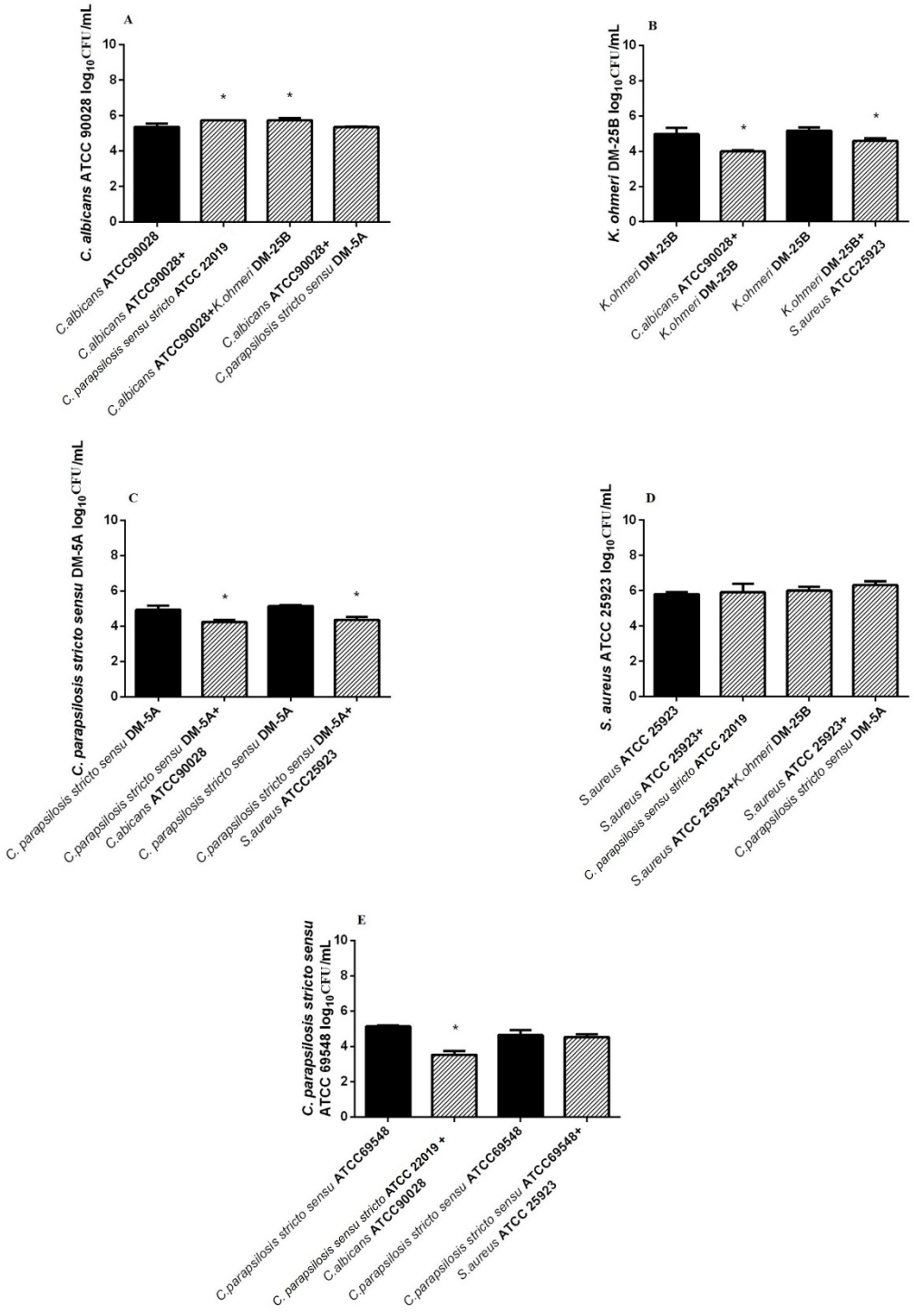
Figure 4 – A Cristal violet assay, determined at absorbance of 550nm, was used as a measure of biofilm ability formation of isolates on 96-well polystyrene plates in YNB media. Biofilms were quantified after 48 h at 37 ºC. Results are expressed as Log_{10} biofilm and considered as statistically significant when $p < 0.05$ (*). Experiments were done in five replicates.



