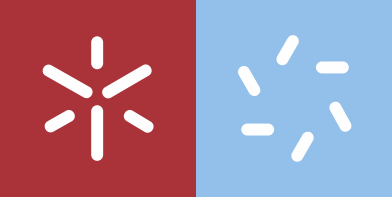




**Elucidating the role of exosomes in
Candida - macrophage interaction**

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**Elucidating the role of exosomes in
Candida – macrophage interaction**

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Great people are those who make others feel that they, too, can become great. - Mark Twain

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RESUMO

Os fungos têm sido identificados como causadores de graves doenças humanas, sendo as espécies do género *Candida* e *Aspergillus* as principais causas de infeções fúngicas oportunistas em todo o mundo. Ao longo dos anos, houve um aumento na incidência destas infeções e no número de espécies resistentes aos medicamentos, o que contribuiu para maiores percentagens de morbilidade e mortalidade, principalmente entre indivíduos imunocomprometidos. Uma vez que as infeções por *Candida* têm um efeito significativo na saúde pública, a necessidade de melhor compreender a sua patogénese de forma a guiar o desenvolvimento de medicamentos antifúngicos otimizados é premente. No decorrer da última década, tem sido dado mais destaque aos tratamentos cujo alvo é específico à interação hospedeiro-patógeno, o que em teoria pode possibilitar um impacto mínimo dos medicamentos na sobrevivência dos organismos comensais, tornando-os menos suscetíveis a conduzir uma resistência universal no microbioma. Assim sendo, o melhor conhecimento sobre as interações hospedeiro-espécies de *Candida* pode levar a novos alvos para os medicamentos antifúngicos e consequentemente auxiliar no controlo destas infeções. Recentemente, as vesículas extracelulares ganharam muito interesse na investigação como um mecanismo onnipresente na transferência de informação entre células e organismos em todos os três domínios da vida. Para além das funções na fisiologia normal, estas partículas têm sido expostas como mediadores de um mecanismo anteriormente não reconhecido para empacotar e proteger carga para absorção e integração noutras células, nomeadamente produtos secretados por patógenos, emergindo assim como um tipo de interação hospedeiro-patógeno funcionalmente diverso. Considerando as potenciais funções relatadas para estas vesículas nas interações hospedeiro-*Candida*, há motivação significativa para aumentar a nossa compreensão sobre a biologia e funções das mesmas, o que pode resultar num melhor entendimento da patogénese de *Candida* e ajudar a elucidar possíveis alvos terapêuticos para o desenvolvimento de vacinas ou imunoterapias contra este tipo de infeções.

O objetivo desta dissertação foi estudar a biologia dos exossomas secretados por *C. albicans* SC5314 e *C. albicans* 124A e as suas funções num ambiente de infeção, através do seu isolamento para posterior caracterização, avaliação de citotoxicidade, análise de fagocitose e produção de citocinas quando em cultura com uma linha celular de macrófagos de murinos. Adicionalmente, este trabalho teve como objetivo estabelecer um modelo de embrião de peixe-zebra para estudar as interações hospedeiro-*Candida*, permitindo estudos adicionais sobre o papel de exossomas na patogénese de *Candida* na escala de um organismo inteiro.

ABSTRACT

Since the 1980s, fungi have been revealed as major causes of human disease, where *Candida* species represent the most common cause of opportunistic fungal infections worldwide. Through the years, there has been an increase in the occurrence of these infections, and in the number of drug-resistant species which contributed to greater percentages of morbidity and mortality, particularly amongst the immunocompromised. Accordingly, *Candida* infections have become a significant influence in public health which leads to a necessity of better understanding their pathogenesis to improve therapeutic options and facilitate the development of enhanced antifungal drugs. Over the past decade, more prominence has been placed on treatments aiming to target specifically the host-pathogen interaction which, in theory, could enable a minimal impact from the drugs on the survival of commensal organisms, being less susceptible to eliciting resistance in the microbiome. Therefore, improved knowledge about host-*Candida* species interactions may identify new targets for the antifungal drugs and consequently assist in controlling these infections. More recently, extracellular vesicles (EVs) have gained much interest in research as a ubiquitous mechanism for transferring information between cells and organisms across all three domains of life. In addition to their influence on normal physiology, these particles have also been pinpointed as mediators of a previously unrecognized mechanism to package and protect cargo for uptake and integration into other cells, namely pathogen secretion products, emerging as a functionally diverse type of host-pathogen interaction. Considering the potential roles reported for EVs in host-*Candida* interactions, improving existing comprehension on the biology and functions of *Candida* EVs could open new views for understanding *Candida* pathogenesis and help elucidate possible therapeutic targets and develop potential vaccines or immunotherapy against this infection.

The aim of this thesis was to study the biology of *C. albicans* SC5314 and *C. albicans* 124A exosomes, and their roles in an infection environment, by isolating them for further characterization, cytotoxicity evaluation, phagocytosis assessment and cytokine production analysis when in culture with a murine macrophage-like cell line. Furthermore, this work aimed to establish an effective zebrafish embryo model to study host-*Candida* interactions, to allow further studies on the role of exosomes in *Candida* pathogenesis in the scale of a whole organism.

