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Evaluation of *Candida albicans* and *C. tropicalis* biofilms life cycle, and their prevalence in bloodstream infections.

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

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Dedicatoria

Con todo mi cariño para mi familia especialmente a mami que me enseñó a leer, a mi hijo que es mi motor y mi fuerza y a mis sobrinas para que sean niñas y mujeres de ciencias.

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RESUMEN

El estudio de las biopelículas es una de las áreas más recientes de la microbiología. Las biopelículas tienen importancia tanto en microbiología ambiental, microbiología de alimentos, así como microbiología médica. Aún más reciente es el estudio de biopelículas formadas por hongos. Estas biopelículas fúngicas toman importancia en microbiología médica porque se asocian a personas con enfermedades que comprometen el sistema inmune.

Hemos encontrado una asociación estadísticamente significativa entre la capacidad de las especies de *Candida* de formar biopelículas y la mortalidad de los pacientes infectados. Al analizar la prevalencia mundial *Candida albicans* y *Candida tropicalis* resultaron ser las especies más prevalentes y se relacionan con alta mortalidad. Al comparar el ciclo de vida de biopelículas de especies de *Candida* formadas *in vitro* como biofilm monoespacie, encontramos que *Candida tropicalis* es una especie con alta capacidad de formar biopelículas.

Hasta donde conocemos este trabajo es uno de los primeros en su campo. A partir de nuestros resultados, quedan sin respuesta nuevas preguntas sobre la fisiología de estas biopelículas y las fuerzas que modulan el comportamiento de la levadura; por lo tanto, futuros estudios deberían analizar la red molecular y metabólica que influye en la evolución del biofilm formado por diferentes especies de *Candida.*

Palabras clave: Biofilms; *Candida* species; Infection; Mortality; Antifungal resistance pattern; Geographical distribution; Meta-analysis.

ABSTRACT

The study of biofilms is one of the most recent areas of microbiology. Biofilms are important in both environmental microbiology, food microbiology, as well as medical microbiology. Even more recent is the study of biofilms formed by fungi. These fungal biofilms are important in medical microbiology because they are associated with people with diseases that compromise the immune system.

We have found a statistically significant association between the ability of *Candida* species to form biofilms and the mortality of infected patients. *Candida albicans* and *Candida tropicalis* are the most prevalent species and are associated with high mortality. When comparing the life cycle of biofilms of these two species of Candida formed in vitro as a monospecies biofilm, we found that *Candida tropicalis* is a species with a high capacity to form biofilms.

To the best of our knowledge, this work is one of the first in its field. From our results, new questions about the physiology of these biofilms and the forces that modulate yeast behavior remain unanswered; therefore, future studies should analyze the molecular and metabolic network that influences the evolution of the biofilm formed by different species of Candida.

Keywords: Biofilms; *Candida* species; Infection; Mortality; Antifungal resistance pattern; Geographical distribution; Meta-analysis.

INTRODUCTION

PREVALENCE OF BIOFILMS IN CANDIDA SPP. BLOODSTREAM INFECTIONS: A META-ANALYSIS

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Abstract

Context: *Candida*-related infections are nowadays a serious Public Health Problem emerging multidrug-resistant strains. *Candida* biofilm also leads to bloodstream and invasive systemic infections.

Objective: The present meta-analysis aimed to analyze *Candida* biofilm rate, type, and antifungal resistance among hospitalized patients between 1995 and 2020.

Data Sources: Web of Science, Scopus, PubMed, and Google Scholar databases were searched for English papers using the following medical subject heading terms (MESH): “invasive candidiasis”; “bloodstream infections”; “biofilm formation”; “biofilm-related infections”; “mortality”; and “prevalence”.

Study Selection: The major inclusion criteria included reporting the rate of biofilm formation and the prevalence of biofilm-related to *Candida* species, including observational studies (more exactly, cohort, retrospective, and case-control studies). Furthermore, data regarding the mortality rate, the

geographical location of the study set, and the use of anti-fungal agents in clinical isolates were also extracted from the studies.

Data Extraction: Independent extraction of articles by 2 authors using predefined data fields, including study quality indicators.

Data Synthesis: A total of 31 studies from publicly available databases met our inclusion criteria. The biofilm formation in the data set varied greatly from 16 to 100% in blood samples. Most of the studies belonged to Europe (17/31) and Asia (9/31). Forest plot showed a pooled rate of biofilm formation of 80.0 % (CI: 67–90), with high heterogeneity (Q = 2567.45, I^2 = 98.83, $\square^2$ = 0.150) in random effects model ($p < 0.001$). The funnel plot and Egger's linear regression test failed to find publication bias ($p = 0.896$). The mortality rate in *Candida*-related bloodstream infections was 37.9% of which 70.0% were from biofilm-associated infections. Furthermore, *Candida* isolates were also characterized in low, intermediate, or high biofilm formers through their level of biofilm mass (crystal violet staining or XTT assays) after a 24h growth. When comparing between countries, statistical differences were obtained ($p = 0.0074$), showing the lower and higher biofilm prevalence values in Italy and Spain, respectively. The prevalence of low, intermediate, and high biofilms were 36.2, 18.9, and 35.0% ($p < 0.0001$), respectively. *C. tropicalis* was the prevalent species in high biofilm formation (67.5%) showing statistically significant differences when compared to other *Candida* species, except for *C. krusei* and *C. glabrata*. Finally, the rates of antifungal resistance to fluconazole, voriconazole, and caspofungin related to biofilm were 70.5, 67.9 and 72.8% ($p < 0.001$), respectively.

Conclusions: Early detection of biofilms and a better characterization of *Candida* spp. bloodstream infections should be considered, which eventually will help preserve public health resources and ultimately diminish mortality among patients.

Keywords: Biofilms; *Candida* species; Infection; Mortality; Antifungal resistance pattern; Geographical distribution; Meta-analysis.

Introduction

Invasive candidiasis is a systemic mycosis caused by *Candida* species, being commonly described as an opportunistic infection. The population group more vulnerable for invasive candidiasis includes patients with critical illness or immunosuppression (such as hematological and solid organ malignancy, hematopoietic cells and solid organ transplantation, recent abdominal surgery, and hemodialysis), or even people with a central venous catheter or parenteral nutrition. In addition, people that received broad-spectrum antibiotics or with drug habits are also susceptible to invasive candidiasis, as well as premature newborns [1]. All these plausible scenarios lead this systemic infection to be nowadays the 4th leading nosocomial infection in the United States, demonstrating mortality of up to 40% [2]. In Europe, Bassetti and colleagues performed a multinational and multicenter study in 2019 reporting 7.07 episodes per 1000 in European intensive care units (ICUs) with a 30-day mortality of 42% [3]. While, in the Asia-Pacific region, Hsueh and colleagues reported a candidemia incidence in ICUs of 5- to 10-fold higher than in the entire hospital and a mortality rate of patients between 35% and 60% [4]. In Latin America, Nucci and colleagues carried out a laboratory-based survey between November 2008 and October 2010 among 20 tertiary care hospitals in seven Latin American countries, reporting an overall incidence of 1.18 cases per 1,000 in general admissions [5]. The mortality associated with invasive candidiasis is similar or even higher in other worldwide countries [6].

To understand the dimension of this infection and its virulence, we must define the term invasive candidiasis as both forms of candidemia detected in the blood and tissues or deep organs under the mucosal surfaces (also known as deep candidiasis). Deep candidiasis can remain localized or spread causing a secondary infection [7]. Patients with a systemic infection induced by *Candida* spp. can be subdivided into three groups: (1) those who present with bloodstream infection (candidemia); (2) those

who develop deep-seated candidiasis (most frequently intra-abdominal candidiasis); and, (3) those who develop a combination of these two groups [8].

The gold standard for the diagnosis of invasive candidiasis is the growth culture, being blood culture commonly used to diagnose candidemia while culture media is applied to diagnose deep candidiasis from tissue biopsies [9]. In this meta-analysis, we only evaluated studies using positive blood cultures to evaluate the biofilm formation and other related factors in candidiasis virulence. More exactly, the selected studies performed an *in vitro* biofilm assay using *Candida* isolates from blood samples of the patients with catheter-related candidemia (CRC) and non-CRC. In cases of patients with CRC, the standard procedure was blood cultures from obtained the catheter and peripheral veins, whereas non-CRC was indicated by the recovery of *Candida* spp. from only blood samples, as previously described by Guembe and colleagues [10].

Nosocomial infections are closely associated with biofilms growing attached to medical devices or host tissues [11]. Biofilms are the predominant growth state of many microorganisms, being a community of irreversible adherent cells with different phenotypic and structural properties when compared to free-floating (planktonic) cells. National Institutes of Health estimated that biofilms are responsible, in one way or another, for more than 80% of all microbial infections in the United States [12]. *Candida* species can produce well-structured biofilms composed of multiple types of cell and even microbial species, leading to an intrinsic resistance against a wide variety of stress factors, such as various antifungal drugs and immune defense mechanisms [13]. Although the dynamics biofilm-host is not yet fully understood, it is well-known that *Candida* biofilms inhibit the innate immune system of the host [14]. Therefore, our main goal was to analyze the relationship between biofilms and mortality in *Candida* spp. related infections, showing a severe menace to the Public Health System with serious outcomes.

RESULTS

Study inclusion criteria and characteristics of the eligible studies

A total of 214 studies were retrieved and 70 full texts were reviewed from publicly available databases (Web of Science, Scopus, PubMed, and Google Scholar). Thirty-one studies met our inclusion criteria (Figure 1). The final data set included studies covering different global regions (most of them in Europe). All available and relevant data were extracted of each study, more exactly, biofilm rate, biofilm type, underlying disease of the patients, *Candida* species reported, and antifungal resistance. The data was then used to create other databases, collecting information of at least five or more papers, and consequently, each paper was cited more than once. These additional databases were chosen to realize subgroup analysis using a random-effect model and to answer relevant questions about *Candida*-related biofilms, such as the mortality rate related to biofilms, the geographical distribution of biofilms, the characterization of biofilm production among *Candida* species, and the correlation between biofilm formation and antifungal resistance (S1 and S2 Files).

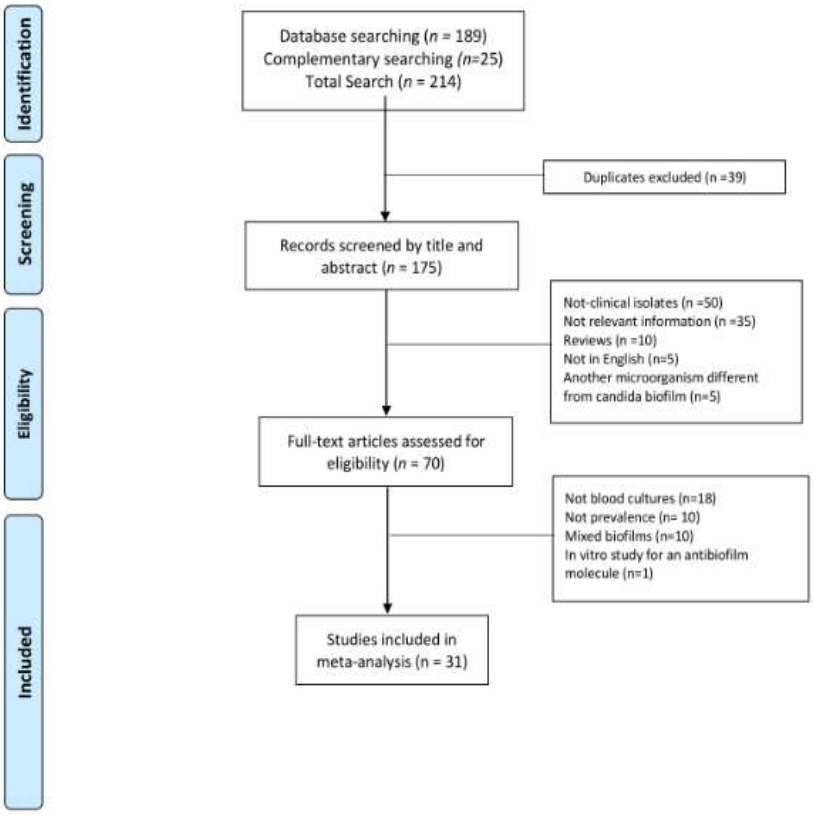

Figure I. 1 Prisma flow chart of included and excluded studies of the selection process.

As shown in Figure 1, a total data set of 31 studies was achieved for the present meta-analysis following the eligibility criteria, screening process, and quality assessment.

Overall effects of *Candida* biofilms

The data set reported biofilm rates of *Candida*-related infections among hospitalized patients between 1995 and 2020 in several countries worldwide. As shown in Table 1, the biofilm formation by *Candida* spp. isolates in the data set varied greatly from 16% to 100% in blood samples from hospitalized patients. Most of the data set belonged to studies realized in Europe (17/31), followed by Asia (9/31), South America (3/31), and North America (2/31).

Table I. 1 General information extracted from the data set selected for the present meta-analysis.

First author	Publication (year)	Region	Country	Biofilm formation, n (%)	Correlation between	Attributable mortality, n

				Methodology to measure biofilm	Biofilm rate, n (%)				biofilm and resistance	(%)
						High	Medium	Low		
Atalay	2015	Asia	Turkey	CV (450 nm)	8/50 (16)				No	
Tumbarello	2007	Europe	Italy	PBS (405 nm) & XTT (490 nm)	80/294 (27.2)				No	56 (70.0)
Tortonaro	2013	Europe	Italy	XTT (490 nm)	160/451 (35.4)	116 (72.5)		44 (27.5)	No	11 (6.9)
Banerjee	2015	Asia	India	Branchini's method	31/80 (38.8)				No	5 (16.1)
Tumbarello	2012	Europe	Italy	PBS (405 nm) & XTT (490 nm)	84/207 (40.6)				No	43 (51.2)
Pongracz	2016	Europe	Hungary	CV (570 nm) & XTT (490 nm)	43/93 (46.2)	12 (27.9)		31(72.1)	Yes	23 (53.49)
Sida	2015	Asia	India	Branchini's method	2/4 (50)				No	
Rodrigues	2019	South America	Brazil	Christensen's method	15/28 (53.8)				No	6 (40.0)
Gangneux	2018	Europe	France	BioFilm Ring Test	181/319 (56.7)	132 (72.9)		49 (27.1)	No	55 (30.4)
Shin	2002	Asia	Korea	DW (405 nm)	58/101 (57.4)				No	
Pannanusorn	2012	Europe	Sweden	XTT (590 nm)	231/393 (58.7)	101 (43.7)		130 (56.3)	No	
Tascini	2018	Europe	Italy	XTT (490 nm)	57/89 (64.0)				No	25 (43.9)
Tobudic	2011	Europe	Austria	CV (630 nm), PBS (405 nm) & XTT (620 nm)	34/47 (72.3)				No	18 (52.9)
Tulasidas	2018	Asia	India	CV (570 nm)	55/74 (74.3)				No	
Pfaller	1995	North America	USA	Branchini's method	13/17 (76.5)	3 (23.1)	6 (46.1)	4 (30.8)	No	
Pham	2019	Asia	Thailand	XTT (490 nm)	38/46 (76.4)	25 (65.8)		13 (34.2)	No	13 (34.2)
Guembe	2014	Europe	Spain	CV (550 nm)	45/54 (76.4)				No	
Kumar	2006	Asia	India	UPW (405 nm)	30/36 (83.3)				No	
Rajendran	2016	Europe	Scotland	CV (570 nm)	245/280 (87.7)	56 (22.9)	44 (17.9)	144 (58.9)	Yes	
Stojanovic	2015	Europe	Serbia	CV (595 nm)	7/8 (87.5)	2 (28.6)	3 (42.8)	2 (28.6)	Yes	
Turan	2018	Asia	Turkey	CV (540 nm)	145/162 (89.5)	37 (25.5)	61 (42.1)	47 (32.4)	Yes	

Tulyaprawat	2020	Asia	India	XTT (490 nm)	45/48 (93.8)	26 (57.8)		19 (42.2)	No	
Muñoz	2018	Europe	Spain	CV (540 nm)	280/280 (100.0)	90 (32.1)	190 (67.9)		No	95 (33.9)
Soldini	2017	Europe	Italy	CV (540 nm)	190/190 (100.0)	84 (44.2)	38 (20.0)	68 (35.8)	No	89 (46.8)
Vitális	2020	Europe	Hungary	CV (550 nm)	127/127 (100.0)	28 (22.0)	69 (54.4)	30 (23.6)	No	70 (55.1)
Prigitano	2013	Europe	Italy	XTT (490 nm)	297/297 (100.0)	96 (32.3)	141(47.5)	60 (20.2)	No	65 (21.9)
Treviño-Rangel	2018	North America	México	CV (595 nm)	89/89 (100.0)				No	32 (35.9)
Marcos-Zambrano	2017	Europe	Spain	CV (540 nm)	22/22 (100.0)		13 (59.1)	9 (40.9)	Yes	3 (13.6)
Marcos-Zambrano	2014	Europe	Spain	CV (540 nm)	564/564 (100.0)	194 (34.4)	187 (33.1)	181 (32.1)	No	
Thomaz	2019	South America	Brazil	CV (595 nm) & XTT (490 nm)	38/38 (100.0)	3 (7.9)		35 (92.1)	No	
Herek	2019	South America	Brazil	CV (570 nm)	13/13 (100.0)	3 (23.1)	7 (53.8)	3 (23.1)	No	

The prevalence of biofilm formation was calculated with 95% CI through random-model and significance level ≤0.05 (*p*-value). The sample size and prevalence were used to calculate the combined biofilm produced. Attribute mortality was calculated by the number of deaths among patients with biofilm in blood samples. The information summarized in the table did not show information on the patients' underlying diseases and resistance. The methodologies used to measure biofilm in the studies were based in the optical density (nm, i.e., wavelength in the assay) of the biomass from growth culture, more exactly: XTT - using micro plate reader with yellow tetrazolium salt; CV - using micro plate reader with crystal violet staining; UPW - using micro plate reader with ultra-pure water; DW - using microplate reader with distilled water; Branchini's method - evaluating the adherent growth of the biofilm's slime production; BioFilm Ring Test - using micro plate reader with a BioFilm Index (BFI) software; and, Christensen's method - evaluating the adherent growth of the biofilm in Falcon tube with safranin or trypan blue staining.