3.7 Multi-species and polymicrobial biofilms

The presence of *C. albicans* ATCC 90028 in mixed biofilm formation decreased the number of viable *C. parapsilosis* (Figure 5C), *K. ohmeri* (Figure 5B) and *C. parapsilosis* ATCC 22019 (Figure 5E) cells when incubated separately for 48 h. However, the number of viable *C. albicans* ATCC 90028 cells in the biofilm increased in the presence of *C. parapsilosis* ATCC 22019 and *K. ohmeri* (Figures 5A). The presence of *S. aureus* ATCC 25923 decreased the number of viable *K. ohmeri* (Figure 5B) and *C. parapsilosis* (Figure 5C) cells when polymicrobial biofilm were formed separately for 48 h. No interference occurred in the number of viable *S. aureus* cells in the polymicrobial biofilm formed together with *C. parapsilosis* ATCC 22019, *C. parapsilosis* or *K. ohmeri* (Figure 5D).

Figure 5. Interference of *Candida albicans* ATCC 90028 and *S. aureus* ATCC 25923 on biofilm formation of *Candida parapsilosis sensu stricto* ATCC 22019, *Candida parapsilosis sensu stricto*, *Candida orthopsilosis* and *Kodamaea ohmeri* isolated from onychomycosis of HIV/AIDS patients, hospitalized in a public network ospital in the city of São Luis, Brazil. **A)** Log_{10} cfu ml^{-1} of *Candida albicans* ATCC 90028 forming multispecies biofilms with the other yeasts. In the presence of *Kodamaea ohmeri* and *Candida parapsilosis* ATCC 22019 there is an increase in *Candida albicans* cells ($p < 0.05$). **B)** The presence of *S. aureus* and *Candida albicans* in multiple biofilms with *Kodamaea ohmeri* decreases the number of *Kodamaea ohmeri* cells in these biofilms in relation to the control ($p < 0.05$). **C)** The presence of *Candida albicans* ATCC 90028 and of *S. aureus* ATCC 25923 in mixed biofilm formation with *Candida parapsilosis sensu stricto* decreased the number of viable cells of *Candida parapsilosis sensu stricto*. **D)** There was no interference of the presence of yeast cells in the formation of multispecies biofilms with *S. aureus* ATCC 25923. **E)** The presence of *Candida albicans* in a mixed biofilm with the reference strain *Candida parapsilosis sensu stricto* decreases the cfu of the same ($p < 0.05$)



4. Discussion

Onychomycosis affects all populations and is the most common fungal infection[25]. Although both *C. albicans* and *C. parapsilosis* have been reported as the leading causes of onychomycosis associated with *Candida*, the role of the emerging species in this condition has not yet been fully elucidated [23,24,25].

This research revealed a high prevalence of emerging yeasts in onychomycosis in HIV/AIDS patients, of which *C. parapsilosis sensu stricto* (46%) was the most frequent. *K. ohmeri* (12%) deserves to be highlighted because it is emerging in onychomycosis. Gupta et al. [5] found a 20% incidence of onychomycosis in HIV+ patients in Brazil, and Cambuim et al. [31] identified *C. parapsilosis* complex as the main agent of onychomycosis in immunosuppressed patients. De Araújo Montarin et al. [32] reported a 69% frequency of yeasts causing onychomycosis in healthy patients, with *C. parapsilosis* complex the most prevalent (54%). These data corroborate our results. *K. ohmeri* is considered an emerging fungal pathogen, isolated from oral infections and fungaemia in immunocompetent and immunosuppressed patients [33,16,34]. However, as far as we know, this is the first report of *K. ohmeri* isolation from onychomycosis in HIV/AIDS patients with a relatively high frequency. Manzano-Gayosso et al. [35] reported *K. ohmeri* as an etiologic agent of onychomycosis (1.2%), however isolated from healthy individuals.

Onychomycosis due to *Candida* is often difficult to treat and, thus, the antifungal susceptibility profile of species in this genus needs to be determined [36]. *Candida* isolates were sensitive to KTC, however, half of them were considered SDD or resistant to one of the tested drugs and most were resistant to TERB. Pakshir et al. [37] found 7.2% of *C. parapsilosis* isolates resistant to FLC. Giacobino [38], studying yeasts from patients with peritonitis who underwent peritoneal dialysis, found that many isolates of *K. ohmeri*, *C. parapsilosis sensu stricto* and *C. orthopsilosis* were resistant to FLC. Our results point to the increase in the resistance to antifungal drugs phenomenon, and, consequently, the need to determine the sensitivity profile and the precise identity of microorganisms present in infections.

The ability of a microorganism to adhere to biotic or abiotic surfaces is a precondition for successful establishment of infection [39]. All isolates analyzed in this work showed an ability to adhere to stainless steel and siliconized latex. Indeed, *C. parapsilosis* has previously been shown to have a strong ability to colonize prosthetic materials and intravascular devices, increasing the risk of infection for patients requiring

prolonged used of a central venous catheter or indwelling devices [22]. As silicone latex and stainless steel are some of the main constituents of hospital devices, our results corroborate this observation and indicate the relevance of *K. ohmeri* isolates as colonizers of medical devices and possible infectious agents in the bloodstream.