TABLE OF CONTENTS

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROSi

STATEMENT OF INTEGRITY ii

AGRADECIMENTOS iii

RESUMO iv

ABSTRACT v

LIST OF FIGURES xii

LIST OF TABLES xiv

Chapter 1. Introduction 1

1. *Candida* and candidiasis 2

1.1 Clinical features of candidiasis 2

1.2 Epidemiology of candidiasis..... 4

1.3 Virulence factors of *Candida* species..... 7

1.3.1 Adhesion 8

1.3.2 Hyphal morphogenesis 9

1.3.3 Hydrolytic enzymes 10

1.3.4 Phenotypic switching 11

1.3.5 Biofilm formation 12

1.4 Challenges in the treatment of candidiasis 13

1.5 Extracellular vesicles and infectious diseases 14

1.5.1 Fungal extracellular vesicles 16

1.6 Models to assay *Candida* virulence 18

1.7 Zebrafish 19

1.7.1 Species characteristics 19

1.7.1.1 Life cycle..... 21

1.7.1.2 Reproduction..... 22

1.7.1.3 Embryonic development.....	22
1.7.2 Zebrafish as a research model.....	24
1.7.3 Modeling human <i>Candida</i> infections in zebrafish.....	26
1.7.4 Fish embryo test (FET)	27
1.8 Outline and aims of the thesis	28
 Chapter 2. Materials and Methods.....	 29
2. Materials and Methods.....	30
2.1 Strains, Media, and Growth Conditions	30
2.2 Obtaining yeast-free supernatant	30
2.3 Exosomes analysis.....	31
2.3.1 Sample size and polydispersity determination by dynamic light scattering	31
2.3.2 Surface charge measurement by electrophoretic light scattering.....	32
2.3.3 Scanning transmission electron microscopy analysis	32
2.3.4 Protein concentration determination	33
2.3.5 Protein gel electrophoresis and Western blot techniques	33
2.4 Macrophage studies.....	33
2.4.1 Cytotoxicity analysis.....	34
2.4.2 Phagocytosis analysis	34
2.4.2.1 Preparation of yeast cells.....	34
2.4.2.2 Preparation of J774A.1 macrophage cell line	35
2.4.2.3 Phagocytosis of <i>Candida</i> cells	35
2.4.3 Cytokine production	35
2.5 Zebrafish studies.....	36
2.5.1 Zebrafish maintenance and husbandry.....	36
2.5.2 Parental zebrafish feeding	36
2.5.3 Stock aquarium maintenance	37

2.5.4 Breeding & Hatching aquaria..... 38

2.5.5 Photoperiod..... 39

2.5.6 Infection of zebrafish embryos with *Candida* cells 39

2.5.6.1 Preparation of yeast cells..... 39

2.5.6.2 FET protocol..... 39

Chapter 3. Results and Discussion 41

3. Results and Discussion 42

3.1 Exosomes isolation and Comparison between concentration systems 42

3.2 Characterization of *C. albicans* exosomes 45

3.3 Analysis of exosomes isolation by Western blotting 50

3.4 The effect of exosomes in cytotoxicity, uptake and cytokine production by macrophages
..... 51

3.5 The effect of zebrafish embryos co-incubation with *Candida* cells on their survival rate 55

Chapter 4. Final Remarks and Future Perspectives 57

4. Final remarks and future perspectives 58

Chapter 5. References..... 60

5. References..... 61

Chapter 6. Supplementary data 74

6. Supplementary data 75

LIST OF ABBREVIATIONS AND ACRONYMS

°C	Degrees Celsius
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
ζ-Potential	Zeta Potential
AIDS	Acquired ImmunoDeficiency Syndrome
Als	Agglutinin-like sequence
BCA	Bicinchoninic Acid Assay
CO₂	Carbon dioxide
CFU	Colony Forming Unit
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dpf	Days post fertilization
ECMM	European Confederation of Medical Mycology
ELS	Electrophoretic Light Scattering
EV	Extracellular Vesicle
FBS	Fetal Bovine Serum
FET	Fish Embryo Test
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
hpf	Hours post fertilization
hpi	Hours post infection
HRP	HorseRadish Peroxidase
ICU	Intensive Care Unit
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
kcps	Kilo counts per second

kDa	Kilodalton
kV	Kilovolt
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
mL	Mililiter
mm	Milimeter
mM	Milimolar
MM	Minimal Medium
MOI	Multiplicity Of Infection
MTT	3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWM	Molecular Weight Marker
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
nm	Nanometer
NMWL	Nominal Molecular Weight Limit
OD	Optical Density
OD640	Optical Density measured at the wavelength of 640 nm
OECD	Organization for Economic Co-operation and Development
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PDI	PolyDispersity Index
PI	Propidium Iodide
PL	Phospholipase
ppm	Parts per million
rpm	Rotations per minute
RT	Room Temperature
RVVC	Recurrent VulvoVaginal Candidiasis
Sap	Secreted aspartyl proteinase
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STEM	Scanning Transmission Electron Microscopy

TCA	Trichloroacetic Acid
TNF-α	Tumor Necrosis Factor alpha
UTI	Urinary Tract Infection
v/v	Volume per volume
w/v	Weight per volume
YEPD	Yeast Extract Peptone Dextrose

LIST OF FIGURES

Figure 1. Main routes of entry into the bloodstream by <i>Candida</i> ..	4
Figure 2. Mechanisms of action of polyenes, azoles, echinocandins, and 5-FC.....	13
Figure 3. Zebrafish adults.	20
Figure 4. Zebrafish embryonic development.	20
Figure 5. Life cycle of zebrafish.....	21
Figure 6. Stages of zebrafish embryonic development.	23
Figure 7. Sequence of the regular flow followed at research trials, from pre-clinical to clinical testing.	25
Figure 8. Schematic representation of a dip cell.....	32
Figure 9. Freshwater aquaria system layout.	36
Figure 10. Stock aquarium.....	37
Figure 11. Breeding and Hatching aquaria.....	38
Figure 12. Scheme of FET to evaluate the effect of zebrafish embryos co-incubation with <i>Candida</i> cells on their survival rate.	40
Figure 13. Representative mean size, PDI and ζ -potential of samples resulting from the two concentration systems tested: Amicon® Ultra-15 Centrifugal Filter Unit and Amicon® Stirred Cell, where <i>C. albicans SC5314</i> free supernatant whose growth was performed in YEPD medium was used.	43
Figure 14. DLS size distributions of samples resulting from the two concentration systems tested: Amicon® Ultra-15 Centrifugal Filter Unit and Amicon® Stirred Cell, where <i>C. albicans SC5314</i> free supernatant whose growth was performed in YEPD medium was used.	44
Figure 15. Representative mean size, PDI and ζ -potential of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where <i>C. albicans SC5314</i> and <i>C. albicans 1244</i> free supernatants whose growth was performed in YEPD medium were used.	46
Figure 16. DLS size distributions of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where <i>C. albicans SC5314</i> and <i>C. albicans 1244</i> free supernatant whose growth was performed in YEPD medium were used.....	47
Figure 17. Representative mean size, PDI and ζ -potential of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where <i>C. albicans SC5314</i> and <i>C. albicans 1244</i> free supernatant whose growth was performed in minimal medium were used.	48
Figure 18. DLS size distributions of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where <i>C. albicans SC5314</i> and <i>C. albicans 1244</i> free supernatant whose growth was performed in minimal medium were used.....	49

Figure 19. Western blot analysis of *C. albicans*-free supernatants concentrated samples alongside with their residues using anti-Hsp70 exosome antibody. 50

Figure 20. Viability of J774A.1 cells after 24 h incubation with *C. albicans SC5314* and *C. albicans 1244*-free supernatants. 52