Although the methodologies to quantify biofilm biomass varied between studies, these methodologies are based on the optical density (OD) obtained by the combination of a certain colorimetric compound or a simple dissolution in a buffer or water with the growth of the isolated *Candida* sp. and then it's compared with reference *Candida* strains in the same growth conditions. The main methodologies in our study set were crystal violet (CV) assays using microplate reader (51.6%; 16/31), assays with tetrazolium dye (2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide, XTT) using micro plate reader (35.5%; 11/31), and Branchini's method (9.7%; 3/31). The Branchini's method, also called slime production method, is based on the production of a viscid slime layer by the growth of the *Candida* isolate in a tube containing Sabouraud broth [15].

Regardless of the applied methodology in the studies, all these authors were able to evaluate biofilm formation among *Candida* isolates. However, only 18 of 31 studies were able to categorize the

biofilm formation, and so just 5 studies were able to evaluate a positive correlation between biofilm presence and increment of antifungal resistance in the treatment. Finally, the incidence of mortality among patients varied considerably among studies, reporting the values of attributable mortality between 6.9 and 70%. All the information extracted is available in the supplementary section.

Analysis of the forest plot was then realized with data set, showing a pooled rate of biofilm formation of 80.0 % (CI: 67–90), as shown in Fig 2. The heterogeneity indices obtained using random effects model ($p < 0.001$) were Q = 2567.45 ($p < 0.001$), $I^2 = 98.83$, and $\square^2 = 0.150$. The pooled rate of biofilm formation obtained needs to be carefully analyzed given the high value of heterogeneity. This will be addressed in our discussion.

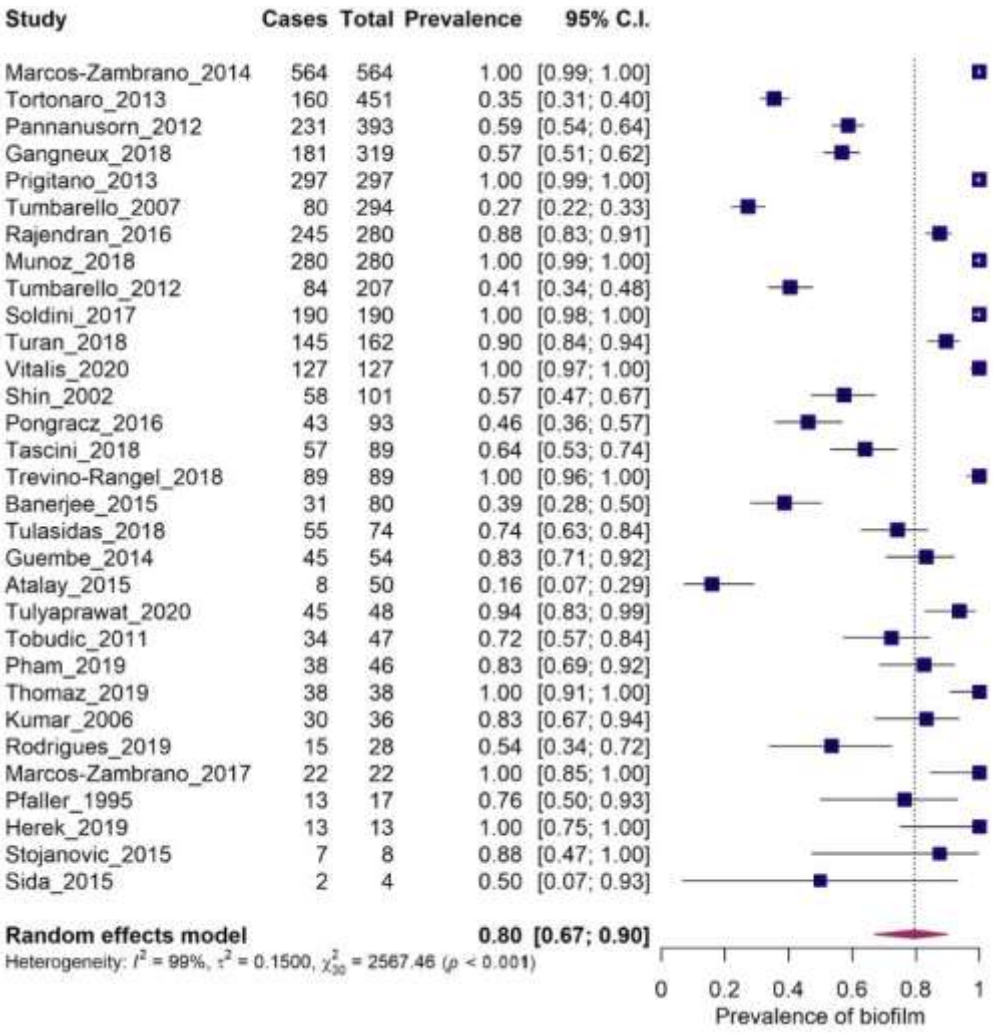

Figure I. 2 Forest plot of the meta-analysis of the prevalence of biofilm formation in *Candida* spp. isolated from blood clinical samples.

A funnel plot was realized to evaluate the existence of publication bias in the final data set (Fig 3). Furthermore, Egger's linear regression test was also used to reveal any publication bias and possible asymmetric data distribution in the funnel plot.

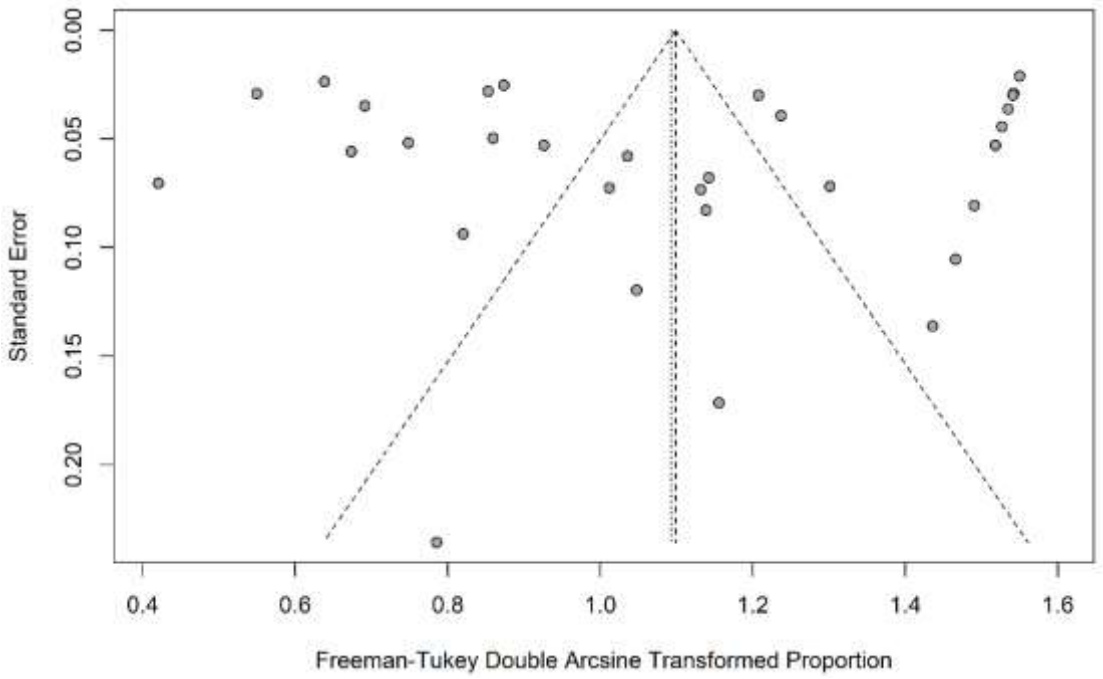

Figure I. 3 Funnel plot of the meta-analysis on the biofilm formation rate in *Candida* spp. isolated from blood clinical samples. Studies are represented by a point. The X-axis represents the effect size (biofilm prevalence), and the Y-axis shows the standard error. Despite some asymmetry revealed by the funnel plot in the data set, Egger's test failed to show publication bias ($p = 0.896$).

No publication bias was identified by the Egger's linear regression test ($p = 0.896$). However, as we will discuss in the next section the qualitative analysis of the funnel clearly suggests some biases from the departure of the geometry from the expected triangular form. The funnel plot of this study illustrates the effect size (biofilm prevalence) on the x-axis and the standard error (SE) on the y-axis. In case of no publication bias in the data set, the studies are distributed evenly around the pooled effect size. The smaller studies should appear near the bottom due to their higher variance when compared to the larger studies, which should be placed at the top of the plot. The diagonal lines show the expected

95% confidence intervals around the summary estimate. In the absence of heterogeneity, the studies of the data set should lie within the funnel defined by these diagonal lines. However, heterogeneity and some asymmetries among the studies of the data set were illustrated by the funnel plot. In our case, we found studies with low errors (similar sizes) but with drastic differences in the biofilm prevalence. This type of pattern probably indicates the presence of confounding variables (sub-groups undelaying structures) which are not included in the global analysis.

Although an obvious biofilm prevalence was found in the data set, the selected studies poorly described the underlying conditions of the patient with biofilm production. The analysis of these conditions among the patients was merely descriptive, as shown in Table 2.

1 Table I. 2 The reported clinical background of the patients with *Candida*-related bloodstream infections in the study set.

Study set	Total	Biofilm	Mortality	Mortality-related biofilm	Adult clinical conditions																								Pediatric clinical conditions			
					CA	IT	MV	CD	Neu	ND	CO	PD	GI	QMT	DI	AL	CRF	UC	CVC	RI	NGT	TPN	GAD	HIV	ANF	ANT	SC	ICU	PCVC	PVC	PB	LWB
Stojanovic et al., 2015	8	7	0	0	4	4	NR	NR	NR	NR	NR	NR	NR	NR	5	2	3	NR	6	NR	4	5	NR	NR	NR	6	4	6	NR	NR	NR	NR
Banerjee et al., 2015	80	31	16	5	11	NR	9	5	6	6	7	11	19	NR	17	16	13	27	58	28	NR	NR	NR	1	NR	42	9	NR	0	19	14	13
Guembe et al., 2014	54	45	0	0	16	NR	NR	6	NR	NR	NR	NR	6	NR	NR	NR	NR	NR	23	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	10
Pongracz et al., 2016	93	43	43	23	25	19	NR	NR	NR	NR	NR	NR	NR	NR	20	NR	NR	NR	NR	NR	NR	22	NR	11	NR	NR	51	NR	NR	NR	NR	NR
Vitalis et al., 2020	127	127	70	70	28	13	87	NR	NR	NR	NR	NR	NR	NR	41	NR	NR	NR	NR	NR	NR	68	NR	13	162	91	8	100	NR	NR	NR	NR
Kumar et al., 2006	36	30	0	0	35	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	NR	NR	NR	NR	NR	NR	NR	NR
Tumbarello et al., 2012	207	84	82	43	42	16	NR	NR	NR	NR	29	17	9	NR	NR	NR	21	NR	56	NR	27	58	NR	1	NR	75	38	NR	NR	NR	NR	NR
Tumbarello et al., 2007	294	80	154	56	88	82	NR	NR	10	NR	NR	NR	16	NR	NR	NR	NR	136	30	NR	NR	72	NR	NR	NR	NR	100	57	NR	NR	NR	NR
Marcos-Zambrano et al., 2017	22	22	0	0	21	13	NR	NR	4	NR	NR	NR	1	NR	76	NR	4	NR	19	NR	NR	13	NR	1	7	NR	4	2	NR	NR	NR	NR
Tortonaro et al., 2013	451	160	13	11	136	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	219	158	NR	NR	17	NR

Muñoz et al., 2018	280	280	0	95	151	22	50	91	18	70	78	59	NR	53	69	NR	61	NR	201	NR	NR	152	NR	6	62	253	136	28	NR	NR	NR	NR
Soldini et al., 2017	190	190	89	89	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	152	NR	NR	132	NR	NR	NR	177	NR	28	NR	NR	NR	NR
Tascini et al., 2018	89	57	42	25	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	47	80	NR	25	62	NR	NR	75	NR	35	NR	NR	NR	NR	NR
Treviño-Rangel et al., 2018	89	89	32	32	NR	NR	24	NR	NR	NR	13	NR	NR	7	NR	NR	NR	37	50	1	NR	30	NR	13	30	53	38	NR	NR	NR	4	NR
Shin et al., 2002	101	58	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	41	NR	NR	35	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Atalay et al., 2015	50	8	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	18	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Gangneux et al., 2018	319	181	105	55	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Herek et al., 2019	13	13	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Marcos-Zambrano et al., 2014	564	564	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Pannanusorn et al., 2012	393	231	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Pfaller et al., 1995	17	13	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Pham et al., 2019	46	38	23	13	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Prigitano et al., 2013	297	297	130	65	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

Rajendran et al., 2016	280	245	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	121	30	153	128	NR	NR	NR	118	NR	123	133	NR	119	NR	40	128	NR	NR	NR	NR	NR	NR	NR
Rodrigues et al., 2019	28	15	13	6	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Sida et al., 2015	4	2	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Thomaz et al., 2019	38	38	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Tobudic et al., 2011	47	34	25	18	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Tulasidas et al., 2018	74	55	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Tulyaprawat et al., 2020	48	45	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Turan et al., 2018	162	145	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

Legend - CA: malignancy; IT: Immunosuppressive Therapy; MV: Mechanical Ventilation; CD: Cardiovascular Disease; Neu: Neutropenia; ND: Neurological Disorders, CO: Corticoids; PD: Pulmonary Disorders; GI: Gastro Intestinal and Hepatically Disease; QMT: Chemotherapy; DI: Diabetes; AL: Alcoholism; CRF: Chronic Renal Failure; UC; Urinary Catheter; CVC: Central Venous Catheter; RI: Renal Insufficiency; NGT: Nasogastric Tube, TPN: Total Parenteral Nutrition; GAD: Genetic Autoimmune Disorders; HIV: Human Immunodeficiency Virus; ANF: Prior Antifungal Therapy; ANT: Prior Antibacterial Therapy; SC: Surgical conditions; ICU: Intensive Care Unit; PCVC: Pediatric Central Venus Catheter; PVC: Peripheric Venus Catheter; PB: Preterm Bird; LBW: Low Weight Bird; NR: Not Reported in the study.

The lack of a detail description of the clinical background and host factors in the patients among the studies represents a main drawback of the present meta-analysis precluding the evaluation of clinical or patient factors and the ability of *Candida* isolates to establish biofilm. Nonetheless, the ability to establish biofilm is a virulence factor by itself and should be evaluate as risk factor in the treatment of patients with *Candida*-related blood infections. As summarized in Table 2, only 16 of 31 studies reported some sort of clinical background of the patients with *Candida*-related bloodstream infections. From this subset of studies, patients evidenced mainly the following clinical conditions: hematological or solid cancer (68.8%, 11/16), surgery interventions (62.5%, 10/16); patients with central venous catheter (56.3%, 9/16); adults under total parenteral nutrition (50.0%, 8/16); patients with human immunodeficiency virus (HIV; 50.0%, and 8/16); patients with diabetes (43.8%; 7/16); patients in the intensive care unit (ICU; 37.5%, and 6/16); patients with immunosuppressive therapy (37.5%, 6/16) and, the remaining clinical backgrounds were only described in 25% or less of the studies in this subset, such as neutropenia (4/16), cardiovascular diseases (3/16), pulmonary diseases (3/16), urinary catheter (3/16), chemotherapy (2/16), and renal insufficiency (2/16). The heterogeneity of the clinical background of the patients and the gap of the host epidemiological factors in these studies excluded further analysis between *Candida*-related biofilm isolates and clinical history.