Carbohydrates such as glucose, sorbitol, sucrose and mannitol were able to intensify *in vitro* adhesion of isolates at rates as high as 60%. The rate of interference was specific to each isolate, as adhesion intensity of the reference strain used in this research decreased in presence of sorbitol. Previous studies have found that *C. parapsilosis* shows advantageous growth in solutions of hyperalimentation rich in sugars [40]. Abu-Elteen [41] evaluated the ability of *Candida* spp to adhere to oral epithelial cells in the presence of carbohydrates and found that fructose, galactose, glucose, maltose, sorbitol and sucrose significantly increased adherence. Sugars can alter the components of the fungal cell wall and such changes may result increase cell wall hydrophobicity, a factor that enhances the adhesion capacity of the microorganism [42,27]. These results are relevant in medical practice because, in addition to demonstrating the high adhesion capacity of the studied isolates, they show that different sugars can modulate adhesion by intensifying the hydrophobic interactions between the surfaces. This finding is alarming and calls attention to a need for more careful selection of catheters in parenteral nutrition.

Another interesting result obtained in this study is that isolates were able to adhere to keratinous nail. In this test, keratin was the only nutritional source available to isolates over a 24 h period, which suggests that these isolates may have an important role in keratolysis of nails. In addition, isolates formed pseudohyphae when adhered to nail, a pattern that may indicate that this adherence favors cell morphologies with the capacity for tissue invasion [10]. This is the first report related to adhesion of *C. othopsilosis* and *K. ohmeri* isolates associated with nail infections to keratinized substrates from human sources.

Fifty percent of the isolates tested formed a biofilm with strong intensity, especially the *C. orthopsilosis* and *K. ohmeri* isolates. Abi-Chacra et al. [43] when testing the biofilm formation capacity of clinical isolates of the *C. parapsilosis* complex, observed that all species comprising the complex could produce mature biofilms on abiotic surfaces. Silva-Dias et al. [44] verified that all clinical isolates of *C. orthopsilosis* and 34% of *C parapsilosis sensu stricto* were biofilm producers. While our findings agree with these two studies, De Toro et al. [45] showed that *C. orthopsilosis* isolates from blood, urine and eyes did not show any biofilm formation ability, contrasting with our

findings. The biofilm formation capacity of *C. parapsilosis* is dependent on the site from which the yeast was isolated, a fact that may explain the divergence between the literature and the data found in this study [46]. Studies with *K. ohmeri* isolates obtained from patients with fungal peritonitis and from the oral cavity of immunosuppressed patients found *K. ohmeri* produced biofilm on abiotic surface [47,38].

As 23% of the collected samples had associations between different species of yeasts or between yeasts and filamentous fungi, we decided to investigate how these species behaved together in the biofilm. The presence of *C. albicans* in multi-species biofilms reduced the number of viable cells of all tested isolates. Interaction of two species of *Candida* during the biofilm formation caused inhibition of growth of one species, likely due to competition for adhesion sites, nutrients, space and quorum sensing. This competitive inhibition may occur during the initial phase of adhesion and even during the development of the biofilm [48,49]. These facts may explain the results obtained in this study. However, the number of viable cells in the *C. albicans* biofilm increased when formed in the presence of *C. parapsilosis sensu stricto* and *K. ohmeri*. Therefore, C. *parapsilosis* and *K. ohmeri* may provide an initial substratum or other factors beneficial to *C. albicans* growth on the polypropylene surface.

Seabra et al. [49] co-cultivated *C. albicans* and *C. parapsilosis* for biofilm formation and found that the number of viable cells of *C. parapsilosis* decreased in the presence of *C. albicans*, which agrees with results from the current study. However, other reports contradict our results, for example Martins et al. [50] found a dominance of non-*albicans* species (*C. glabrata*, *C. kruzei*, *C. parapsilosis*, *C. rugosa* and *C. tropicalis*) when cultivated together with *C. albicans*. Also, a study by Thein et al. [51] on mixed biofilms of *C. albicans* and *C. krusei* on acrylic prostheses, revealed an antagonistic interaction between the species that form the biofilm, since there was a low abundance of *C. albicans* hyphae formation in mixed biofilm compared to simple biofilm. It was verified that in mixed biofilm, both species had morphology of blastospores.