Figure 21. Quantification of macrophage phagocytosis of yeast cells evaluated by fluorescence microscopy, where macrophages from test conditions were previously incubated with *C. albicans SC5314* and *C. albicans 1244*-free supernatants for 24 h. 53

Figure 22. Illustration of macrophage phagocytosis of yeast cells by fluorescence microscopy in the control and the test condition where macrophages were previously incubated with *C. albicans SC5314*-free supernatant for 24 h..... 53

Figure 23. Production of TNF- α by J774A.1 cells after 24 h incubation with *C. albicans SC5314* and *C. albicans 1244*-free supernatants, and after 45 min incubation with *C. albicans SC5314*-yeast cells. 54

Figure 24. The survival rates of zebrafish embryo infected with different doses of *C. albicans SC5314* along 80 hpf in three independent replicates 56

LIST OF TABLES

Table 1. Agents of opportunistic mycosis.....	5
Table 2. Species distribution of <i>Candida</i> bloodstream isolates..	6
Table 3. Mortality rates of <i>Candida</i> bloodstream infections.	7
Table 4. Strains and media used in this study.	30
Table 5. Concentrations achieved in the two different concentration systems.	42
Table 6. Concentration achieved in the Amicon® Ultra-15 Centrifugal Filter Unit.....	45
Table 7. Count rates and attenuators of samples obtained from the two concentration systems.	75
Table 8. Count rates and attenuators of samples obtained from the Amicon® Ultra-15 Centrifugal Filter Unit which growth was performed in YEPD medium.....	75
Table 9. Count rates and attenuators of samples obtained from the Amicon® Ultra-15 Centrifugal Filter Unit which growth was performed in minimal medium.....	75

Chapter 1. Introduction

1. *Candida* and candidiasis

Since the 1980s, fungi have been revealed as major causes of human disease, particularly in immunocompromised individuals and with critical diseases. These infections are associated with considerable percentages of morbidity and mortality, making it clear that fungal diseases have a significant influence on public health [1]. The main concerns about this situation are related to the growing number of fungal infections, as well as the diversity and complexity associated to the etiological agents involved [2]. This increased incidence is associated with several factors, such as a greater use of antibiotic drugs, frequent invasive medical procedures, and an increased number of immunocompromised individuals due to mucosal or cutaneous barrier disruption, neutropenia, qualitative neutrophil dysfunction, defective cellular immunity, metabolic dysfunction, and extremes of age [3], [4]. These immunodeficiencies are commonly attributed to cytotoxic and mucotoxic chemotherapy, radiation therapy, transplantations, and steroids administration, that further increase the risk for different fungal pathogens infections [3].

The most common fungi causing life-threatening infections are *Candida*, *Cryptococcus* and *Aspergillus* species. In addition to these, there are many other agents that can cause human disease, such as *Saccharomyces*, *Coccidioides*, and *Histoplasma* species [5]. Despite the vast list of pathogenic agents, *Candida spp.* remain the most regular cause of human fungal infections [1]. This genus consists of approximately 200 species, of which more than 30 have been identified as causes of human infection such as *C. albicans*, *C. parapsilosis*, *C. auris*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and the list continues to extend [6]. These are opportunistic fungal pathogens that commonly live as commensals in several locations of the human body, such as the oral cavity, the gastrointestinal tract, and the genitourinary system. In the commensal state, these species live as benign members of the microflora of healthy individuals, causing no disease. However, in response to disorders of the normal flora balance or changes in host physiology that compromise the immune defenses that normally suppress the growth of these commensals, they become able to invade tissues and cause opportunistic infections, denominated candidiasis [7].

1.1 Clinical features of candidiasis

Most infections due to *Candida* species are endogenous, meaning that are caused by prior colonization of locations of the human body, such as the mouth, skin, gastrointestinal tract, and vagina. The infection is asymptomatic until some disorder in the normal flora balance or change in host physiology occurs and

compromises the immune system, which allows the infection to become symptomatic [7]. Additionally, candidiasis can also be acquired by exogenous sources, mainly in healthcare environments. Several *Candida* species have been isolated from different sources in healthcare facilities, such as the floor, countertops, portable equipment, doorknobs, and many other surfaces [8], [9].

Candida species can cause a wide spectrum of infections, from superficial lesions in mucosa and skin that are frequently community acquired and responsible for substantial morbidity to deep-seated, invasive, and systemic infections that are typically nosocomial in origin [10]. Superficial candidiasis is the most common form of candidiasis. It is frequent in individuals with prior colonization, emerging when the immune defenses become compromised and primarily affecting the mucosa of the human oral cavity and the vagina [11]. Some of the most common clinical manifestations of this type of candidiasis include oral candidiasis, vulvovaginal candidiasis and candiduria [12], [13], [14]. *Candida* species are commonly present in the mouth of healthy individuals as commensals, with carriage rates ranging from 20% to 75% [15]. However, changes in the oral cavity environment can induce the overgrowth of *Candida*, which can lead to local discomfort, alteration of the taste sensation, and dysphagia. Oral candidiasis is the most common human fungal infection especially in early and later life. Other predisposing factors include diabetes mellitus, immunocompromised individuals such as patients diagnosed with HIV/AIDS or leukemia, those using broad spectrum antibiotics, and individuals in critical conditions [12]. Vulvovaginal candidiasis is the second most common vaginal infection, which affects 75% of the female population at least once in their life span [14]. In addition, approximately 50% of these women will experience a single recurrence, and 5% – 8% will experience recurrent vulvovaginal candidiasis (RVVC), which is defined as four episodes, at least, of infection per year and requires extensive treatments [16]. Possible risk factors for RVVC include treatment-resistant *Candida* species, use of antibiotics, immunodeficiencies, use of contraceptives, sexual activity, and inadequate treatment for the vulvovaginal candidiasis [17]. Candiduria, the presence of *Candida* species in urine, is a common clinical finding, particularly in hospitalized patients. Multiple studies indicate that more than 10% – 15% of hospital acquired urinary tract infections (UTIs) are caused by *Candida* species, being that all common *Candida* species can cause this type of infection [13]. Invasive candidiasis only manifests itself in severe cases of debilitation or when the immune system is critically compromised. In this type of infection, *Candida* species may enter the bloodstream, which is defined as candidaemia, and penetrate multiple organs of the human body like the lung, liver, kidneys, and bone. The mortality rate among individuals with invasive candidiasis is around 40%, even when antifungal therapy is prescribed. Particularly, candidaemia is associated with the highest crude mortality of all bloodstream infections, being that in several studies, the crude mortality of candidaemia exceeds 50% even in non-neutropaenic individuals [18], [19].