Mortality among patients with *Candida* biofilm

Further subgroup analysis using a random-effect model was realized to differentiate the *Candida*-related mortality rates between bloodstream infections with planktonic cells and biofilm formation. From the initial data set, only 15 studies evaluated the mortality among patients with *Candida*-related bloodstream infections. As shown in Table 3, the pooled mortality rate due to *Candida*-related bloodstream infections was 37.9% (95% CI: 26.2-50.2) of which the mortality associated with biofilm-forming infections was 70.0% (95% CI: 52.8–84.8).

Table I. 3 Pooled mortality rates in bloodstream infections due to *Candida* spp.

	k	*Mortality rate (95% CI) (%)*	*Random model*			
			Q	I^2	□	***p***
All Candida spp. bloodstream infections	*15*	37.9 (26.2 – 50.2)	493.82	97.2	0.237	< 0.0001
Biofilm-forming	*15*	70.0 (52.8 – 84.8)	345.47	95.9	0.331	< 0.0001

k, Number of studies; Q, I^2 and , Heterogeneity indexes; *p*, Random effect model significance level. Mortality rates were estimated within 30 days after diagnosis and confirmation of *Candida* spp. bloodstream infection. The studies considered (k = 15) were those in which a sample corresponded to an individual and reported deaths related to biofilm-formers strains.

In both scenarios, the mortality rate was statistically incremented among hospitalized patients ($p < 0.0001$). However, biofilm-related infections evidenced almost the double value of mortality rate in patients, when compared to all *Candida*-related bloodstream infections.

Geographical distribution of biofilm-forming *Candida* spp. isolates

The prevalence rate of biofilm-related infections significantly varied among studies of different countries and regions. Therefore, a subgroup analysis was realized between the biofilm formation rates and the geographical region to evaluate possible statistically significant differences (Table 4). Subgroup analysis evaluated the biofilm prevalence between regions and countries with a minimum of published studies, at least two and three studies per region and country, respectively. However, Egger's test was not applied due to the low number of studies in this analysis.

Table I. 4 Subgroup analysis for different geographical regions and countries.

Subgroups	**k**	**Prevalence (95% CI) (%)**	**Random model**			
			Q	**I^2**	□	***p****
Region						
Europe	17	81.0 (63.3 – 94.0)	2267.21	99.3	0.407	
Asia	9	67.9 (48.1 – 85.0)	171.49	95.3	0.283	0.4049
South America	3	91.6 (50.7 – 100.0)	31.83	93.7	0.387	

North America	2	94.0 (55.1 – 100.0)	12.94	92.3	0.319	
Country (≥3 studies)						
Italy	6	69.1 (32.0 – 95.8)	1095.33	99.5	0.471	0.0074
India	5	72.3 (46.2 – 92.7)	55.54	92.8	0.267	
Spain	4	98.9 (93.5 – 100.0)	33.85	91.1	0.126	
Brazil	3	91.6 (50.7 – 100.0)	31.83	93.7	0.387	

k, Number of studies; Q, I^2 and , Heterogeneity indexes; *p**, Significance level in subgroup analysis.

Although the biofilm prevalence varied among regions, there were no statistically significant differences ($p = 0.4049$). Europe reported a greater number of studies and showed an intermediate biofilm prevalence among *Candida* spp. infections. Meanwhile, when comparing prevalence rates between countries, a statistically significant value was obtained ($p = 0.0074$). In the pairwise comparison analyses, Spain was significantly superior to Brazil ($p < 0.0001$), Italy ($p = 0.0263$), and India ($p = 0.0030$).

Biofilm-forming capability in *Candida* spp. isolates

Candida spp. isolates vary in their ability to form biofilms, being usually categorized as low (LBF), intermediate (IBF), and high biofilm formers (HBF) according to biomass production (S1-3 Figs). Briefly, biofilm forming capacity was assessed using the crystal violet or XTT assays, measuring the biofilm mass. *Candida* isolates were cultured in 96-well plates at 37°C for 24 h and the biomass of each isolate was measured. Then, isolates were grouped based on their level of biomass, more exactly: low biofilm formers (LBF) showed a biomass production below the 1st quartile (Q_1; $Abs_{isolate} < 0.432$), intermediate biofilm formers (IBF) evidenced a biomass production in the 2nd quartile (Q_2; $0.432 < Abs_{isolate} < 1.07$), and high biofilm formers (HBF) demonstrated a biomass production higher the 1st quartile 3rd quartile (Q_3; $Abs_{isolate} > 1.07$), as previously described by Monfredini et al. [16] and Vitális et al. [17]. Eighteen studies reported this biofilm classification and so a subgroup analysis was realized (Table 5).

Table I. 5 Overall effects in subgroups based on biofilm-forming capability.

Biofilm-forming capability	k	Prevalence (95% CI) (%)	Egger's test	Random model			
			p	Q	I^2	□	*p**
High (HBF)	18	35.0 (26.6 – 43.9)	0.768	313.94	94.58	0.177	< 0.0001
Intermediate (IBF)	18	18.9 (7.8 – 33.1)	0.457	1074.52	98.42	0.334	< 0.0001
Low (LBF)	18	36.2 (24.7 – 48.5)	0.370	623.25	97.27	0.253	< 0.0001

k, Number of studies; Q, I^2 and □□, Heterogeneity indexes; *p**, Random effect model significance level in subgroup analysis. The selected studies (k=18) categorized the strains according to their biofilm-forming capability using only methods based on biomass quantification through spectrophotometric measures.

Statistically significant differences were found among *Candida* isolates according to their biofilm-forming capability ($p < 0.0001$), evidencing a low number of *Candida* isolates related to intermediate biofilms. No publication bias was detected in both subgroups according to Egger's linear regression test.

Evaluation of biofilm formation between different *Candida* species

Although *Candida* spp. isolates vary in their ability to form biofilms, little is known about this biofilm-forming ability among *Candida* species. Each category of biofilm was further evaluated among *Candida* species to evaluate the most virulent *Candida* species (S1 Table). When analyzing HBF (Table 6), *C. tropicalis* was the most prevalent HBF overpassing *C. albicans* and *C. parapsilosis* by a factor of 2. More precisely, the HBF prevalence of *C. tropicalis* was the highest showing statistically significant differences with the other *Candida* species, except for *C. krusei* ($p = 0.5477$) and *C. glabrata* ($p = 0.0896$).

Table I. 6 Subgroup analysis between different *Candida* species.

Species	k	BF strains (n)	Prevalence of HBF % (95% CI)	Random model			
				Q	I^2	□	*p**
C. albicans	22	1461	30.3 (20.5-41.0)	225.66	95.6	0.173	0.0454[a]

non-*albicans* *Candida* species	26	1868	43.6 (34.5- 52.9)	306.69	87.6	0.230	
C. albicans	22	1461	30.3 (20.5-41.0)	225.66	95.6	0.173	
C. glabrata	17	387	37.6 (0.1 – 71.0)	95.0	95.8	0.325	
C. tropicalis	17	331	67.5 (58.3 – 76.3)	11.71	31.7	0.069	
C. parapsilosis	20	744	29.6 (20.3 – 39.9)	69.9	84.3	0.154	
C. krusei	10	68	52.8 (0.1 – 94.9)	30.12	83.4	0.409	< 0.0001[b]
**** Other species**	20	338	40.7 (26.5 – 55.6)	22.49	60.0	0.139	

k, Number of studies; Q, I^2 and □ □, Heterogeneity indexes; *p**, Random effect model significance level in subgroup analysis.

[a] Comparison between *C. albicans* and non-*albicans Candida* species. [b] Comparison between all *Candida* species

** Other species includes *C. dublinensis* (n=12), *C. quilliermondi* (n=25), *C. lusitaniae* (n=10), *C. haemulonii* (n=4), *C. lypolitica* (n=1), *C. pelliculosa* (n=1) and unreported species (n=285).

In order to comprehend how these two major factors: countries and *Candida* species could actually explain the high heterogeneity showed in our data, we carried out a meta-regression analysis. The inclusion of both variables as interacting variables in a multiplicative model (R^2 = 59.13%, p < 0.0001) explained more than an additive model (R^2 = 43.48%, p < 0.0001), regarding the prevalence of biofilm formation.

Evaluation of antifungal resistance pattern among *Candida* isolates

Multiple antifungal resistance among candidiasis has become a serious public health issue, leading to clinical complications and expensive costs. A subgroup analysis based on antifungal resistance was also realized among our study set. Due to the different methodologies used to test susceptibility, the number of studies not enough to analyze statistically antifungal resistance rates between *Candida* species. As shown in Table 7, the rates of antifungal resistance to fluconazole, voriconazole, and caspofungin related to biofilm-forming strains were 70.5, 67.9, and 72.8%, respectively.

Table I. 7 Summary of subgroup analysis for antifungal resistance in *Candida* spp. isolates.

Studies	*k*	Antifungal resistance rate % (95% CI)		
		Fluconazole	**Voriconazole**	**Caspofungin**
Mixed/Planktonic cells	*3*	15.1 (0.7-41.2)	1.6 (0.1-4.4)	3.1 (0.0 – 20.76)
Biofilm-forming strains	*2*	70.5 (54.6-84.5)	67.9 (51.8-82.3)	72.8 (55.1 – 87.8)
Cochran's Q*		11.68	85.15	22.88
p-value**		0.0006	< 0.0001	< 0.0001
Not reported/ Other methods	*26*	-	-	-

k, Number of studies; Q*, Test of heterogeneity between groups; *p***, Random effect model significance level in subgroup analysis. Subgroup analysis based on antifungal resistance contains k = 5 studies. Egger's test may lack the statistical power to detect bias when the number of studies is small (i.e., k < 10).

When comparing to planktonic cells, all *Candida*-related biofilm isolates showed a statistical increment of resistance against the three antifungals evaluated in the study ($p < 0.001$).

Discussion

The present study evaluated a possible relationship between *Candida*-related biofilm formation, bloodstream infections, and mortality among hospitalized patients. Invasive mycoses are responsible every year for more than two million infections worldwide and for, at least, as many deaths as tuberculosis or malaria. Candidiasis, aspergillosis, cryptococcosis, and pneumocystosis cause more than 90% of reported deaths associated with invasive mycoses [18]. Among them, the most frequent mycosis is invasive candidiasis causing high morbidity in critically ill patients [19].

Overall effects of *Candida* biofilms in infections and mortality

As previously referred, around 70.0% of candidemia reports were caused by biofilm-forming strains. However, its biofilm formation was less than in isolates from urogenital infections [20–23] and even respiratory tract infections [22,23]. Still, the rate of candidemia-associated biofilm infections was higher than oral-related biofilm infections [24] and more than invasive infections [25]. These findings

are in agreement with the Institute of Health in the United States, which estimates that biofilms are responsible, in one way or another, for over 80% of all microbial infections [12]. Yet, the reports of *Candida*-associated biofilm infections varied greatly between published studies possibly due to the lack of differentiation between *Candida* species, the experience of the researchers, the number of *Candida* isolates in the study set, and the diversity of biofilm detection and quantification methodologies and its subsequent classification within the study set, such as crystal violet assay, biomass measure, XTT reduction assay, and microtiter plate method [8,12].

Another issue concerns the lack of differentiation between planktonic and biofilm-related *Candida* infections in the diagnosis of the clinical laboratories at public health system [19,26]. The traditional clinical microbiology laboratories have focused on testing planktonically isolated microorganisms and reporting the susceptibility to various antimicrobials under planktonic growth conditions [27]. While the authors from the studies of this meta-analysis applied a further analysis by evaluating the ability of biofilm production in *Candida* isolates through an *in vitro* biofilm assay. In *Candida* biofilms, traditional techniques require device removal followed by culture or microscopy of a catheter segment, while catheter-sparing diagnostic tests include paired quantitative blood cultures. However, as previously indicated by Høiby et al. (2015) and Bouza et al. (2013), the number of positive peripheral blood cultures also seems to be a promising diagnostic tool to diagnose catheter-related candidemia without directly removing the catheter [27,28]. Therefore, an implementation of a new gold standard methodology is vital to a better characterization of microbial-associated infections avoiding unproductive treatments among hospitalized patients. The mortality rate caused by biofilm formation in *Candida*-related infections was almost double when compared to planktonic infections. Other studies already stated the burden of invasive candidiasis and its severe outcomes [1,29], indicating biofilm formation and antifungal resistance as main risk factors among patients. Moreover, we report a pooled attributable mortality of 37.9% to *Candida*-related bloodstream infection with planktonic cells, which is in agreement with previous reports [1,18,30,31]. These studies reported a

mortality range between 25 and 40%, showing a higher mortality incidence among ICU or burn patients, and immunocompromised patients [32]. While the mortality associated with biofilm-forming strains was 70.0% in *Candida*-related bloodstream infections. However, this correlation has been debated by several authors [10,16,33,34], reporting different mortality rates (25 - 70%).

It is also important to mention that the ability to quicky proliferate and to establish biofilm is not exclusively dependent of the type of *Candida* species and even strains in a blood-related infection, but it is also influenced by their interaction with host homeostasis and variations (mucosal pH shifts or nutritional changes), previous use of antibiotics, and immune system alterations (such as secondary effect of stress or immunosuppressant therapy) [35].

The I^2 observed in the forest plot indicate a high heterogenic data. The I^2 is a measurement of the heterogeneity that is not caused by variations in the sample size considered in each study. Therefore, this high value and also the geometry of the funnel plot indicates the possibility of major sources of variation across the studies. Some of the sources of variations can clearly be related with the differences previously described (i.e., methodology, *Candida* species, etc.) and consequently the pooled effect around the 80% need to be considered with caution. Several factors can be modulating this pooled effect leading to higher and/or lower values. In this context, the present meta-analysis was unable to study any correlations between clinical or epidemiological factors and mortality in patients with biofilm-related blood infections. These heterogeneity and gaps on the selected studies constitute the main drawback of our study. However, it is also well-known that the ability to establish biofilms among *Candida* species is an important virulence factor contributing to a more severe infection in patients [36] and it is worth to be studied. The observed heterogeneity was the leading cause to consider the effect of several variables like geographical distribution and *Candida* species. However, the missing information in the consulted scientific literature can be an important source of unexplained variation.

Geographical distribution of *Candida* biofilm-related infections

World incidence of invasive candidiasis is difficult to estimate because the criteria used for diagnosing and categorizing invasive candidiasis are quite different [6,8,9]. Also, most studies restricted many factors in their group set, such as the range age of patients and their health status. The present meta-analysis recollected data from diverse study sets demonstrating the *Candida*-related biofilm infections as a main nosocomial infection, but only 16 of 31 studies partially reported the clinical background of the patients (Table 2), such as patients suffering from immunodeficiency, receiving organ transplantations, under major surgery, or treated with cancer chemotherapy and different primary hospitalizations, and no epidemiological factors were available. Only a study realized in a tertiary care hospital of southern India reported the clinical backgrounds in adult and pediatric patients [37], evidencing central venous catheter and low weight at birth as the most prevalent risk factors in these population sets, respectively.

Generally, the number of patients in surveillance studies is very low and there are many gaps in our knowledge on the true epidemiology of invasive candidiasis in many regions of the world [19]. As expected, around 55% of our data set belonged to European studies (17/31), where the rate of biofilm-related infections varied greatly among countries showing Spain with statistical differences in the incidence of *Candida*-related biofilm infections in hospitalized patients in comparison with other countries. However, Cesta and colleagues recently reported Italy as the one region with a higher number of deaths caused by antibiotic-resistant bacteria and biofilm-related infections [38]. Due to European Centre for Disease Prevention and Control (ECDC) reported a spread of multi-drug resistant strains (MDR) in Italy, in particular of the bacterial species of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Acinetobacter baumannii* [38], it is plausible that the *Candida*-related biofilm incidence among hospitalized patients in Italy had been underrated. Likewise, only two and three studies in our data set belong to North and South America, respectively. All three studies of South America were indeed from Brazil, demonstrating one of the highest *Candida*-related biofilm incidences

among hospitalized patients (91.6%). However, no further information was available in the remaining Latin-American countries with the criteria selection of the present meta-analysis.

We can notice in the meta-analysis that the values of I^2, Q and other indicators also suggest a high heterogeneity within each group. It is an indicator that other factors can be involved. For example, if we consider only the articles from Italy, we can notice that the sample size in 5 of 6 studies do not considerably differ but the effect size is quite different (this will impact directly in the funnel plot geometry as presented in Fig 3). In three studies, we found a low prevalence of biofilm formation [33,39,40] while in other two articles we found a high prevalence of biofilm formation [41,42]. This distribution suggests that factors quite beyond the geography are possible causes of heterogeneity within groups.

Association between different *Candida* species in biofilm and infections

The number of *Candida* species with clinical importance in humans is relatively small, more exactly, *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis,* and *Candida dubliniensis* [43]. *C. albicans* is the most reported *Candida* species worldwide in different ethnic populations [34,44–47], being responsible for the majority of oral and systemic candidiasis cases. However, there has been an increase in the number of reports about non-*albicans Candida* infection in the last years and even surpassing *C. albicans* in terms of incidence and attributable mortality [25,31,34,42,48–51]. This new scenario could be attributed to the implementation of better molecular techniques in the identification of *Candida* species [21,29,52].