When we analyzed the polymicrobial biofilm of *Candida* isolates in the presence of *S. aureus* ATCC cells, a decrease of *C. parapsilosis sensu stricto* and *K. ohmeri* viable cells was observed. On the other hand, no interference occurred in the number of viable cells of *S. aureus* biofilm in the presence of *C. parapsilosis sensu stricto* or *K. ohmeri* cells. Zago et al. [52] observed that *C. albicans* and *S. aureus* could co-inhabit a biofilm, since the number of both microorganisms does not decrease over time in the polymicrobial biofilm. However, in the presence of *C. albicans*, the number of bacterial

cells increased over specific time periods, when compared to the bacterial monomicrobial biofilm. It is suggested that in polymicrobial biofilms, particularly formed by *C. albicans* and bacterial species, the physiological nature of bacteria modulates the biofilm and complex relationships that may affect yeast morphogenesis develop between bacterial species [53]. Harriott and Noverr [54], while monitoring the growth of fungi and bacteria in biofilms by the cfu count assay using selective plaques to determine the number of fungal and bacterial cells, found that *C. albicans* cell levels remained the same in monomicrobial and polymicrobial biofilms. However, *S. aureus* levels were significantly higher in the polymicrobial biofilm, compared to the *S. aureus* monomicrobial biofilm.

Taken together, our results show that *C. parapsilosis sensu stricto, C. orthopsilosis* and *K. ohmeri* are emerging as etiological agents of onychomycosis, and have high adhesion capacity to keratinous substrates. In addition, the isolates showed adhesion capacity to medical devices and many were good biofilm producers, data considered relevant in clinical practice. *C. albicans* and *S. aureus*, both microorganisms present on the mucosal and skin microbiota, regulate the formation of mixed and polymicrobial biofilms, decreasing the number of cells of non- *albicans* species and of *Candida* spp, respectively. Nonetheless, the presence of *C. parapsilosis sensu stricto* and *K. ohmeri* in mixed biofilms increased the number of viable cells of *C. albicans*. Besides the virulence manifested by the isolates, half were SDD or resistant to one of the tested drugs, which corroborates the infectious capacity and survivability of these isolates.

Conflict of Interest

The authors declare that the research was conducted in the absence of any conflict of interest.

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Table 1. Combination of species for multispecies and polymicrobial biofilms.

Multispecies combinations		
Candida albicans ATCC 90028	+	*Candida parapsilosis sensu stricto* ATCC 22019
Candida albicans ATCC 90028	+	*Candida parapsilosis sensu stricto* clinical isolate
Candida albicans ATCC 90028	+	*Kodamaea ohmeri* clinical isolate
Staphylococcus aureus ATCC 25923	+	*Candida parapsilosis sensu stricto* ATCC 22019
Staphylococcus aureus ATCC 25923	+	*Candida parapsilosis sensu stricto* clinical isolate
Staphylococcus aureus ATCC 25923	+	*Kodamaea ohmeri* clinical isolate

Table 2. Identification of isolated yeast from onychomycosis of HIV/AIDS patients hospitalized in a public network hospital in the city of São Luis, Brazil.

SAMPLES	VITEK2 SYSTEM	SEQUENCING (% identity)
DM-3A	*Candida parapsilosis*	*Candida parapsilosis sensu stricto* (100%)
DM-4	*Candida parapsilosis*	*Candida parapsilosis sensu stricto* (99%)
DM-4C	*Candida parapsilosis*	*Kodamaea ohmeri* (99%)
DM-5A	*Candida parapsilosis*	*Candida parapsilosis sensu stricto* (100%)
DM-9A	*Candida parapsilosis*	*Candida orthopsilosis* (100%)
DM-9B	*Candida parapsilosis*	*Candida orthopsilosis* (100%)
DM-25B	*Candida parapsilosis*	*Kodamaea ohmeri* (100%)
DM-26B	*Candida parapsilosis*	N.I
DM-29A	*Candida parapsilosis*	*Kodamaea ohmeri* (100%)
DM-29A1	*Candida parapsilosis*	*Candida parapsilosis sensu stricto* (100%)
DM-29A3	*Candida parapsilosis*	*Candida parapsilosis sensu stricto* (100%)
Candida parapsilosis ATCC® 22019	*Candida parapsilosis*	*Candida parapsilosis sensu stricto* (100%)

Note: DM = samples identification; N.I- not identified.

Table 3. Minimum inhibitory concentration of *Candida parapsilosis sitricto sensu, Kodamaea ohmeri* and *Candida orthopsilosis* isolated from onychomycosis of HIV / AIDS patients, hospitalized in public hospital in the city of São Luis, Brazil.