Candida cells can enter the bloodstream through three main routes. The entry can occur by the natural way, where yeast cells penetrate epithelial cells from mucosal surfaces into deeper tissues and blood capillaries or vessels, or by the iatrogenic way, through medical devices (catheters, artificial joints, and prosthetic heart valves) in which biofilms play an important role, or through the damage of defense barriers due to surgeries, polytraumas or drug treatment (**Figure 1**) [19]. Although *Candida* species can infect several locations in the human body, it is known that the immune protection against them is site-specific. In animal models of candidiasis, the protection against systemic infections does not show results against superficial infections. T-cell immune responses are key in protection against superficial candidiasis but resistance to systemic candidiasis is regularly associated with a functional phagocytic response involving neutrophils or mononuclear phagocytes [20].

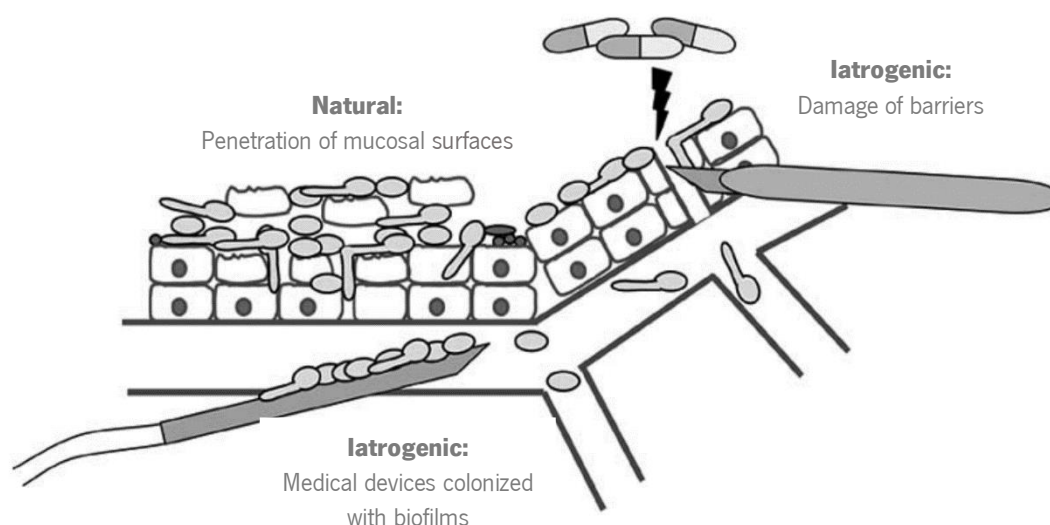


Figure 1. Main routes of entry into the bloodstream by *Candida*. Adapted from [19].

1.2 Epidemiology of candidiasis

Fungal infections are major causes of human disease, particularly among critically ill or immunocompromised individuals, with substantial rates of morbidity and mortality associated. Additionally, *Candida* species are the most common cause of opportunistic fungal infections worldwide (**Table 1**) [1].

Table 1. Agents of opportunistic mycosis. Adapted from [1].

Organism (s)	No. of cases/million/yr	Case/fatality ratio (%)
Yeasts		
<i>Candida</i> species	72.8	33.9
<i>Cryptococcus</i> species	65.5	12.7
Other yeasts		
Hyaline molds		
<i>Aspergillus</i> species	12.4	23.3
Zygomycetes	1.7	30.0
Other hyaline molds	1.2	14.3
Dematiaceous molds	1.0	0
<i>Pneumocystis jiroveci</i>		

Over the last decades, several epidemiologic studies have been conducted to assess the incidence of superficial and invasive candidiasis. Regarding UTIs, studies indicate that at least 10% - 15% of hospital-acquired UTIs are caused by *Candida* species. Candiduria occurs most commonly in adult patients present in intensive care units (ICUs) who often have multiple predisposing factors such as diabetes mellitus, indwelling urinary catheters, and exposure to antimicrobials, as well as in neonates and children present in ICUs, particularly in premature infants [13]. Moreover, *Candida* species are considered the secondary cause for onychomycosis, being responsible for 2% - 11% of the cases [21], can cause at least one episode of vulvovaginal candidiasis in the life span of 75% of female population [16], and are also responsible for 13.2% of all intra-abdominal infections, which are considered the most common type of deep-seated candidiasis [22], [23].

The incidence of invasive *Candida* infections has been studied by several multi-institutional surveys, such as the European Confederation of Medical Mycology (ECMM) survey [24], and the National Epidemiology of Mycosis Survey [25]. These studies demonstrated that candidaemia frequency among hospitalized patients has doubled over the past two decades, being now considered the fourth most common nosocomial bloodstream infection in the United States and the fourth most common catheter-related bloodstream infection in Europe [19]. Data from ECMM indicate that *C. albicans* is the species most frequently isolated from the blood of patients diagnosed with invasive fungal infection [24]. Accordingly, it is considered the most prevalent and the most pathogenic of all *Candida* species, being responsible for most systemic and oral candidiasis manifestations along with community-onset and nosocomial candidaemia [26]. However, over the years there has been a significant increase in the incidence of infections caused by other *Candida* species,

which may be explained by the introduction of fluconazole in 1990 [27]. This agent had good activity against *C. albicans* with reduced toxicity when compared to the other anti-fungal that existed, so it became the agent of choice for many fungal infections as well as for prophylactic purposes. Although there is no conclusive evidence, many specialists in the field consider that it was the selective pressure applied by this therapeutic concept that caused changes in the epidemiology [28]. The most common *non-albicans Candida* species are *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, although the incidence rates can slightly vary according to the population in analysis (**Table 2**) [19].

Table 2. Species distribution of *Candida* bloodstream isolates. Adapted from [19].