Our results demonstrated *C. tropicalis* as the most prevalent HBF evidencing statistical dominance among *Candida* species. Although *C. tropicalis* is described as a species with normal to high biofilm-forming capacity [36], it is commonly related to infections in prosthetic joints, endodontic issues, ulcerative colitis [53–55]. *C. tropicalis* biofilm is characterized by chains of cells with thin, but large, amounts of extracellular matrix material with low sums of carbohydrate and protein [36,40].

Furthermore, Silva and colleagues showed that matrix material extracted from biofilms of *C. tropicalis* and *C. albicans* contained carbohydrates, proteins, hexosamine, phosphorus and uronic acid [55]. However, hexosamine was the major component quantified in *C. tropicalis* biofilm (27%). *C. tropicalis* biofilms are described as a dense network of yeast cells with evident different filamentous morphologies [36].

After *C. tropicalis*, the present meta-analysis showed *C. krusei* and *C. glabrata* as the second and third most prevalent HBF among *Candida* species, more exactly, 52.8 and 37.6%, respectively. *C. krusei* is characterized by a thick multilayered biofilm of pseudohyphal forms embedded within the polymer matrix [56], being categorized with a high ability to establish biofilm [36]. Several mucosal infections and pneumonia are caused by *C. krusei* [23,56]. Although *C. glabrata* is known to develop less biofilm, it is characterized to produce high content of both protein and carbohydrate [40,57]. *C. glabrata* is commonly associated with infections among patients with total parenteral nutrition, periodontal disease, ventilator-associated and non-healing surgical wounds [58]. *C. glabrata* biofilms are structured on multilayers of blastospores with high cohesion among them [55]. The elucidation of these biofilm-forming abilities and properties among *Candida* species could provide a promising step toward the improvement of treatments.

Until this point, we have showed that *Candida* species and geographical distribution can be related with our data heterogeneity. The actual combination of both variables in a multiple meta-regression model as interacting variables explained more than the 50% of the global variability. The lack of clinical information and many other discussed variables are probably related, at least partially,

with the remained variability. Unfortunately, as previously explained, this information is not accessible for most of the studies and constitute by itself a recommendation in further studies.

Antifungal resistance among *Candida*-related biofilm infections

Candida spp. infections had successfully become more difficult to treat in the last decade due to the growth of immunogenic diseases, the disproportionate use of immunosuppressive drugs, malnutrition, endocrine disorders, the widespread use of indwelling medical devices, broad-spectrum antibiotics, aging, and an increase of the number of patients among the population [36,59]. Thus, the morbidity and mortality associated with candidiasis are still very high, even using the actual antifungal drugs [59]. The main antifungal drugs applied to *Candida* infections are azoles, polyenes, and echinocandins [60]. Briefly, azoles (such as fluconazole and voriconazole) block ergosterol synthesis by targeting the enzyme lanosterol 14α-demethylase and leading to an accumulation of toxic sterol pathway intermediates. While echinocandins (such as caspofungin) aim for the synthesis of 1,3-β-glucan (a cell wall component), being the ideal antifungal drug of choice in severe cases of candidemia [61,62]. As previously referred, the rates of antifungal resistance to fluconazole, caspofungin, and voriconazole in biofilm cells surpassed planktonic cells by a factor of 4.7, 23.5, and 42.4, respectively. Despite the number of studies comparing resistance between planktonic and biofilm cells among *Candida* species is still scarce, these results are in agreement with the literature postulations [36,63]. Numerous reasons are attributed to this enormous resistance against antifungal drugs in *Candida*-related biofilms, such as high cell density, growth rate reduction, nutrient limitation, matrix extracellular production, presence of persister (dormant and non-dividing) cells, phenotypic shift, and high sterols content on membrane cell [36,59,63]. So, the treatment for *Candida*-biofilm infections requires a comprehensive knowledge of the complex mechanisms underlying the interaction between a biofilm and its host.

Although no efficient treatment for *Candida* biofilms has been found yet, several promising strategies are being explored. New therapeutic targets, such as the genes involved in biofilm development and the quorum-sensing systems, are considered an alternative treatment to the currently antifungal drugs.

Conclusions

In summary, several studies on the prevalence of *Candida* biofilms in bloodstream infections have been published across the world, allowing some conclusions on its mortality, species, and virulence in different geographic regions. However, a lot of information is missing, such as the lack of a thorough clinical background from the patients and the diversity of the primary infections from the patients. Further studies are needed to close gaps in our understanding of the incidence of *Candida* biofilms and to monitor trends in antifungal resistance and species shifts.

To the authors' best knowledge, this meta-analysis is one of the few that explored the association of biofilm production among different *Candida* species in bloodstream infections [64–67], using data published worldwide and adhering to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guideline. Although the present meta-analysis was performed methodically, there are some limitations of this study: (1) heterogeneity exists in some subgroup and overall analyses; (2) relationship between mortality and each *Candida*-related biofilm species could not be assessed; and, (3) a detailed analysis of antifungal resistance in *Candida* biofilms was not possible. These limitations are due to a lack of sufficient published data. Therefore, early detection of biofilms and a better characterization of *Candida* spp. bloodstream infections should be considered, which eventually will help preserve public health resources and ultimately diminish mortality among patients.

Materials and Methods

Data selection, search strategy, and study guidelines

This study was conducted following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) strategies (S1 File) [68]. Web of Science, Scopus, PubMed, and Google Scholar databases were searched for English papers using the following medical subject heading terms (MESH): "invasive candidiasis"; "bloodstream infections"; "biofilm formation"; "biofilm-related infections"; "mortality"; and, "prevalence".

In each electronic database, a combination of MESH terms was used to conduct the search applying the following strategy (in the MEDLINE for example): ''(*Candida*) AND (biofilm [Title/Abstract]) AND (mortality).'' All studies published until 30th July of 2020 were retrieved. The articles reporting the prevalence of bloodstream infections biofilm-related, the mortality rates, and the species identification of *Candida* isolates were included. The references of these articles were also checked for finding additional records. The data selection was limited to human clinical isolates and studies in English. All references were compiled into a database Zotero Library and then managed using Excel.

Screening process

Duplicates were initially identified and eliminated in Zotero after entering all the recognized studies into an Excel self-created database (S2 File). All articles were assessed by two reviewers (MBA-C and FSC-M) by screening titles, abstracts, topics, and finally full texts. At each level, the reviewers independently screened the articles and finally merged their conclusions. An additional examination of the selected articles was realized by a third author (AM) focused on the homogeneity of the eligibility criteria of previous reviewers in the initial data set. Discrepancies were resolved by

discussion before finalizing the records for the evaluation of eligibility criteria. In case of disagreements, the third assessor (AM) was assigned to make a final decision.

Eligibility criteria

The major inclusion criteria included reporting the rate of biofilm formation and the prevalence of biofilm-related to *Candida* species, including observational studies (more exactly, cohort, retrospective, and case-control studies). Furthermore, data regarding the mortality rate, the geographical location of the study set, and the use of anti-fungal agents in clinical isolates were also extracted from the studies.

All studies without information about biofilm formation or clinical *Candida* isolates were consequently excluded. The method to quantify biofilm biomass was not a criterion to include or exclude any paper in this meta-analysis. Concerning antifungal resistance rate, only studies that used the standard susceptibility tests according to the Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing EUCAST were selected for the present study.

Reviews, editorials, congress or meeting abstracts, literature in languages other than English, case reports, and letters to editors were excluded from the final data set. Finally, articles without full text available, duplicate reports on different databases, and studies with unclear or missing data were also omitted.

Data extraction and quality assessment

Methodological quality assessment of the studies was performed using a checklist for necessary items as outlined in the Critical Appraisal Skills Programmed checklists [69]. For each article, a series of critical questions were asked. If the pertinent data were presented, the question was scored ‘‘yes.’’ If there was any doubt or no information in the study, that question was marked as ‘‘no’’. A data

extraction form was designed to extract the relevant characteristics of each study (S1 and S2 Files). The extracted information included the first authors' names, time of the study, year of publication, location, sample size, biofilm formation rate, *Candida* species and its categorization (as *C. albicans* and non-*albicans Candida* species), the correlation between biofilm formation and antifungal resistance, and the type of biofilm. The type of biofilm was categorized as low biofilm formers (LBF), intermediate biofilm formers (IBF), and high biofilm formers (HBF). The initial two authors (MBA-C and FSC-M) extracted all data, further confirmation and final evaluation were realized by the lead authors (AM and ET).

Data analysis and statistical methods

Meta-analysis was performed using several R packages ("meta" [70], "metafor" [71], "poibin" [72], and "stringr" [73]) of R version 3.4.3 [74] and RStudio version 1.3.1073 [75] (S3 File). The rate of biofilm formation was computed, and values were reported with confidence intervals (CI) of 95%. The heterogeneity was assessed by the Cochrane Q and I^2 tests. The I^2 metric indicates the amount of heterogeneity that is not related with sampling size variation. Moreover, it is also independent of the number of studies included in the meta-analysis (in contrast to the Cochrane Q metric). Considering the heterogeneity indices, the random-effects model was then used for meta-analysis of the selected studies, and the Freeman-Tukey transformation was also applied to calculate the pooled frequencies. To estimate the between-study variance in a random-effects model we use tau-squared, and its square root is the estimated standard deviation of underlying effects across studies. Subgroup analyses were conducted based on the type of biofilm, biofilm-related species, geographical regions, and antifungal resistance rates. Outliers' analysis was done with the Baujat diagram, while quantitative Egger weighted regression test and Funnel plot were used to evaluate the eventual existence of publication bias. In statistical analysis, p-values <0.05 were considered as significant statistical results. We used the multiple meta-regression analysis with the "metareg" function from "meta" to explore the

contribution to model heterogeneity of several variables. In this approach, the maximum-likelihood method was used.

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ORIGINAL ARTICLE

EVALUATION OF THE BIOFILM CYCLE LIFE BETWEEN *CANDIDA ALBICANS* AND *CANDIDA TROPICALIS.*

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Abstract: *Candida tropicalis* is an emergent pathogen with a high rate of mortality associated to its biofilm formation. Biofilm formation has important repercussions to public health system. However, little is still known about its biofilm cycle life. The present study analyzed the biofilm cycle life of *Candida albicans* and *Candida tropicalis* during time (24, 48, 72 and 96h) though biomass assays, colony-forming unit (CFU) counting, and epifluorescence and scanning electron microscopies. Our results showed a significant difference between *C. albicans* and *C. tropicalis* biofilms in each biomass and viability assays (Friedman test $p< 0.05$). All-time samples in the biomass and viability assays confirmed statistical differences between *Candida* species through pairwise Wilcoxon tests ($p< 0.05$). *C. albicans* demonstrated a lower biomass growth but reaching nearly the same level of *C. tropicalis* biomass at 96h; while, CFU counting assays exhibited a superior number of viable cells within the *C. tropicalis* biofilm. Statistical differences were also found between *C. albicans* and *C. tropicalis* biofilms from 48 and 72h microscopies, demonstrating *C. tropicalis* with a higher number of total cells within biofilms and *C. albicans*' cells with a superior cell area and higher matrix production. This study proved the higher biofilm production of *C. tropicalis*.

Keywords: biofilms; cycle life; *Candida albicans*; *Candida tropicalis*

1. Introduction

Candida species are widely distributed in nature, normally as a part of commensal mammalian microbiota [1]. However, alterations in the host environment including disruptions on commensal microbiota might trigger the transition from the commensal to a pathogenic phase [2]. In last decades, fungal infections in humans are becoming an emergent problem to public health system and considered by many authors as a neglected infectious disease [3–5]. Nowadays more than 200 species of *Candida* have been described [2]. Although *Candida albicans* still remains as the most prevalent fungal pathogen, the morbidity and mortality caused by non-*albicans Candida* (NAC) species are increasing [2]. Besides *C. albicans*, there are four emerging NAC species, more exactly, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, and *Candida krusei* [2]. Among these, *C. tropicalis* is now considered the most important emerging fungal pathogen, and recent reports have identified several strains resistant to standard empirical treatments, such as fluconazole [6].

Both *Candida albicans* and *Candida tropicalis* are known to possess a broad range of virulence factors and commensal characteristics conferring the ability to colonize and invade host tissue [7]. These factors include the expression of adhesins and invasins on the cell surface, ability to damage host cells, thigmotropism (contact sensing), phenotypic switching, secretion of hydrolytic enzymes, and formation of biofilms [7]. Although it is well-known that biofilms represent the most prevalent growth form of microorganisms [8,9] and biofilm formation among *Candida* species confers significant resistance to antifungal therapy [10,11], little is still known about the biofilm cycle life of *C. tropicalis*. In 2017, Kawai and colleagues [12] evaluated the *C. tropicalis* biofilm formation and the antifungal efficacy of liposomal amphotericin B using time-lapse imaging, showing C. *tropicalis* as a fast-growing type and forming aggressive biofilms. However, Kawai and colleagues only analyzed *C. tropicalis* biofilms during 24h [12].

The ability to establish biofilm is considered a main virulence factor among pathogens, limiting the penetration of substances through the matrix and protecting cells from host immune responses [10,11]. In addition, mature biofilms are able to evade the host immune system [13,14]. It is assumed that the formation of mature biofilms and subsequent production of extracellular matrix is strongly dependent on species, strain and environmental conditions (such as pH, medium composition, oxygen, among others) [15,16]. Therefore, it is important to evaluate the biofilm cycle life among pathogens, such as *C. albicans* and *C. tropicalis*.

Our recent meta-analysis on the prevalence of *Candida* biofilms in bloodstream infections showed that 70.0% of the mortality rate were from biofilm-associated infections [17], evidencing *C. tropicalis* as the prevalent species and overpassing *C. albicans* in these infections. So, the present work aimed to compare the biofilm cycle of life between *C. albicans* and *C. tropicalis* and characterize their biofilm production in *in vitro* conditions. This study analyzed biofilms of these *Candida* species during time (24, 48, 72, and 96h) by biomass growth assays (optical density measurement at 630 nm using crystal violet staining and PBS suspension), colony-forming unit (CFU) counting, epifluorescence microscopy (EM), and scanning electron microscopy (EM).

2. Materials and Methods

2.1 Fungal Isolates and Growth Conditions

Two *Candida* species, *C. albicans* of the American Type Culture Collection ATCC® 10231™ [18], and *C. tropicalis* isolate from the microbial collection of the Institute of Microbiology Universidad San Francisco de Quito (designated as IMUSFQ-V546), were selected for the present study. *C. tropicalis* isolate IMUSFQ-V546 was previously recovered from a patient with invasive candidiasis and identified through DNA sequences at multiple loci and biochemical properties in the National Institute for Research in Public Health (INSPI) [19]. Strains were stored at -80°C and at -20 °C, 24 hours previously each assay a new culture in Sabouraud dextrose agar (SDA; Dipco Cía. Ltda., Quito, Ecuador) was made to avoid natural mutants due to multiple passages [20]. After 24h at 37 °C of growth, yeast cells were harvested and suspended in phosphate-buffered saline (PBS) to obtain cellular density using a UV-Vis spectrometer (GENESYS™ 20 Thermo

Scientific™, Waltham, Massachusetts, USA). At 540 nm, cellular density was adjusted at 1×10^7 colony forming units (CFU) per ml. Cellular density was obtained with a growth curve of the two strains [21,22] (see Supplemental Material).

2.2 Biofilm Formation

The appropriate inoculum in PBS was centrifugate 400 rpm, 10 minutes and the pellet resuspended in sterile Sabouraud dextrose broth (SDB; Dipco Cía. Ltda., Quito, Ecuador), to each well of the 6-well plate containing a sterile coverslip, add 3 mL of primary biofilm inoculum ($1x10^7$ CFU per mL), blank control were prepared in the same plate, which also contained a cover slip but placed in sterile medium [22]. Plates were wrapped in plastic film to avoid evaporation and taken to incubator 37 °C, the biofilms were washed, and media was changed every 24 four hours until complete the period of growth (24, 48, 72, and 96 hours) [23]. Each assay was performed with at least three replicates per strain, and growth period. Also, in each replicate assay, we prepared two samples of biofilm by strain to perform the biomass assays separately.

2.3 Biomass Quantification

To screen the strain's capability to form biofilm, we used an optical density assay with crystal violet (CV) staining and phosphate-buffered saline (PBS) suspension using a modified version of the method suggested by Gulati et al. [24]. Briefly, each optical density assay is described below.

2.3.1 Crystal Violet Staining

After a certain period of growth, the biofilm samples were carefully washed with 3 mL of sterile PBS removing the remaining growth medium and the planktonic cells in each well. Then, the coverslips containing the biofilm sample were translated to a clean 6-well plate and stained with 3 mL of crystal violet 1% (v/v) for 45 minutes and the excess of staining was carefully removed from the wells. 3 mL of alcohol 96% (v/v) was placed into each well for 5 minutes and, finally, 200 µL of the biofilm sample was placed in a 96-well plate and read in the ELISA Elx808 spectrophotometer (BioTek, Winooski, USA) at 630 nm. All biofilm samples,

the blank controls, and also a well with pure alcohol was included in the lectures of the 96-well plate, measuring at least two times [24].