		MIC μg ml^{-1}				
SPECIES	**SAMPLES**	**KTC**	**FLC**	**ITC**	**AMB**	**TERB**
Candida parapsilosis sensu stricto	DM-3A	<0.05	2	0.125	1	0.5
	DM-4	<0.05	1	0.062	2	2
	DM-5A	<0.05	8	0.062	1	4
	DM-26B	<0.05	16	0.5	2	0.5
	DM-29A1	<0.05	16	0.5	0.5	2
	DM-29A3	<0.05	8	0.5	2	2
	ATCC® 22019	<0.05	16	0.125	0.5	2
Candida orthopsilosis Kodamaea ohmeri	DM-9A	<0.05	16	0.25	2	8
	DM-9B	<0.05	4	0.062	2	8
	DM-4 C	<0.05	8	0.125	0.5	2
	DM-25B	<0.05	8	0.062	0.5	4
	DM-29A	<0.05	8	0.125	1.0	2

Legends: DM - samples identification; MIC - Minimum inhibitory concentration; KTC - Ketoconazole; FLC - Fluconazole; ITC- Itraconazole; AMB- Amphotericin B and TERB- Terbinafine. For FLU, MIC values ≤ 8 μg ml^{-1} were considered susceptible (S), 16– 32 μg ml^{-1} were considered as susceptible dose-dependent (SDD), and ≥ 64 μg ml^{-1} as resistant (R). For AMB, MICs ≤ 1 μg ml^{-1} were considered to be S and >1 μg ml^{-1} were R. For ITC MICs ≤0,125 μg ml^{-1} were considered to be S, 0. 25-0.5 μg ml^{-1} were SDD and ≥1 μg ml^{-1} as R. For KTC MICs >16 μg ml^{-1} were considered (R). For TERB MICs > 1.4 μg ml^{-1} were considered (R).

Table 4. Classification of yeasts species in relation to the production and intensity of biofilms. Optical density (OD) values lower than controls were considered as non-producers. OD values higher than controls were considered as producers. Assays were carried out in five replicates and were repeated on different days.

Samples	Species	Mean Biofilm score ± SD	Biofilm intensity
DM-3A	*Candida parapsilosis sensu stricto* (100%)	0.0318 ± 0.001	Non-producer
DM-4	*Candida parapsilosis sensu stricto* (99%)	0.0332 ± 0.001	Non-producer
DM-4C	*Kodamaea ohmeri* (99%)	0.6474 ± 0.38	Producer
DM-5A	*Candida parapsilosis sensu stricto* (100%)	0.859 ± 0.05	Producer
DM-9A	*Candida orthopsilosis* (100%)	0.0328 ± 0.002	Non-producer
DM-9B	*Candida orthopsilosis* (100%)	0.7412 ± 0.12	Producer
DM-25B	*Kodamaea ohmeri* (100%)	0.858 ± 0.10	Producer
DM-26B	N.I	0.0362 ± 0.002	Non-producer
DM-29A	*Kodamaea ohmeri* (100%)	0.0336 ± 0.007	Non-producer
DM-29A1	*Candida parapsilosis sensu stricto* (100%)	0.6456 ± 0.05	Producer
DM-29A3	*Candida parapsilosis sensu stricto* (100%)	0.0308 ± 0.004	Non-producer
Candida parapsilosis ATCC® 22019	*Candida parapsilosis sensu stricto* (100%)	0.793 ± 0.08	Producer
Candida albicans® ATCC 90028	*Candida albicans*	0,7092 ± 0.08	Producer

DM – samples identification; Non-Producer: ODi < ODc; Weak producer: ODc < ODi ≤ (2× ODc); Moderate Producer: (2× ODc) < ODi ≤ (4× ODc); Strong Producer: (4× ODc).
ODc indicates Optical Density of control (value = 0.0898 ± 0.14)
ODi indicates Optical Density of isolate

Table 5. Chi-square independence test for biofilm formation of *Candida parapsilosis sensu stricto*, *Candida orthopsilosis* and *Kodamaea ohmeri* isolated from onychomycosis of HIV/AIDS patients, hospitalized in a public network hospital in the city of São Luis, Brazil. Optical Density (OD) values lower than controls were considered as non-producers. OD values higher than controls were considered as producers. Assays were carried out in five replicates and were repeated on different days.

Species	**Classification**				***p***
	Non-producer	**%**	**Strong**	**%**	
Candida parapsilosis sensu stricto ATCC 22019	0	0	5	100	0.019
Candida parapsilosis sensu stricto	20	66.7	10	33.3	
C. orthopsilosis	5	50.0	5	50.0	
K. ohmeri	5	33.3	10	66.7	