<i>Candida</i> species	Candidaemia, Europe
<i>C. albicans</i>	49 – 53%
<i>C. parapsilosis</i>	11 – 21%
<i>C. glabrata</i>	10 – 12%
<i>C. tropicalis</i>	6 – 11%
<i>C. krusei</i>	1 – 9%
Others	1 – 10%

C. parapsilosis incidence has dramatically increased over time, being frequently reported as the second *Candida* species most frequently isolated from blood cultures [29]. It is generally a commensal of human skin whose pathogenicity is limited by an intact integument. This species is well known for its ability to grow in total parenteral nutrition, the formation of biofilms on catheters and other implanted devices, for nosocomial spread by hand transferences, and for its persistence in the hospital environment [30]. This fungal pathogen is particularly important since its incidence is much higher among young children, specifically those aged <2 years, when compared with adults [31]. *C. glabrata* is often described as the second or third most common cause of candidiasis, being commonly isolated from immunocompromised patients such as intensive-care, neutropenic, postsurgical, and bone marrow transplant patients [32]. This species is of special importance due to its innately reduced susceptibility to antifungal agents, specifically the azoles, which results in harder to treat infections [33]. *C. tropicalis* is considered the third most frequently isolated *non-albicans Candida* species from blood and urine cultures, being commonly associated with patients with malignancy and neutropenia [34]. Lastly, *C. krusei* is described as the fifth most commonly isolated *Candida* species from bloodstream isolates. Nonetheless, this species is usually intrinsically resistant to fluconazole and can cause serious infections in susceptible patients, which results in a significant challenge to clinicians [35].

The mortality rate from candidaemia ranges approximately from 30% to 60% in European surveys, depending on the species and geographic location in analysis (**Table 3**) [36]. Similar studies have been conducted in the United States, which revealed slight differences in the incidence of candidiasis when compared to Europe. This variability is likely due to differences in patient demographics and comorbidities, as well as distinct medical practices [1]. In addition to high mortality and morbidity rates, candidaemia also extends hospitalization times of those surviving these infections, which consequently increases medical care costs. Systemic *Candida* infections have been associated with an attributable intensive care unit cost of US \$21,590 [24].

Table 3. Mortality rates of *Candida* bloodstream infections. Adapted from [36].

<i>Candida</i> species	ECMM survey	
	No. of episodes	Mortality rate, % (range)
<i>C. albicans</i>	1090	38.5 (29 – 45)
<i>C. glabrata</i>	269	45.0 (25 – 64)
<i>C. parapsilosis</i>	263	25.9 (0 – 34)
<i>C. tropicalis</i>	140	41.4 (20 – 44)
<i>C. krusei</i>	38	55.3 (20 – 67)
Total	1942	37.9 (27 – 40)

a Range reflects data from all participating countries.

b Includes 142 other less common *Candida* species not reported in detail.

1.3 Virulence factors of *Candida* species

To develop an infection, opportunistic pathogens must be able to colonize a host, permeate its surface, evade the immune system, endure and divide in the specific host environment, and disseminate to other tissues [37]. As common commensals of the normal flora, *Candida* species are adapted to and specialized for survival on host surfaces. Throughout the transition to superficial and invasive infections, these pathogens need to adapt to drastically changing environments with extreme changes in several parameters, such as oxygen and carbohydrate levels, pH, osmolarity, availability of nutrients and temperature. The capacity of causing infections at such a vast range of body sites demonstrates the need of specific factors that are adapted and essential to each stage of infection to ensure survival and proliferation. The mechanisms required

to surpass all these adaptations are designated as virulence factors [19]. The virulence factors required by *Candida* species to cause infections may differ depending on the type of infection, the site and phase of infection, and the nature of the host response. Many factors have been proposed to be virulence attributes for *Candida*, yet the most studied are hyphal formation, adhesion capacity, phenotypic switching, hydrolytic enzyme production, and biofilm production [38].

1.3.1 Adhesion

An effective *Candida* infection begins with the adherence of the pathogen to specific host cells and/or to the surface of medical devices. Therefore, adhesion is an essential step in the infection process, and its extension is dependent on microbial, host and abiotic surface properties, such as cell-surface hydrophobicity and cell-wall composition [39]. The presence of specific cell-wall proteins, commonly referred to as adhesins, is considered a significant factor in the adhesion ability of *Candida* species. Adhesins are defined as biomolecules that promote the adherence of *Candida* species to host cells or host-cell ligands, and various have already been identified and characterized [20], [39]. Mutants deficient in genes encoding these adhesins not only manifested attenuated adherence to host substrates *in vitro* but also a corresponding reduction in virulence in multiple experimental models of *Candida* infection. The scope of these reductions has been rather variable, which is thought to be caused by other adhesins actions with redundant or overlapping functions [40]. The majority of the *Candida* adhesins are glycoproteins, being the most studied from the agglutinin-like sequence (Als) family. The Als protein family is encoded by eight genes, ALS1 – 7 and ALS9, and expresses three common domains amongst the belonging proteins [37]. The central domain contains variable numbers of a tandem repeat sequence which is highly glycosylated, and the N-terminal domain consists of highly variable sequences which is a feature thought to be responsible for differences in Als proteins' functions and interactions with the host [19]. For example, Als1p has an adhesin function to human buccal epithelial cells important during the early stage of infection, while Als5p provides adherence to fibronectin, collagen, bovine serum albumin, and laminin [37], [40]. Furthermore, the ALS genes are differentially expressed among *Candida* species and depend on the growth conditions, for instance ALS3 is hypha specific while ALS4 expression is associated with growth phase [19]. Multiple strains of *C. albicans* express all eight ALS genes, nonetheless only five ALS genes were detected in *C. parapsilosis* and three in *C. tropicalis* and *C. dubliniensis* [39], [41]. Additional adhesins have been identified, including the epithelial adhesin family in *C. glabrata*, the glycoposphatidylinositol-anchored protein 30 in *C. parapsilosis*, and the Ala1 and Hwp1 in *C. albicans* [19], [39].