2.3.2 Phosphate-Buffered Saline Suspension

After the biofilm formation assays, the second set of biofilm samples were also carefully washed with 3 mL of sterile phosphate-buffered saline (PBS) to remove the growth medium and the planktonic cells. Then each coverslip containing the biofilm sample was placed in a sterile plastic flask with 3 ml of sterile PBS, and vortexed at maximum velocity for five minutes to ensure the biofilm remotion of the coverslip into the PBS solution [24]. For each sample, 200 µL of the previous suspension was placed in 96 well plate and read in the ELISA Elx808 spectrophotometer at 630 nm. Likewise, all biofilm samples, the blank controls, and sterile PBS were measured at least two times [24]. The remaining PBS suspension was used for viability quantification assays.

2.4 Viability Quantification

To evaluate the viability of biofilm samples in different times of growth, we applied two methodologies, more exactly: cell enumeration using colony forming units (CFU), and dead-alive fluorescent microscopy assays.

2.4.1 Colony forming Units Counting

To enumerate culturable sessile cells, colony-forming unit (CFU) counting assay was used. At least five individual PBS suspensions of each biofilm sample were used in a serial tenfold dilution, by adding 100 µL of sample in 900 µL of sterile PBS. Each dilution was thoroughly vortexed and pipette tips were changed before the next dilution or experimental step. Dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated on SDA by triplicated resulting in 15 plates per biofilm sample, plates were incubated for 24 h at 37°C, after which colonies were counted [25]. The experiments were performed at the same time as biomass experiments; thus, three CFU assays by each dilution were available for analysis, and data was collected. For statistical analysis, the dilution with a growth between 25 and 250 CFU was chosen according to previous studies [26,27].

2.4.2 Fluorescence Staining

After the evaluation of CFU counting and biomass quantification assays, 48 and 72h time samples were chosen to be analyzed by fluorescence staining. After each biofilm formation in 6-well plates, any remaining broth in the wells was removed and tree coverslips were transferred to a fresh 6-well plate. A working solution of fluorescent stains was prepared by adding 10 µL of SYTO® 9 stain and 10 µL of propidium iodide stain (FilmTracer™ LIVE/DEAD® Biofilm Viability Kit, Invitrogen, Carlsbad, California, USA) to 10 mL of filter-sterilized water in a foil-covered container (dead-alive working solution). In addition, another working solution was prepared using 20 µL of 4′,6-diamidino-2-phenylindole stain (DAPI, Sigma Aldrich #10236276001, St. Louis, Missouri, USA) in 10 mL of filter-sterilized water in a foil-covered container (DAPI working solution). These two working solutions were stored at -20°C. About 200 µL of dead-alive working solution was added onto each coverslip (biofilm sample), gently so as not to disturb the biofilm. Samples were incubated for 30 min at room temperature, protected from light, before being rinsed with 200 µL of PBS. Then, 200 µL of DAPI working solution was also added in the previous biofilm sample and incubated for 10 min at room temperature, protected from light, and finally washed again with 200 µL of PBS. Each coverslip was then placed face up onto a clean, dry microscope slide and a drop of mounting medium added (ProLong Gold Antifade, ThermoFisher Scientific, Massachusetts, USA). An autoclavable 22-mm diameter glass coverslip (Dipco Cía. Ltda., Quito, Ecuador) was used to fix the sample in place. Samples were stored protected from light at room temperature (25 °C) until epifluorescence microscopy was realized within the first hour [28,29].

2.5 Epifluorescence Microscopy

Epifluorescence microscopy (EM) was carried out using Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan) equipped with 100x oil immersion objective. Images were captured with AmScope Digital Camera MU633-FL (AmScope, California, USA) and digitalized with AmScope software version 1.2.2.10. As previously described by Rosenberg and colleagues [28], for counting purposes at least 12 images were taken per sample on the 22-mm diameter glass coverslip at random locations. For more reproducible result presentation, cell/yeast counts are given per cm^2. Briefly, the number of *Candida* cells was

counted from each field to obtain the average number of cells over the total area of the abiotic surface. Briefly, the coverslip area (4.84E + 08 μm^2) was divided by the area of the photograph picture (12880 μm^2) and the average of cells from microscopic fields was multiplied by the previous ratio, obtaining the total number of cells over the abiotic glass surface. These results were expressed as the number of cells ± standard deviation per cm^2 (N. of cells/cm^2 ± SD) by dividing the previous total number of cells and their deviation over the glass surface area in cm^2 (4.84 cm^2). In EM, the percentages of dead and alive cells within images were measured through ImageJ by Fiji [30] (version 1.57) using the macros Biofilms Viability checker proposed by Mountcastle et al. [29] and the plugin MorphoLibJ [31]; while, the total cells counting in DAPI images were processed by a sequence of modules forming a pipeline designed for this purpose in Cell Profiler software [32] an open-source software version 4.2.1 (available from the Broad Institute at www.cellprofiler.org), which the applied pipeline can be revised in supplemental material. DAPI images were used to obtain total cells per image and the average was then calculated the mean of yeasts per cm^2.

2.6 Scanning Electron Microscopy

Times samples of 48 and 72h were also selected to be examined by scanning electron microscopy (SEM). For SEM analysis, 22-mm circular cover glasses (Heathrow Scientific, Vernon hills, Illinois, USA) were placed in 6-well plate and *Candida*-related biofilms were formed as previous described. Pre-formed biofilms were fixed adding in the wells a solution of PBS concentration adjusted to pH = 4,7 containing glutaraldehyde at 4% for 1 h. Post-fixation was carried out with 1% osmium tetroxide in cacodylate buffer for 1 h. Subsequently, the samples were treated with 1% tanic acid for 1 h. The samples were dehydrated with a series of ethanol washes of 30 min each with solutions containing 30%, 50%, 70%, 80%, 90%, and 100% of ethanol in distilled water; samples were further dried with CO_2 in a critical point dryer (Balzers CPD 030, Schalksmühle, Germany) [33,34]. Finally, discs with biofilm were coated with gold and the morphological analysis was elaborated using a Tescan Mira 3 scanning electron microscope equipped with a Schottky Field Emission Gun (Schottky FEG-SEM, MIRA III TESCAN, Kohoutovice, Czech Republic) at the Centro de Nanociencia y Nanotecnología of the Universidad de las Fuerzas Armadas ESPE [35]. Morphology of the

yeast was also obtained from the best images by Fiji ImageJ [30] (version 1.57), which the mean of yeast area (μm^2) was measured through the average area of *Candida* cells obtained by each picture from triplicate assays in SEM analysis.

2.7 Statistical Analysis

All data of the present study was obtained from at least triplicate assays realized in different days. In case of biomass growth and CFU counting assays, each assay was realized with five replicates. In addition, raw results from biomass growth assays were subtracted by the negative OD control values. Then, the standard deviation (SD) was determined for each data set of results. To evaluate data distribution, normality assessment was realized through Shapiro-Wilk tests, if p-value was equal or less than 0.05 it was classified as a non-normal distribution and non-parametric statistical analysis should be performed. Medians were compared by using the Kruskal Wallis nonparametric test, followed by Dunn's test using a Benjamini–Hochberg adjustment for multiple comparisons test at $\alpha = 0.05$ between results obtained in the same *Candida* species (intraspecies). While, between *Candida* species (interspecies), the results were compared globally using the Friedman test and then pairwise comparisons were performed with the Wilcoxon test. All data analysis was realized in R studio version 4.0 [36] using several R packages ("ggpubr", "rstatixs", "openxlsx" and the "tidyverse" set of packages [37,38]), where analysis was executed individually for results presented on each plot, table and supplemental material. Finally, the significance level for *p* values was considered as <0.05.

3. Results

The present study analyzed the cycle life of the monospecies biofilms formed by *Candida albicans* and *Candida tropicalis* during time, more exactly, 24, 48, 72 and 96h. For this goal, biofilm biomass was evaluated trough two methodologies of optical density measurement at 630 nm (more exactly, crystal violet staining and PBS suspension), while viable cells within the biofilm was analyzed though colony-forming unit counting to describe the number of viable microorganisms. Through this preliminary data, an initial analysis evaluated the distribution of the results to select the more appropriate statistical analysis. Finally, epifluorescence and

scanning electron microscopies were realized to analyze the percentage of alive and dead cells and structural morphology of the biofilms, respectively.

3.1. Quantification of the *C. albicans* and *C. tropicalis* Biofilms and their Normality Assessment

The biofilm capability of *C. albicans* ATCC® 10231™ and *C. tropicalis* V453 was determined comparing biomass against viability (Table 1). A normality assessment, through Shapiro-Wilk test and histogram examination, was also applied to the obtained data (see Supplemental Information).

Table II. 1 Summary of the results and statistical analysis obtained from biomass and viability assays of the biofilm growth with *Candida albicans* and *Candida tropicalis* species.

		Candida albicans			*Candida tropicalis*			Normality Assessment	
Time Points	**Variable**	**Total samples [a]**	**Mean (SD)**	**Median [Min - Max]**	**Total samples [a]**	**Mean (SD)**	**Median [Min - Max]**	**Shapiro-Wilk test [b]**	
								Statistics	***P*-value**
24h	Biomass PBS A_{630}	20	0.280 (0.127)	0.277 [0.101 – 0.596]	20	0.667 (0.160)	0.702 [0.362 – 0.952]	0.9521432	8.99E-02
	Biomass CV A_{630}	20	0.056 (0.014)	0.058 [0.023 – 0.077]	20	0.151 (0.018)	0.146 [0.117 – 0.185]	0.8819685	5.95E-04
	Viability CFU/mL	20	1.22E+08 (2.23E+07)	1.13E+08 [8.00E+07 – 1.93E+08]	20	9.89E+08 (1.60E+08)	1.03E+09 [5.30E+08 – 1.39E+09]	0.8163214	1.51E-05
48h	Biomass PBS A_{630}	15	0.562 (0.184)	0.539 [0.271 – 0.928]	20	0.936 (0.126)	0.968 [0.708 – 11.455]	0.9457164	8.36E-02
	Biomass CV A_{630}	15	0.168 (0.015)	0.175 [0.145 – 0.194]	20	0.270 (0.026)	0.270 [0.194 – 0.303]	0.8670426	5.76E-05
	Viability CFU/mL	15	2.49E+08 (4.59E+07)	2.30E+08 [1.33E+08 – 4.67E+08]	20	2.33E+09 (2.79E+08)	2.12E+09 [1.80E+09 – 3.47E+09]	0.8419846	1.60E-04
72h	Biomass PBS A_{630}	15	0.822 (0.169)	0.819 [0.548 – 1.116]	15	1.170 (0.090)	1.184 [0.919 – 1.131]	0.6378376	2.18E-11
	Biomass CV A_{630}	15	0.269 (0.011)	0.269 [0.221 – 0.291]	15	0.328 (0.038)	0.333 [0.257 – 0.384]	0.9499481	1.68E-01
	Viability CFU/mL	15	4.13E+08 (1.09E+08)	3.93E+08 [2.10E+08 – 5.93E+08]	15	2.99E+09 (3.09E+08)	2.93E+09 [2.07E+09 – 4.13E+09]	0.8090880	9.64E-05
96h	Biomass PBS A_{630}	15	1.045 (0.109)	1.033 [0.885 – 1.306]	15	1.290 (0.130)	1.419 [1.085 – 1.141]	0.251759	4.81E-11
	Biomass CV A_{630}	15	0.314	0.318	15	0.338	0.347	0.9697337	5.32E-01

		(0.031)	[0.264 – 0.369]		(0.045)	[0.223 – 0.423]		
Viability CFU/mL	15	4.77E+08 (3.05E+07)	4.67E+08 [4.23E+08 – 5.83E+08]	15	5.58E+09 (8.22E+08)	6.00E+09 [3.87E+09 – 6.80E+09]	0.7540960	1.06E-05

Legend- Evaluation of biofilm life cycle of two *Candida* species *in vitro* assays, where values of biomass assays are shown without the absorbance values obtained in the negative controls of each individual same assay, as described in Material and Methods section. [a] At least five samples per assay and each assay was realized in triplicate in three different days. [b] Shapiro-Wilk tests show the data evidence a non-normal distribution, more exactly, if *p*-value is equal or less than 0.05 it is a non-normal distribution and non-parametric statistical analysis must be performed.

As shown in Table 1, *C. tropicalis* biofilms clearly proved a higher capacity to produce biomass and colony-forming unit counting *in vitro* during all time samples when compared to *C. albicans* biofilms. To further evaluate statistical differences and in each species and between them, a normality assessment of the results was made, and Shapiro-Wilk tests evidenced a non-normal distribution, showing *p*-values < 0.05 apart from two samples times in CV assays (72 and 96h) and one sample time in PBS assays (24h). Therefore, most of the data showed a non-normal distribution among the results and, consequently, a non-parametrical statistical analysis was selected to future evaluation.

3.2. Evaluation of the Intraspecies Biofilm Growth

The knowledge of biofilm growth during time allows to better characterize the cycle of life of opportunistic pathogens, such as *C. albicans* and *C. tropicalis*, and to better understand their eradication on abiotic and biotic surfaces. Biofilm biomass and viable cells within the biofilm were quantified and further analyzed for statistical differences intraspecies during time (Fig. 1). Kruskal Wallis (KW) test demonstrated a significant effect between time samples in the biomass growth and viability of biofilms in both *Candida* species (Supplementary Material Information S1). More exactly, *C. albicans* biofilms exhibited a $p< 0.0001$ in both PBS assays (KW test $p= 4.33E-11$), CV assays (KW test $p= 2.19E-12$), and CFU counting assays (KW test $p= 4.59E-10$); likewise, *C. tropicalis* showed a $p< 0.0001$ in both PBS assays (KW test $p= 6.10E-12$), CV assays (KW test $p= 1.58E-11$), and CFU counting assays (KW test $p= 4.20E-13$). Then, as illustrated in Fig. 1, pairwise comparisons using the Benjamini–Hochberg p-adjustment method were applied to identify these intraspecies statistical differences among time samples (24, 48, 72, and 96h).