1.3.2 Hyphal morphogenesis

The ability to undergo a morphological switch between the unicellular budding yeast form and the filamentous forms, namely the pseudohyphal form characterized by chains of elongated yeast cells, and a hyphal form characterized by branching chains of tubular cells without constrictions at the sites of septation, is considered a crucial virulence factor in the infection process of some *Candida* species [42]. The formation of true hypha is observed in species such as *C. albicans*, *C. dubliniensis* and *C. tropicalis*, although the last two species have a reduced efficiency and a more confined range of conditions in which they are capable of proceeding with the switch. The majority of other *Candida* species are only able to transition between yeast and pseudohyphal states, like *C. parapsilosis*, *C. lusitaniae* and *C. krusei* [43]. *C. glabrata* is typically described as a non-dimorphic yeast, however some studies report the ability of pseudohyphae formation in certain strains and in response to some stimuli [44], [45]. Several environmental stimuli are described to promote the yeast-to-hypha transition in *Candida* species, including the presence of serum at 37 °C, neutral or alkaline pH, carbon starvation, nitrogen starvation, low oxygen, elevated CO_2 , among others. This transition is mediated by several signal transduction regulators and is accompanied by the increased expression of a large number of genes, which includes many transcription factors such as Cph1, Efg1 and Hms1 [42]. Nonetheless, a core set of induced genes common to many hyphal induction modes can be specified, which includes genes encoding hyphal cell surface components such as the Hwp1 and Als3 proteins, suggesting these as a prerequisite for hyphae invasion [46]. Another common hallmark in hyphae penetration consists in the secretion of enzymes able to degrade proteins, lipids, and other cellular components easing the infiltration into solid substrates and tissues [47]. Hyphal morphogenesis is the most prominent morphological change in *Candida*'s life cycle, specifically *C. albicans*. This feature has been suggested to comprise important functions in the establishment of infections, including the ability to transition from candidemia to the subsequent tissue invasion, since *Candida* cells are found predominantly in the hyphal form in tissue samples of candidiasis patients, and the capacity to escape phagocytosis and kill macrophages [42]. Hyphae development is considered an essential virulence factor in *Candida* pathogenesis, nonetheless most lesions are colonized by either morphological form, which indicates that both have a role in the establishment and progression of infection [20]. Yeast cells have been proposed to be better adapted for dissemination and damage while hyphae are vital for tissue and organ invasion, and for adaptation to the various host niche's conditions [47].

1.3.3 Hydrolytic enzymes

In recent years, the secretion of hydrolytic enzymes has gained attention for their potential role throughout the progression of candidiasis as a virulence feature for degradation of host surfaces to aid adhesion and invasion, resistance to the host's immune system by destroying the host immune factors, and nutrient takeover. In *Candida* species, the most significant secreted hydrolases include families of secreted aspartyl proteinases (Saps), phospholipases, and lipases [19]. The Saps are considered the most studied type of secreted hydrolases. The virulence mechanism of these enzymes consists of assisting invasion and colonization of the host tissues by disrupting host mucosal membranes and degrading crucial immunological and structural defense proteins, such as immunoglobulin G heavy chains, C3 proteins, lactoperoxidase, collagen, fibronectin, among others [29]. The Sap proteins are encoded by a family of 10 SAP genes, which exhibit differential expression profiles under multiple conditions, in different *in vivo* models and in candidiasis patients [19]. For example, it was demonstrated that SAP genes are differentially expressed in the different morphological states of *Candida*. Expression of SAP1-3 is predominant within the cell wall and cytoplasmic membrane of yeast cells and has been indicated as crucial for superficial infections in experimental mucosal and cutaneous candidiasis, whereas SAP4-6 are suggested to be important for systemic disease since they were localized at the tips of hyphae during invasion [48]. It has been suggested that Sap5 may be required for organ invasion whilst Sap2 is necessary in a later stage of infection to degrade host proteins for nutrition [19]. Moreover, SAP8 and SAP9 transcripts are mainly detected in yeast cells grown at 25 °C and in later growth phases, respectively [49]. Differences in size and optimal pH are also exhibited in between these enzymes. The variability among Sap proteins may prove vital to the success of *Candida* as an opportunistic pathogen by enabling the fungus to survive and cause infections on a variety of different tissues [38], however the expression of the SAP genes vary according to the *Candida* species. The expression of all ten SAP genes is exclusively detected in *C. albicans* strains, while in *C. parapsilosis* and *C. tropicalis* strains only three (SAP1-3) and four (SAP1-4) SAP genes have been detected, respectively [39]. In *C. glabrata*, *C. krusei*, *C. lusitanae*, and *C. guilliermondii* some proteinases are detected, however the number and roles of these have not been well established [37]. Although Saps are considered virulence-associated factors in this model, the variability of SAP genes found among *Candida* species indicates that other factors must be contributing to invasion and cell damage. This was demonstrated by the results obtained by Correia *et al.* (2010) that used sap-null mutant *Candida* strains to infect murine models and assess the differences between those infected with wild-type strains [50]

Phospholipases (PLs) are enzymes capable of hydrolyzing one or more ester linkages in glycerophospholipids, and are classified into PLs A, B, C, or D depending on the different cleaved ester bonds.

The presence of these enzymes throughout the infection could conceivably contribute to host cell-membrane damage, which could promote cell damage and expose receptors to ease the adherence of *Candida* [39]. Gahnoun (2000) demonstrated that many blastospores developed hyphae with phospholipase activity concentrated at the growing tip during invasion, and that the highest activity was where the hyphae were in direct contact with the host cell membrane. Mostly, only hyphae could invade the membrane successfully, which confirmed that PLs are involved in the pathogenesis of candidiasis by easing the invasion of tissue [51]. The production of all classes of PLs has been defined for *Candida* species. In *C. albicans*, phospholipase A, B, C, and D activities have been detected, however only proteins encoded by the phospholipase B family (PLB1-5) seem to be extracellular [19]. Several studies have indicated that *C. tropicalis* and *C. parapsilosis* can produce extracellular PLs, although at much lower levels compared with *C. albicans*. Regarding *C. glabrata*, few studies were performed and no PL activity has been reported [39].

Lipases catalyze both the hydrolysis and synthesis of triacylglycerols and are characterized by their stability at elevated temperatures and in organic solvents, and their resistance to proteolysis. The roles of these enzymes in the progression of infection include the digestion of lipids for nutrient acquisition, adhesion to host cells and tissues, and initiation of inflammatory processes by affecting immune cells [29]. Their significance was demonstrated by Gácsér *et al.* (2007) considering that their results showed that the use of lipase inhibitors substantially reduces tissue damage during infection in reconstituted human tissue models [52]. Lipases are encoded by a gene family – LIP1 to LIP10, and in a similar manner to the SAP gene family, the LIP genes are differentially expressed at different stages and sites of infection [19]. In *C. albicans* and *C. tropicalis* the ten LIP genes were detected, while for *C. parapsilosis* only two LIP genes, LIP1 and LIP2, have been reported. No studies have been conducted concerning the expression of LIP genes in *C. glabrata* and *C. krusei* [39].