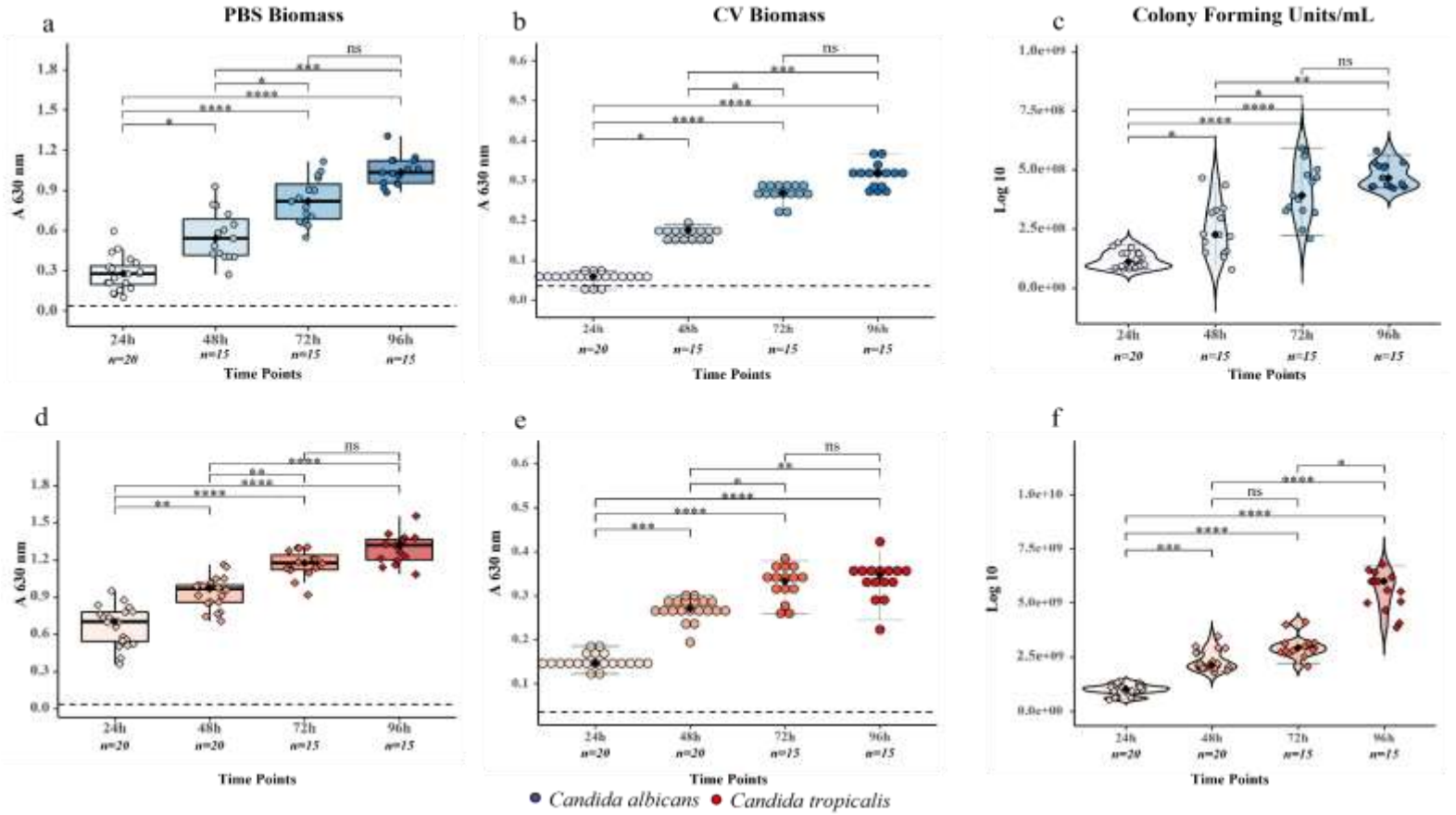

Figure II. 1 Evaluation of the biofilms formed by *Candida albicans* and *Candida tropicalis* during time (24, 48, 72, and 96h) through biomass and viability analysis. Biofilm biomass was evaluated trough two methodologies of optical density measurement while viable cells within the biofilm was analyzed though colony-forming unit counting to describe the number of viable microorganisms. All these results were grouped and plotted for each specie, where color dots represent individual result of each assay (blue dots for C. *albicans* and red dots for C. *tropicalis*), overlaid box plots cover the upper and lower interquartile ranges, whiskers extend to extreme datapoints, black diamond represents median (data shown in Table 1). (a) Biomass of C. *albicans* biofilm by phosphate buffered saline (PBS) method, (b) Biomass of C. *albicans* biofilm by crystal violet (CV) method, (c) Viability of C. *albicans* biofilm by colony-forming unit counting, (d) Biomass of C. *tropicalis* biofilm by phosphate buffered saline (PBS) method, (e) Biomass of C. *tropicalis* biofilm by crystal violet (CV) method, and (f) Viability of C. *tropicalis* biofilm by colony-forming unit counting. The illustrated statistical analysis between time in biomass and viability in each individual *Candida* species was realized through Post-Hoc Dunn's test using a Benjamini–Hochberg adjustment (all data shown in Supplementary Material Information S1), more exactly: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Candida albicans biofilms *demonstrated* statistical differences between all-time samples in biomass growth and viable cells within biofilm, excepting the period between 72 and 96h and thus evidencing a decrease in biofilm growth after 72h. Concerning *Candida tropicalis*, similar statistical significances were found in the time samples of these biofilms demonstrating also a diminution in biomass growth after 72h; however, in contrast, *Candida tropicalis* biofilms did not show significant growth of viable cells between 48 and 72h but also evidenced a significant *p*-value in the period between 72 and 96h ($p< 0.05$). It is worth to mention that the increment in viable cells within the *C. tropicalis* biofilm during this period was surprisingly superior to the previous periods, despite the stationary biomass growth in *C. tropicalis* biofilm.

3.3. Evaluation of the Interspecies Biofilm Growth

Both species of *C. albicans* and *C. tropicalis* are well-known to establish invasive candidiasis among vulnerable population but the differences between them in biofilm formation remain little understood. Therefore, the comparison of the interspecies biofilm growth was realized. As data were not distributed normally, a non-parametrical statistical analysis was also realized using the Friedman test for global comparison and then pairwise comparisons were performed though Wilcoxon tests between *Candida* species at each time sample of biomass and viability assays (Table 2).

Table II. 2 Evaluation of the statistical differences in biofilm growth between *Candida albicans* and *Candida tropicalis* from biomass and viability assays.

Biofilm growth		***C. albicans***			***C. tropicalis***		**Non-parametrical statistical analysis Interspecies**	
Assays	**n[a]**	**Mean**	**SD**	**n[a]**	**Mean**	**SD**	**Friedman test *p*-value**	**Wilcoxon test *p*-value**
Biomass PBS A_{630}								
24h	4	0.280	0.127	4	0.667	0.160	2.20E-16	5.41E-09
48h	3	0.562	0.184	3	0.936	0.126		9.79E-07
72h	3	0.822	0.169	3	1.170	0.090		1.33E-05
96h	3	1.045	0.109	3	1.290	0.130		3.35E-05
Biomass CV A_{630}								
24h	4	0.056	0.014	4	0.151	0.018	2.20E-16	6.71E-08
48h	3	0.168	0.015	3	0.270	0.026		6.73E-07
72h	3	0.269	0.011	3	0.328	0.038		8.34E-04
96h	3	0.314	0.031	3	0.338	0.045		4.87E-02
Viability CFU/mL								
24h	4	1.22E+08	2.23E+07	4	9.89E+08	1.60E+08	2.20E-16	6.68E-08
48h	3	2.49E+08	4.59E+07	3	2.33E+09	2.79E+08		6.17E-07
72h	3	4.13E+08	1.09E+08	3	2.99E+09	3.09E+08		3.37E-06
96h	3	4.77E+08	3.05E+07	3	5.58E+09	8.22E+08		3.27E-06

Legend – The standard deviation (SD) and the mean were calculated by the average values of the total number of assays (five samples per assay). The data set showed a non-normal distribution among the results and, consequently, a non-parametrical statistical analysis was selected to evaluate statistical differences in biofilm growth between *C. albicans* and *C. tropicalis*. More exactly, the results between *Candida* species were compared globally on each assays using the Friedman test and then pairwise comparisons were performed using the Wilcoxon test. [a] Number of assays realized in different days for each time sample.

Friedman test evidenced a significant difference between *C. albicans* and *C. tropicalis* biofilms in each biomass and viability assays (p= 2.20E-16). Likewise, all-time samples in the biomass growth and viability assays confirmed statistical differences between *Candida* species through multiple pairwise comparisons with Wilcoxon tests (Table 2). All p-values were below 0.0001, except in two-time samples of 72 and 96h in CV assays showing p-values below 0.001 and 0.05, respectively. Interestingly, it is possible to observe that both PBS and CV assays showed the same biomass growth tendency, where *C. albicans* demonstrated a lower biomass growth but reaching nearly the same level of *C. tropicalis* biomass at 96h. However, CFU counting assays did not show the same conduct, exhibiting constantly a superior number of viable cells within the *C. tropicalis* biofilm (approximately a CFU counting difference around 1.00E+01 CFU/mL in all-time samples).

Live/Dead Cells and Cell Morphologies of the *C. albicans* and *C. tropicalis* Biofilms

Following the biomass and viability evaluation during time, we decided to analyze the amount of live/dead cells and cell morphologies within biofilms between *Candida* species (Table 3). For this purpose, two time points were chosen in order to evaluate exponential phase and initial stationary phase of the biofilms in both species, more exactly, 48 and 72h, respectively. Epifluorescence microscopy (EM) was applied to visualize dead/alive cells using DAPI (4′,6-diamidino-2-phenylindole) fluorescent stain and a Live/Dead Staining Kit (Fig. 2) and scanning electron microscopy (SEM) was used to observe cell morphologies (Fig. 3). As shown in Table 3, neither methodology evidenced significant effect between time samples in both *Candida* species using Kruskal Wallis (KW) test (Intraspecies analysis). However, when applied non-parametrical statistical analysis in interspecies, Friedman tests showed statistical differences in 48 and 72h biofilms from *C. albicans* and *C. tropicalis* of either methodology, more exactly, EM (Friedman test p= 3.89E-03) and SEM (Friedman test p= 4.30E-12). Thoroughly, in EM with dead-alive staining, Wilcoxon tests evidenced p-values of 3.03E-13 and 3.20E-05 in 48 and 72h, respectively, between *C. albicans* and *C. tropicalis* biofilms. *C. tropicalis* demonstrated a higher number of total cells within biofilms at 48 and 72h, when compared to *C. albicans*

biofilms and as expected from previous CFU counting assays. However, the percentage of dead and alive cells were practically equal in both *Candida* biofilms at 48 and 72h, ranging between 4.30-17.40 and 82.60-95.70%, respectively. Therefore, no statistical differences were found in the percentage of dead and alive cells within the biofilm between these *Candida* species. When evaluating cell morphologies, Wilcoxon tests showed *p*-values of 3.05E-05 and 3.00E-05 in 48 and 72h, respectively, between *C. albicans* and *C. tropicalis* biofilms. Interestingly, the mean size of *C. albicans*' cell area at 48 and 72h were 1.80E-02 and 1.60E-02 μm^2, respectively, evidencing a non-significant decrease in the stationary phase but still demonstrating a significant and superior cell area when compared to *C. tropicalis*' cells in both time samples.

Table II. 3 Evaluation of dead and alive cells and cell morphologies between *Candida albicans* and *Candida tropicalis* from biofilms of 48 and 72h.

Legend – 48 and 72h time samples were selected to compare the number of alive and dead cells, as well as biofilm structure for each *Candida* species using the dead/alive assay in epifluorescence microscopy (EM) and the scanning electron microscopy (SEM), respectively. In EM, the percentages of dead and alive cells within images were measured through ImageJ by Fiji [30] (version 1.57)

Epifluorescence microscopy (EM) with Dead-Alive Staining

		C. albicans					*C. tropicalis*				Non-parametrical statistical analysis Interspecies		
Assay	**n [a]**	**Mean of yeasts/frame (SD) [b]**	**Mean of yeasts/cm² [c] (SD)**	**Dead (SD) %**	**Alive (SD) %**	**KW *p*-value**	**Mean of yeasts/frame (SD)**	**Mean of yeasts/cm² (SD)**	**Dead (SD) %**	**Alive (SD) %**	**KW *p*-value**	**Friedman test [d] *p*-value**	**Wilcoxon test [d] *p*-value**
48h	3	1.30E+03 (8.50E+02)	1.01E+07 (6.59E+06)	5.00 (0.53)	95.00 (1.00)	3.17E-01	1.94E+04 (3.90E+03)	1.51E+08 (3.03E+07)	4.30 (0.50)	95.70 (0.20)	3.17E-01	3.89E-03	3.03E-13
72h	3	4.20E+04 (2.09E+04)	3.26E+08 (1.62E+08)	17.00 (3.18)	83.00 (3.00)		6.15E+04 (2.20E+04)	4.77E+08 (1.71E+08)	17.40 (4.50)	82.60 (5.00)			3.20E-05

Scanning Electron Microscopy (SEM) with Morphology of yeasts

		C. albicans		*C. tropicalis*		Non-parametrical statistical analysis Inter-species	
Assay	**n [a]**	**Mean size of yeast cell area, µm² (SD) [e]**	**KW *p*-value**	**Mean size of yeast cell area, µm² (SD) [e]**	**KW *p*-value**	**Friedman test [d] *p*-value**	**Wilcoxon test [d] *p*-value**
48h	3	1.80E-02 (3.00E-03)	2.00E-01	1.00E-02 (2.00E-03)	4.00E-01	4.30E-12	3.05E-05
72h	3	1.60E-02 (2.00E-03)		1.10E-02 (2.00E-03)			3.00E-05

using the macros Biofilms Viability checker (see methods); while, the total cells counting in DAPI images were processed by a sequence of modules forming a pipeline in Cell Profiler software [32], which the applied pipeline can be revised in supplemental material. DAPI images were used to obtain total cells per image and the average was then calculated the mean of yeasts per cm^2. Morphology of the yeast was also obtained from the best images by Fiji ImageJ [30] (version 1.57). Kruskal Wallis (KW) test was applied to evaluate statistical differences between 48 and 72h samples in each *Candida* species (Intraspecies). [a] Number of assays realized in different days; in each assay, we collected at least 12 photographs for cells counting. [b] Average of *Candida* cells obtained by picture from triplicate assays and their standard deviation (SD). [c] The estimation of yeast/cm^2 was calculated by the following formula: average of *Candida* cells (SD)* (1E+08/12880). [d] In non-parametrical statistical analysis (Interspecies), Friedman test and Wilcoxon test were calculated using the results of mean of yeast/cm^2 from *C. albicans* and *C. tropicalis*. [e] The mean of yeast area (μm^2) was measured through the average area of *Candida* cells obtained by each picture from triplicate assays and their standard deviation (SD) in SEM analysis.

When observing the biofilms of 48 and 72h by EM (Fig. 2), both *Candida* species showed an increase of cells and higher density within the biofilms during time, although no significant differences were obtained between time samples (Table 3). No visual differences were detected in the amount of dead and alive cells between *Candida species* during time, in concordance with the previously statistical analysis, and showing a homogenous distribution of dead cells within the biofilms of both *Candida* species (see merged pictures on Fig. 2).

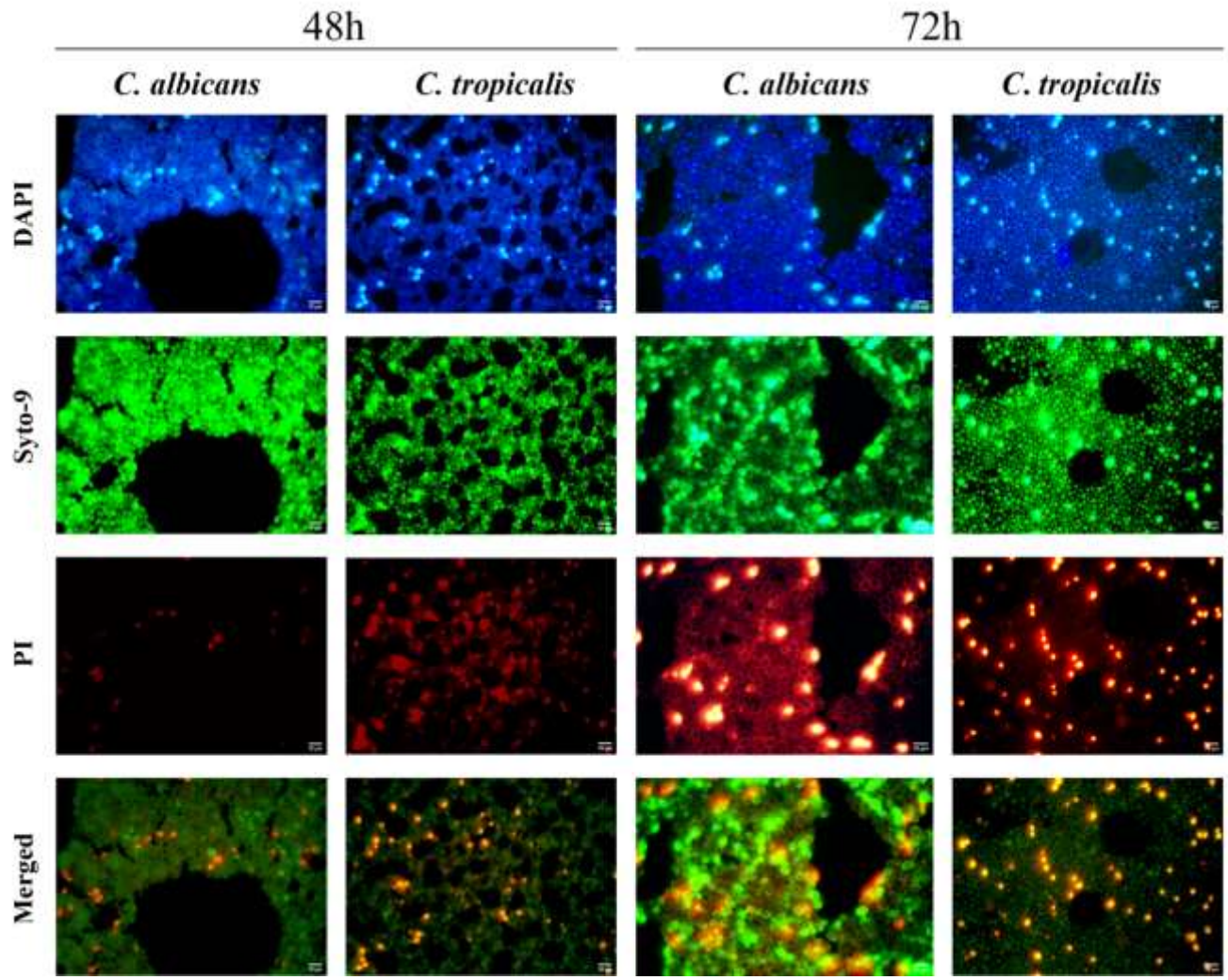

Figure II. 2 Illustration of the biofilms of *C. albicans* and *C. tropicalis* at 48 and 72h of growth culture by epifluorescence microscopy using DAPI (4′,6-diamidino-2-phenylindole) fluorescent stain and a Dead-Alive Staining Kit. Time samples of 48h and 72h were used to compare the structure and density of the biofilms using a Olympus BX50 microscope and the pictures were obtained by an AmScope software at 100x magnification and then pictures from each filter were merged in Fiji-ImageJ [30] (version 1.57).

In addition, both *C. albicans* and *C. tropicalis* exhibited mature biofilms with a multilayer growth during time (at least 48 and 72h), which made difficult to evaluate the average size of

cell area in each biofilm and to compare it between *Candida* species. Therefore, SEM analysis was applied to study cell morphologies in each individual biofilm in these two time points (Fig. 3).