1.3.4 Phenotypic switching

The colonies of *Candida* species are capable of reversibly switching between different morphologies, a process known as phenotypic switching. This aptitude is thought to assist survival in various microenvironments, evasion of the host's immune response, and to influence adhesion, hyphal formation, and sensitivity to neutrophils [19]. Nonetheless, the fundamental mechanism of this virulent factor and its role in the pathogenesis of *Candida* species remains open to interpretation [20]. Of all the switch phenotypes reported, the most studied is the white-opaque transition in the strain WO-1 of *C. albicans*, characterized by smooth and white colonies switching to flat and gray colonies, also referred to as opaque. Multiple differences

can be observed between these two colony types, including cell shape (white cells are round-ovoid and opaque cells are elongated or bean-shaped), cell surface structures (pimples are noticed on opaque cells only) and germination at 37 °C, pH 6.7 by white cells but not opaque cells [53]. Differences in gene expression are also exhibited with many genes being expressed in only one of the colony types, for example SAP3 is shown to be opaque-cell-specific while SAP2 is white-cell-specific [20]. These variations suggest that phenotypic switching may affect the way *Candida* cells behave according to the conditions encountered. This is supported by some experiments results, for instance, it was demonstrated that opaque phase cells have higher ability to colonize the skin while white cells are more virulent in a systemic animal model [54]. The phenotypic switching is most studied in *C. albicans*, however other *Candida* species also exhibit this virulent factor, such as *C. parapsilosis*, *C. tropicalis* and *C. glabrata*. Laffey & Butler (2005) identified four stable and heritable phenotypes in *C. parapsilosis*, nominated as crepe, concentric, smooth, and crater, along with evaluating their effect on biofilm formation [55]. In *C. glabrata*, four phenotypes were defined that are distinguishable by coloration: white, light brown, dark brown, and very dark brown [44]. *C. tropicalis* also revealed the existence of four phenotypes distinguishable by the shape of the colonies, nominated as smooth, rough, ring, and semi-smooth [56].

1.3.5 Biofilm formation

Candida infections frequently arise with the attachment of the pathogen to the host or/and the surface of medical devices followed by cell division, proliferation, and subsequent biofilm development. Biofilms are surface-associated microbial communities embedded within an extracellular matrix and are reviewed as the most prevalent growth form of microorganisms [39]. Particularly, *Candida* biofilms are composed of multiple layers of microbial cells and a matrix of extracellular material that may differ among species, which comprises polysaccharides, mannans, and proteins [43]. The development of biofilm is considered an important virulence factor for the progression of infections by several *Candida* species, since it provides significant resistance to antifungal therapies, by restricting the penetration of substances through the matrix, and protects the embedded cells from the host immune responses [29]. Moreover, the presence of biofilm throughout the infections has been associated with higher mortality rates, although it has been noticed that the biofilm formation is strongly dependent upon species, strains, and environmental circumstances such as pH, medium composition, and oxygen [39]. *C. albicans* is the species most commonly associated with biofilm formation, yet many other non-*albicans Candida* species also exhibit this capacity throughout the process of infection, including *C. tropicalis*, *C. parapsilosis*, *C. auris*, and more. The aptitude of various *Candida* species

to transition between different morphological states, and the substantial number of surface adhesins associated particularly with the hyphal form, such as ALS3 and HWP1, both perform key roles in sustaining biofilm formation [43]. Filamentous cells assist in structural integrity and strength to a biofilm and act as a support for yeast cells to adhere, while yeast cells may enable for dissemination of biofilm cells within a host [57], [58]. Estivill *et al.* (2011) analyzed the biofilm formation by five species of *Candida*, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* on three different clinical materials. According to the results obtained, *C. albicans* was not the species that showed greater biofilm formation on polyurethane, but *C. tropicalis*, while *C. parapsilosis* exhibited the greatest biofilm formation on the other two materials, PVC, and Teflon. *C. glabrata* was the species that showed lower biofilm formation capacity on all three materials. Participation of *C. krusei* in this study was limited since the isolation of these cells is rare, nonetheless all the existing isolates formed biofilms with no important variations among the varied materials [59].

1.4 Challenges in the treatment of candidiasis

The increasing incidence of fungal infections, particularly *Candida* infections, and the increasing variety and complexity among pathogenic species, have substantially contributed to great morbidity and mortality rates, particularly amongst immunocompromised individuals [1], [2]. Aiming to reverse the significant influence of these infections in public health, several antifungal agents have been formulated. These are classified into different groups according to the mechanism of action, namely polyenes, azoles, echinocandins and others antifungal agents (**Figure 2**) [60].

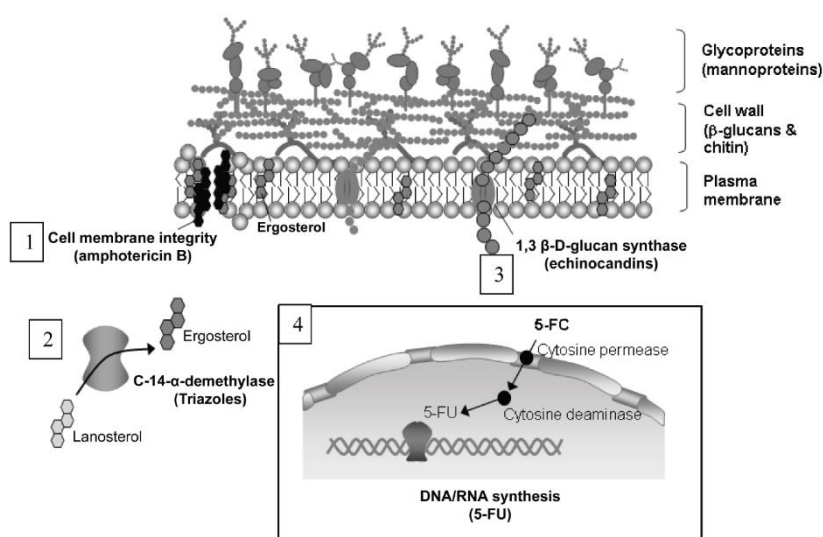


Figure 2. Mechanisms of action of (1) polyenes, (2) azoles, (3) echinocandins and (4) 5-FC. Retrieved from [165].