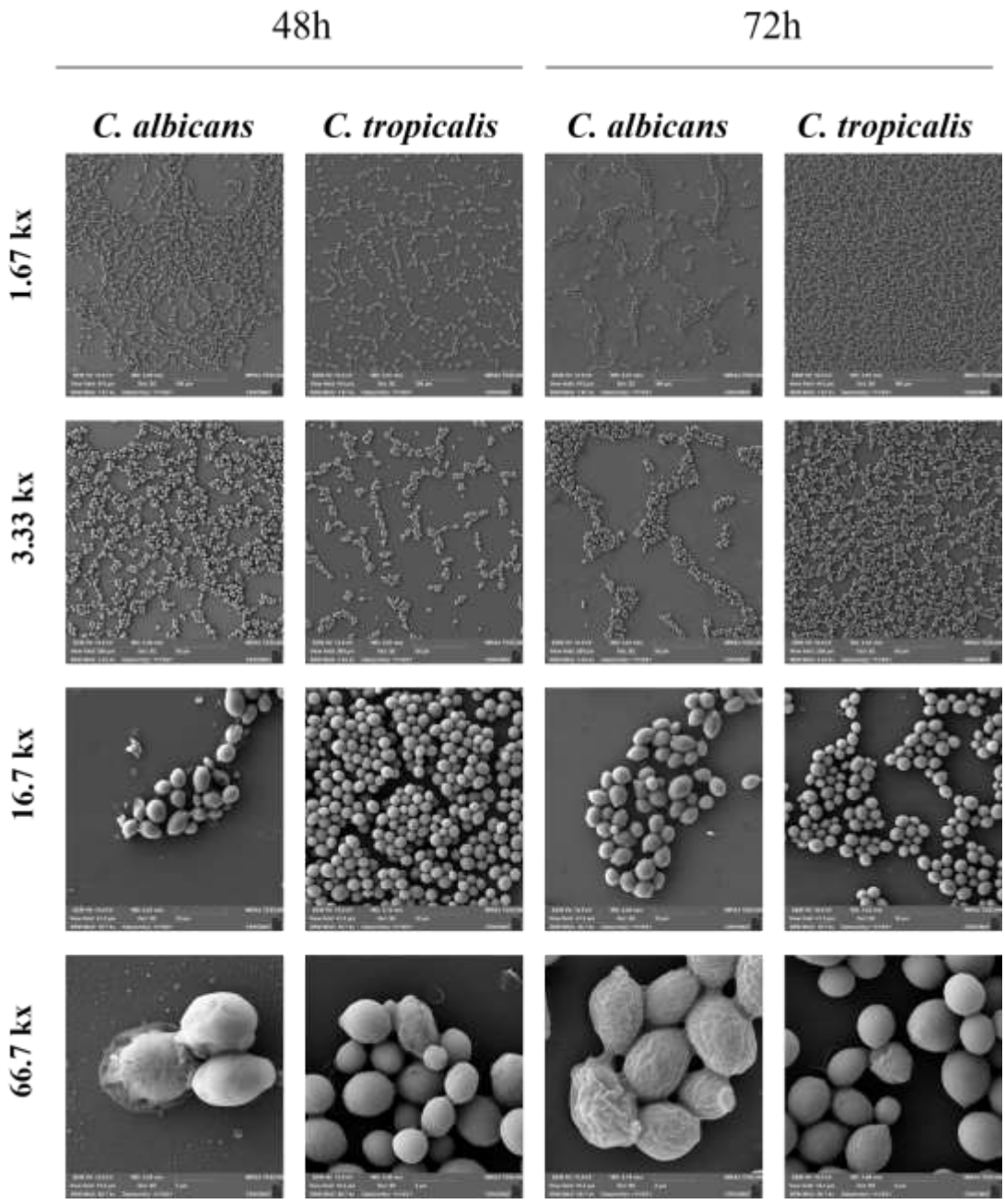

Figure II. 3 Illustration of the cells within biofilms of *C. albicans* and *C. tropicalis* at 48 and 72h of growth culture by scanning electron microscopy using different magnifications (1.67, 3.33, 16.7, and 66.7 kv). Time samples of 48h and 72h were used to compare the cell morphology and area of the biofilms using a using a Tescan Mira 3 scanning electron microscope equipped

with a Schottky Field Emission Gun (Schottky FEG-SEM). Yeast cell area was calculated from the best pictures by Fiji ImageJ [30] (version 1.57).

As shown in Fig. 3, biofilm comparisons between *Candida* species at 48h and 72h time points using SEM analysis were also realized through different magnifications (1.67, 3.33, 16.7, and 66.7 kv). In the lower magnifications (1.67 and 3.33 kv), it was possible to observe biofilms with highly ordered structure of cell assemblages, multilayer growth and exhibiting an interconnectivity between cell assemblages. At 48h, both *Candida* biofilms evidenced larger spaces without adhered cells that became shorter in their biofilms of 72h, achieving a mature phase of biofilm. However, at 72h, the density of cells within the biofilm was notoriously higher in biofilms of *C. tropicalis*. Meanwhile, in the higher magnifications (16.7 and 66.7 kv), we were able to analyze the cell areas and morphologies in both biofilms, perceiving visible differences in cell area and matrix production between *Candida* species. As previous indicated in Table 3, *C. albicans* showed a significant and superior cell area when compared to *C. tropicalis*' biofilm cells in both time samples. Moreover, the irregular texture on the surface of *C. albicans*' cells clearly reflected the higher production of matrix or extracellular polymeric substance (EPS) by the biofilm stage, especially at 72h. However, *C. tropicalis* biofilm cells did not evidence the same rate of EPS production, at least visually, as *C. albicans*.

4. Discussion

The ability to establish biofilm is a well-known property among several microorganisms and by itself is considered a main virulence factor among infections due to several intrinsic biofilm-associated factors, such as antimicrobial resistance, immune system evasion, and horizontal gene transfer (HGT) mechanisms in multispecies biofilms [11,39–41]. The formation of *Candida* biofilms had been observed multiple surfaces, since blood, mucosal surface and most medical devices (i.e., nonliving objects in contact with patients' body) [11,42,43]. In fact, both *Candida albicans* and non-*albicans Candida* (NAC) species have been

found in developed biofilm stage at several medical devices, such as stents, shunts, implants, endotracheal tubes, pacemakers, and multiple types of catheters [2]. Recently, our metanalysis on the prevalence of *Candida* biofilms in bloodstream infections showed that the mortality rate in these infections was 37.9% of which 70.0% were from biofilm-associated infections [17], showing *Candida tropicalis* as the prevalent species in high biofilm formation (67.5%) and overpassing *C. albicans* (30.3%). Therefore, the present study aimed to compare the biofilm cycle of life between *C. albicans* and *C. tropicalis* and evaluate their ability to establish biofilm through multiple methodologies. To the authors' best knowledge, this is the first study to analyze biofilms of these *Candida* species during time by biomass assays (crystal violet staining and PBS suspension), colony-forming unit counting, epifluorescence microscopy, and scanning electron microscopy.

4.1 Biofilm Growth of the *C. albicans* and *C. tropicalis*

The standard optical density measurement assays offer a quick and relatively high-throughput way to screen microorganisms with the ability for biofilm formation with minimal equipment requirements [23]. Therefore, we applied this methodology through biomass assays (crystal violet staining and PBS suspension) in 6-well plates to reduce the standard deviation in the obtained results and compare the *in-vitro* biofilm results between these *Candida* species [44]. In 2022, Castro and colleagues already demonstrated the usefulness applications of biomass assays (such as crystal violet staining) on biofilms, reporting the acuteness in the results obtained in monospecies biofilms and the lack of flawlessness in the assessment of synergism or antagonism in multispecies biofilms [45].

As expected, both *Candida* species clearly exhibited their ability to form biofilms. When evaluating their biofilms, statistical differences in biomass growth were identified through non-parametrical tests, showing the greatest biomass growth until 72h in both *Candida* species. Our

results are in accordance with the typical life cycle described in yeasts biofilms [46], more exactly: (1) attachment and colonization of round yeast cells to a surface; (2) growth and proliferation of yeast cells creating a basal layer of anchoring cells; (3) growth of pseudohyphae (oval yeast cells joined end to end) and hyphae (long cylindrical cells) accompanying the production of the extracellular matrix; and eventually (4) dispersal of cells from the biofilm to find new sites to colonize.

Regarding to interspecies comparison, it was noted that the biofilm forming ability of *C. tropicalis* was significantly higher than *C. albicans*, showing a Friedman test *p*-value of 2.20E-16 (Table 2). Our results agreed with the observations from previous studies [47,48]. Shin et al. [47] reported that biofilm positivity occurred most frequently in isolates of *C. tropicalis*, and furthermore Konečná et al. [48] described that *C. tropicalis* could be categorized as a strong biofilm producer due to the biomass production observed in this species [48]. Likewise, the results of the present study are in agreement with our meta-analysis [17]. In these studies, *C. tropicalis* was associated with a higher mortality rate when compared with *Candida albicans* and other NAC species. This propensity of *C. tropicalis* for dissemination and higher mortality rate could be related to its high biofilm formation, as one of the main intrinsic virulence factors exhibited by this species [21,42]. The higher biomass growth of *C. tropicalis* biomass could also provide an advantage to the cells within the biofilm enabling a better protection against antifungal or antimicrobial agents [49].

Moreover, colony-forming unit (CFU) counting assay was used, as a basic and gold standard method to correlate the biomass assays with a given number of viable cells within biofilms [23,50]. In fact, the CFU counting assay is also known to quantify viable cells even with low metabolic activity [50]. In the present study, it is possible to observe that CFU counting results showed a significantly increasing of viable cells over the time in *C. albicans* (KW test *p*-value = 4.59E-10) and in *C. tropicalis* (KW test *p*-value= 4.2E-13) biofilms.

However, it is worth mentioning that only biofilms of *C. tropicalis* demonstrated a significant increment of viable cells between 72 and 96h (Wilcoxon test *p*-value= 3.27E-06), evidencing an active cell proliferation within the stationary biomass of the biofilm and suggesting a longer biofilm cycle of life in *C. tropicalis* species. Therefore, further studies should be realized to confirm the extension of *C. tropicalis* biofilm cycle of life (such as, dispersal of cells from the biofilm) and to evaluate the survival rate of cells when exposed to several antifungal or antimicrobial treatments, as previously realized by Khot et al. [51] in *C. albicans* biofilms.

Between these *Candida* species, *C. tropicalis* biofilms exhibited a continuously higher CFU counting when compared with *C. albicans* (approximately a CFU counting difference around 1.00E+01 CFU/mL in all-time samples), displaying a significant difference during time (Table 3). These results collaborated with the classification of *C. tropicalis* as a strong biofilm producer and its association with a higher mortality rate in hospitalized patients. The infective ability of yeasts depends on specific virulence mechanisms that confer the ability to colonize host surfaces, to invade deeper host tissue or to evade host defenses [43,52]. *C. tropicalis* ability as a strong biofilm producer demonstrated an important clinical impact once biofilm-associated infections are currently difficult to treat, representing a seriously source of reinfections [43,52]. However, concerning the difficulties and cons of the applied methodologies, it is important to point out that these analyses are quite laborious and time consuming due to the *in vitro* assays before biofilm evaluation. So, these methodologies cannot be applied as routine diagnostic tests but should be considered in the monitoring of hospitalized patients with high risks of biofilm-associated infections (such as, patients with critical illness or immunosuppression, or even people with a central venous catheter and parenteral nutrition).

4.2 Live/Dead Staining and Cell Morphologies of the *C. albicans* and *C. tropicalis* Biofilms

Epifluorescence microscopy (EM) and scanning electron microscopy (SEM) findings together with the previous results confirmed the higher biofilm production of *C. tropicalis* when compared with *C. albicans*. DAPI staining assays evidenced statistically significant differences between *C. albicans* and *C. tropicalis* biofilms at 48 and 72h, showing Wilcoxon tests *p*-values of 3.03E-13 and 3.20E-05, respectively. Once again, *C. tropicalis* demonstrated a higher number of total cells within biofilms at both time samples and as expected from previous CFU counting assays. Meanwhile, live/dead staining provided information on how many of the total cells were dead and alive within biofilms though their capacity to exclude, accumulate, and metabolize the fluorophores Syto-9 and propidium iodide (PI) [53]. The principle of these fluorophores is based on the detection of membrane integrity. The ability of PI stain to intercalate DNA with no sequence preference (such as DAPI but without its high permeability) allows him to release an enhanced fluorescence of 20- to 30-fold when bound to DNA. This fluorophore is frequently used for identifying dead cells and as counterstain in multi-dyes fluorescence assays due to its ability to penetrate only cells with disrupted membranes. In contrary, the Syto-9 stain can enter in both live and dead cells. However, when both fluorophores are present, PI exhibits a stronger affinity for nucleic acids than Syto-9 and hence Syto-9 is usually displaced by PI [53]. As previously described by several authors, when live/dead staining with Syto-9 and PI is performed, several factors need to be considered, such as the bleaching effect of Syto-9, different binding affinity of Syto-9 to dead and alive cells and background fluorescence [53–55]. Therefore, the live/dead staining is the most variable of the methodologies used in this work because there are not unified protocols for *Candida* biofilms [54,55]. Although several staining techniques have been evaluated and even considered appropriate alternatives to plate counting, there are no homologies in results among several

studies with the exception for samples with 100% dead cells [55,56]. As expected in the present study, no statistical differences were found in the percentage of dead and alive cells within the biofilm between *C. albicans* and *C. tropicalis*. So, further studies should be realized to minimize the previous cited limitations and optimize the resolution of this methodology.

It is well-known that biofilms formed by different *Candida* species may vary in morphology and density, showing a polymeric extracellular matrix that protects the biofilm cells and water channels, as previously described in bacterial biofilms [57–59]. The extracellular matrix components also differ from those found in the *Candida* cell wall, and these moieties are proposed to modulate host recognition by concealing the cell wall that typically interact with the immune system [14,60]. In SEM analysis, it was possible to observe the ultrastructure of the *Candida* biofilms and also their cell morphologies exhibiting the characteristics for each specie. In general, two species strongly adhered to the abiotic surfaces (glass slide), and then subsequently developed into a mature biofilm within 48 h (Fig. 3). Neither *Candida* species evidenced statistically significant effect between time samples (48 and 72h) suggesting the achievement of the mature stage of these biofilms. However, when comparing cell morphologies between these *Candida* species, statistically significant differences were found in the mean size of cell area in both time samples, where *C. albicans* demonstrated a superior cell area when compared to *C. tropicalis*' cells. Apparently, another visual difference was the irregular texture on the surface of *C. albicans* biofilm cells indicating a higher production of extracellular polymeric substance (EPS) when compared to *C. tropicalis* at 72h. Although *C. albicans* visually showed a higher amount of EPS, both *Candida* species evidenced a confluent basal blastospore layer covered by a matrix of EPS and few hyphal elements, similar to the findings reported by Kuhn and colleagues [61]. These hyphal elements are believed to play an important role in fungal infection, as previously described in *C. albicans* [23,24], being also identified on biofilms of *C. tropicalis* in the present study. It important also

to mention that the observations obtained in SEM analysis could be affected by the methodology applied. More exactly, the present study used Sabouraud media without any supplemental ingredient, and the literature suggested the application of media with supplements to promote the gemination of the yeast and hyphal formation *in vitro* conditions [20,24]. The success of *C. albicans* as a pathogen depends largely on its ability to generate diversity not only at the genetic level but also at the morphological and physiological level to suit pH conditions, carbon sources, and among other factors [62,63].

Some authors reported that *Candida* biofilms at 72h begin to disintegrate [64], however we observed mature biofilms without signs of disintegration and with low number of dead cells within biofilm. Moreover, *C. albicans* formed thick, spatially organized biofilms comprising a multitude of blastospores and pseudohyphae forms partially embedded in a strong extracellular matrix, as previously described [64]. While *C. tropicalis* developed into strong and compact biofilms exclusively of blastospores embedded within a thin extracellular matrix. Concerning cell morphologies, it is important their identification because yeasts are also capable of differentiation and forming specialized cell-types that exist either as individuals or as constituents of organized multicellular populations [65]. Cell differentiation to opposite mating types and switching from yeast form to filamentous form (hyphae or pseudohyphae) are examples of individual yeast cell differentiation. Both processes have been investigated using different yeast species, and they can contribute to the virulence and invasiveness of pathogenic yeast [65]. Differentiate yeast are usually specifically localized within the structure; they can perform specific tasks and can even mutually interact [65]. In this study, it was only possible to analyze the adhered cells that formed a multilayer consortium in the biofilm, and therefore the lack of yeast gemination (pseudohyphae/hyphae forms) constituted one limitation of the present work that should be rectified in further studies by optimizing several factors (such as pH conditions and carbon sources).

5. Conclusions

The present study attained to compare the biofilm cycle of life between *C. albicans* and *C. tropicalis* and characterize their biofilms through multiple methodologies. To the authors' best knowledge, this is the first study to simultaneously analyze biofilms of these *Candida* species during time (until 96h) by biomass assays (crystal violet staining and PBS suspension), colony-forming unit counting, epifluorescence microscopy, and scanning electron microscopy. We were able to validate findings in *C. albicans* biofilms previously described by other authors, and prove the higher biofilm production of *C. tropicalis* at same *in vitro* conditions. From our results, new questions about the physiology of these biofilms, and the forces that modulate yeast behavior remain unanswered; therefore, furthers studies should analyzed the molecular and metabolic network that influences the evolution of the biofilm formed by different *Candida* species.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Supplementary Material Information S1 (Summary of material and methods used to grow *Candida* biofilms and analysis of them, Normality assessment study, Supplementary Table S1, and Description of procedure for image counting and programs used), Supplementary File S2 (Excel file crude data and cured process), and Supplementary File S3 (Excel file cells counting).

Author Contributions: Conceptualization, A.M.; methodology, A.M.; software, M.B.A.-C.; validation, A.M., M.B.A.-C. and F.S.C.-M.; formal analysis, M.B.A.-C.; investigation, M.B.A.-C. and F.S.C.-M.; resources, A.M. and A.D.; data curation, M.B.A.-C.; writing—original draft preparation, A.M. and M.B.A.-C.; writing—review and editing, A.M., A.D., M.B.A.-C. and F.S.C.-M.; visualization, A.M., A.D., M.B.A.-C. and F.S.C.-M.; supervision, A.M.; project administration, A.M.; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data presented in this study are available on request by contacting the corresponding author (A.M.).

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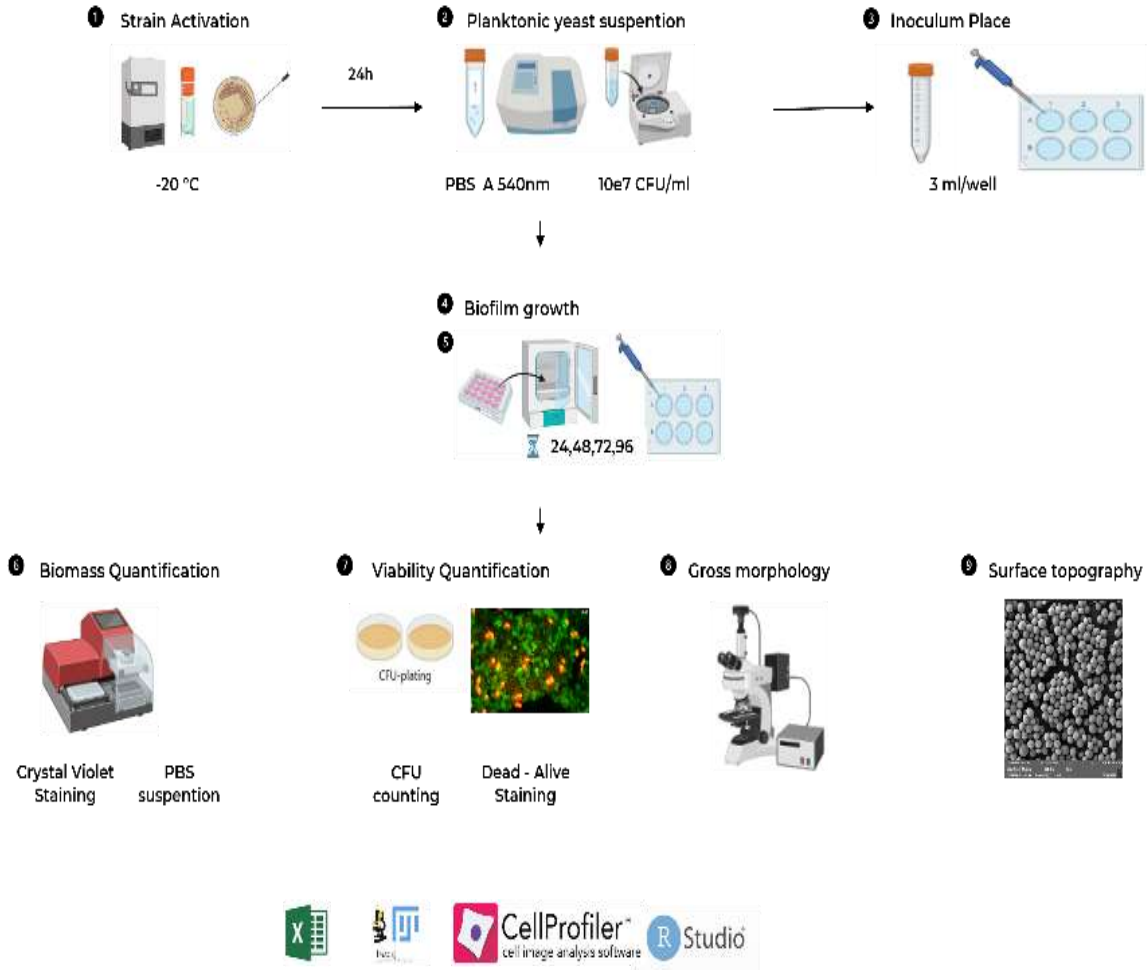
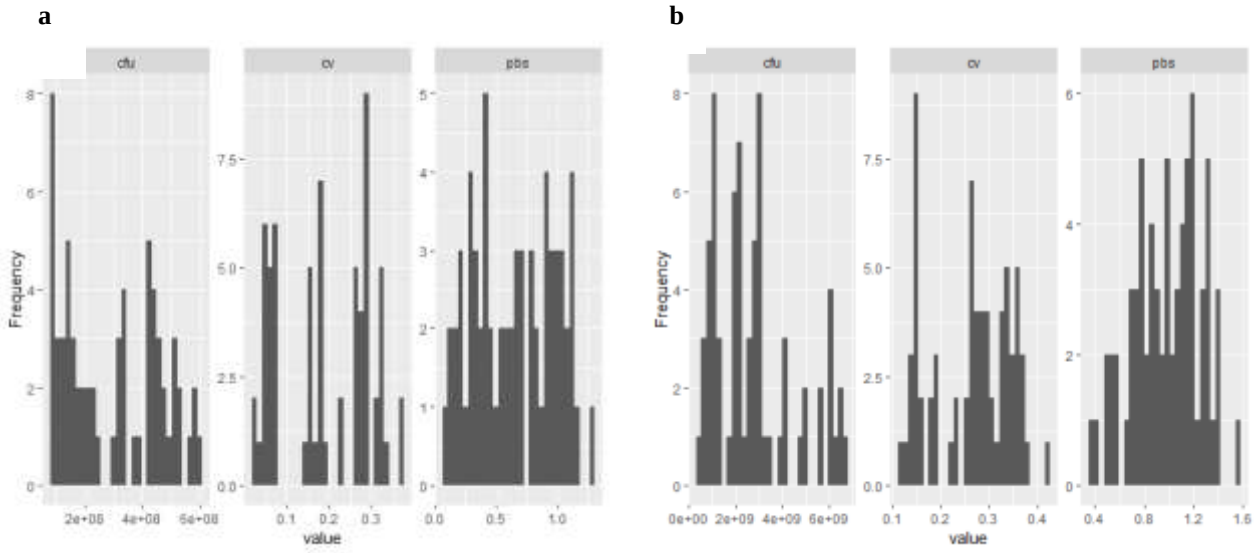
ÍNDICE DE ANEXOS

ANEXO A: SUPPLEMENTARY FIGURE S1: SUMMARY OF MATERIAL AND METHODS USED TO GROW *CANDIDA* SPECIES BIOFILMS AND ANALYSIS OF THEM.

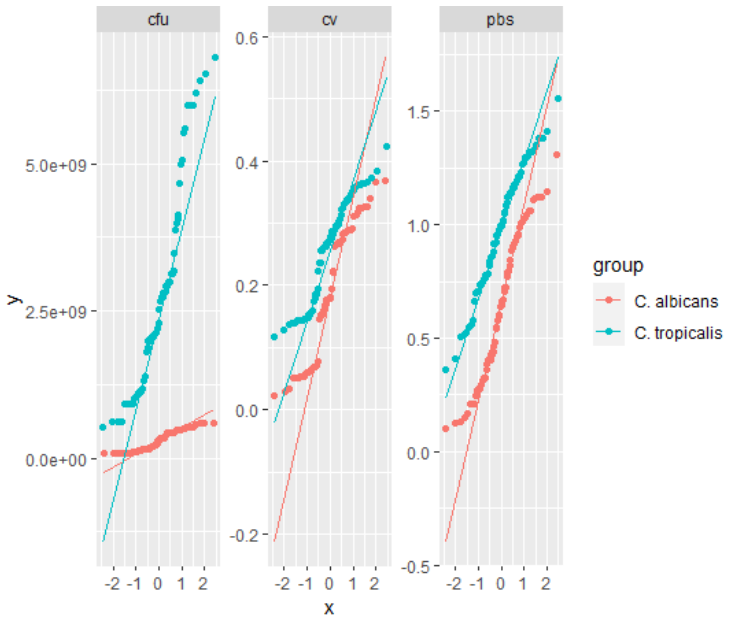


Supplementary Figure S1: Summary of material and methods used to grow *Candida* species biofilms and analysis of them.

ANEXO B: NORMALITY ASSESSMENT



Supplementary Figure S2: Histograms of total data for two species of Candida. Each histogram displays the distribution of data values for continue variables (tree assays). Vertical axis represents the count (frequency), and the horizontal axis represents the possible range of the data values. This information was used in conjunction whit Shapiro Wilk results and qq-plot to evaluate normality in data.



Supplementary Figure S3: quantile-quantile plots for data distribution of *Candida* spp. biofilms growth divide by specie. Plots shows non normality distribution and could be beneficial transform CFU scale in logarithmical scale.

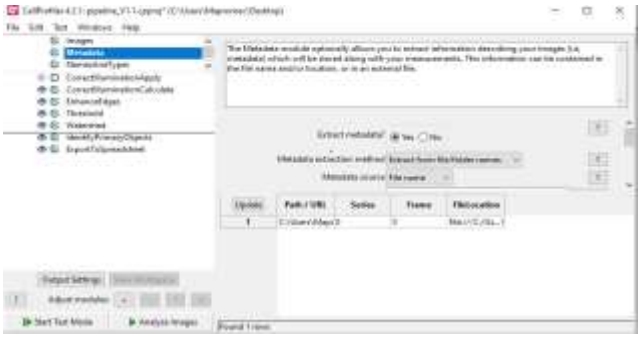
ANEXO C: COMPARISON INTRASPECIES OF BIOMASS AND VIABILITY ASSAYS BY TIME POINTS THROUGH KRUSKAL WALLIS NONPARAMETRIC TEST, FOLLOWED BY DUNN'S TEST USING A BENJAMINI–HOCHBERG ADJUSTMENT FOR MULTIPLE COMPARISONS.

Species	**Type of assays**	**Kruskal Wallis test** ***p-value***	**group1**	**group2**	**n1**	**n2**	**Benjamini–Hochberg p-adjustment method** ***p-value***
Candida albicans	PBS Biomass	4.33E-11	24	48	20	15	1.62E-02
			24	72	20	15	5.55E-06
			24	96	20	15	7.63E-11
			48	72	15	15	4.55E-02
			48	96	15	15	1.57E-04
			72	96	15	15	6.10E-02
	CV Biomass	2.19E-12	24	48	20	15	1.01E-02
			24	72	20	15	2.06E-07
			24	96	20	15	1.59E-11
			48	72	15	15	1.44E-02
			48	96	15	15	1.23E-04
			72	96	15	15	1.34E-01
	CFU viability	4.59E-10	24	48	20	15	1.55E-02
			24	72	20	15	6.59E-07
			24	96	20	15	6.13E-09
			48	72	15	15	1.55E-02
			48	96	15	15	1.42E-03
			72	96	15	15	3.87E-01
Candida tropicalis	PBS Biomass	6.10E-12	24	48	20	15	7.04E-03
			24	72	20	15	2.83E-07
			24	96	20	15	6.50E-11
			48	72	15	15	7.04E-03
			48	96	15	15	5.05E-05
			72	96	15	15	1.73E-01
	CV Biomass	1.58E-11	24	48	20	15	5.61E-04
			24	72	20	15	8.69E-09
			24	96	20	15	1.18E-09
			48	72	15	15	1.21E-02
			48	96	15	15	4.04E-03
			72	96	15	15	6.90E-01
	CFU viability	4.20E-13	24	48	20	15	7.05E-04
			24	72	20	15	2.28E-06
			24	96	20	15	3.14E-13
			48	72	15	15	8.77E-02
			48	96	15	15	3.60E-05

			72	96	15	15	1.89E-02

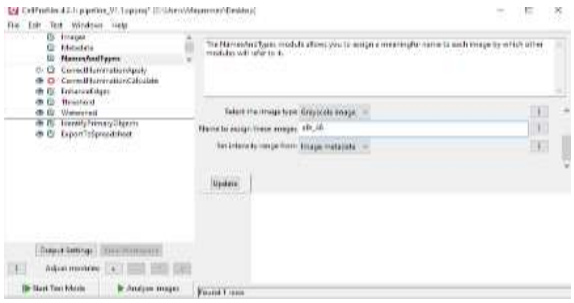
ANEXO D: COUNTING SUPPLEMENTARY INFORMATION

DAPI photographs for each time point (12 per day ,3 different days) was analyzed whit the pipeline to obtain total cells per image, the average and SD was then calculated. The pipeline used to execute the total counting of yeast was constructed in Cell Profiler following the tutorials available in the web site https://cellprofiler.org. The procedure is detailed in the following images.



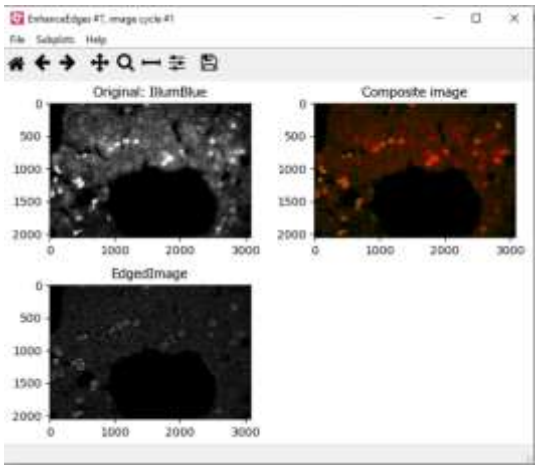
1.- Charge the images dragging into the space designed by default.

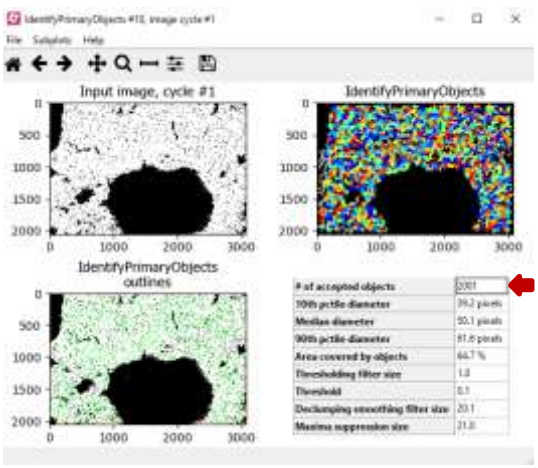
2.- Allow the software access to metadata of the images



3.- Assign one name for all your bundle of pictures

After step 3 pipeline will execute without problems, the process will depend on the ram memory of your computer. Result of counting

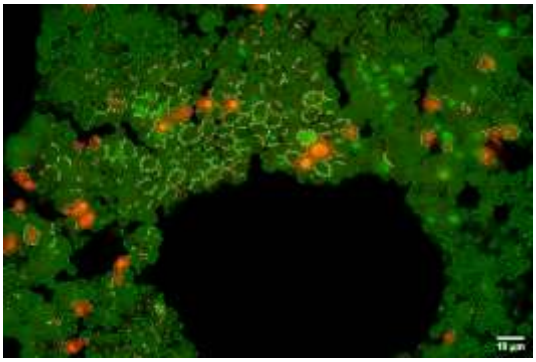




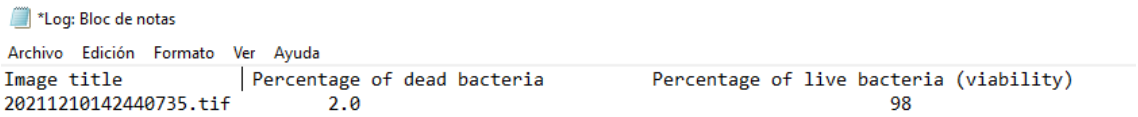
Red arrows show the counting for this image, in our case we process 12 images in bundle, so we use the terminal and see the results in a excel sheet at the final of process.

ANEXO E: DEAD-ALIVE PROCESS

To dead alive we follow the process exactly as describe in the paper “Biofilm viability checker: An open-source tool for automated biofilm viability analysis from confocal microscopy images” [12]. The following image is an example of the overlayed image output from the macro, as well as the results of the analysis of the sample image.



Macros Result. This image is generated automatically by the program and save in the chose location



Result generate automatically and save in the same place of the image above.