Polyenes are the major class of antifungal agents and are isolated from *Streptomyces* spp. Their mechanism of action is based on their interaction with ergosterol components of the fungal membrane, resulting in a polyene-sterol complex that provides an aqueous pore and affects cell permeability, which consequently causes cell leakage and cell death. Azoles are the second most studied antifungal agents and their mechanism of action is based in the inhibition of ergosterol biosynthesis. In more detail, exposure of fungal species to azoles leads to the inhibition of the ergosterol enzymatic pathway, especially the enzyme cytochrome P450 sterol 14 α -demethylase. This inhibition promotes the disruption of the membrane structure as well as its functions in nutrient transport and chitin synthesis, reducing fungal growth [60]. The echinocandins are lipopeptide molecules that act as inhibitors of the synthesis of β -1,3-D-glucan, an important component of the fungal cell wall, by blocking the action of a pathway enzyme, β -1,3-D-glucan synthase [61]. The absence of this component destabilizes the integrity of the fungal cell wall and promotes the osmotic instability and cell death [62]. In addition to these three major classes of antifungal agents, other compounds have also been used, including allylamines, flucytosines, griseofluvin, sordarins, nikkomycins, and ciclopiroxolamines [60].

Despite these formulations, several species remain problematic to treat due to delayed diagnostics, drug toxicity, drug bioavailability, lack of oral or intravenous preparations, and antifungal drug resistance [63]. Particularly, the extent of drug-resistant *Candida* species is increasing and persisting as a serious concern in the clinical setting. Considering that *Candida* species are responsible for most human fungal infections, it is of major importance to obtain a greater understanding of *Candida* species pathogenesis, in order to improve therapeutic options and facilitate the development of new antifungal drugs [64].

Over the past decade, more prominence has been placed on treatments aiming to target specifically the host-pathogen interaction, either through products of the host or the pathogen. This type of approach, in theory, would allow the new drugs to have a minimal impact on the survival of commensal organisms, being less prone to lead to a universal resistance in the microbiome [65]. Therefore, an improved understanding of the interactions between host-*Candida* species may identify new targets for the antifungal drugs and consequently assist in controlling these fungal infections.

1.5 Extracellular vesicles and infectious diseases

Most pathogens at some phase in their life cycle depend on the capacity to communicate with each other and with their hosts, however, the mechanisms that support this communication are still being revealed [66]. This communication can be mediated by direct cell-to-cell contact, but it is frequently directed by soluble

factors such as hormones, cytokines, and inflammatory mediators that are able to communicate with several cells either locally or at dispersed sites [67]. Research on this topic has been largely focused on soluble proteins secreted by pathogens, which have been proposed to down-modulate the host immune response, and to be involved in metabolic adaptation to the host environment and tissue invasion [68], [69]. More recently, extracellular vesicles (EVs) have gained much interest in cell biology and biomedicine as a ubiquitous mechanism for transferring information between cells and organisms across all three domains of life, including fifteen different fungal genera [66]. The term EVs is used to describe cell-derived double-layer phospholipid membrane particles that reach the extracellular environment, and these are highly heterogeneous, differing in several aspects such as biogenesis pathways, size, cargo, membrane composition, and biological functions. The cargo of EVs consists of a diverse suite of molecules including proteins, lipids and nucleic acids, some of which are reported to have immunomodulatory properties. EVs control various biological roles through the delivery of effectors to target cells, from the regulation of physiological events to the response to specific environmental conditions [70]. In addition to their influence on normal physiology, these particles have also been pinpointed as mediators of a previously unrecognized mechanism to package and protect cargo for uptake and integration into other cells, namely pathogen secretion products, thus emerging as a functionally diverse type of host-pathogen interaction. Pathogen-derived EVs are known to mediate highly complex interkingdom communications by transporting molecules from pathogens to hosts, spreading antigens, as well as infectious agents, and transferring genetic material to host cells or other pathogens [66]. Accordingly, these have been reported to have various functions in immune cell activation and suppression, and to play a role in tissue homeostasis and disease development, such as cancer and various infectious diseases [71], [72], [73], [74].

Based on their biosynthetic pathways, EVs are typically classified into two broad classes: microvesicles (also entitled ectosomes or microparticles) and exosomes [70]. Microvesicles are generally larger vesicles, ranging in size from 50 nm to 1000 nm in diameter but can have up to 10 μ m in the case of oncosomes. These particles are generated by the direct outward budding and fission of the plasma membrane and the subsequent release of vesicles into the extracellular space. Exosomes are smaller vesicles derived from the endocytic pathway, ranging in size from 30 to 150 nm in diameter. Their biogenesis initiates by inward budding when endosomes mature to form multivesicular bodies [75]. These structures can fuse with the hydrolytic lysosome, where the vesicle cargo is subsequently degraded or can fuse directly with the plasma membrane, releasing their intraluminal vesicles, now known as exosomes, into the extracellular space. Consequently, exosomes express markers of their parent cells, but are also enriched in other molecules associated with their biogenesis or that are selectively packaged into them, while microvesicles incorporate certain lipids, surface proteins, and other molecules before fission [66]. Nowadays, it is widely accepted that

cells from almost every type of organism are capable of secreting these nano- to micrometer-scale vesicles [76], however EVs are dynamic and strongly dependent on the source cell, cell topography, state, and environmental conditions. These factors determine their functionality and target specificity, meaning that the same cell type may secrete distinct types of vesicles depending on the specific factors it is exposed to [77].

1.5.1 Fungal extracellular vesicles

Fungal diseases result from a combination of fungal virulence factors and the defective host's capacity to control the fungal growth successfully, in which EVs are known to play a role in both of these factors [78]. The first fungal EVs to be isolated and described were those of *Cryptococcus neoformans* in 2007, and ever since they have been indicated as a crucial mechanism for molecular export in a variety of fungal species [79]. Nowadays, several fungal species' EVs have been characterized including those of *C. albicans*, *C. glabrata*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus*.

In *C. albicans*, the existence of vesicle-like compartments was demonstrated for the first time in cell wall pimples from opaque cultures of *C. albicans* cells in 1990 [80]. *C. albicans* EVs were first isolated and observed by transmission electron microscopy in 2008, an analysis that demonstrated the presence of bilayered compartments similar to those initially described for *C. neoformans* and *H. capsulatum* [81]. These were later further analyzed to unravel their characterization, composition and implications for human immune responses in wild-type and mutant strains [82]. *C. albicans* EVs were defined as being EVs typical of fungi, containing proteins, lipids, nucleic acids and carbohydrates. Microscopical observations reveal spherical structures with heterogeneous sizes, with the majority of the vesicles having sizes between 50 and 100 nm, and a smaller percentage having larger sizes reaching more than 200 nm, being that the distribution of EV particle size differs slightly for different strains [82] [83]. Several studies on *C. albicans* EVs attributed different roles to these in an infection situation. EVs from *C. albicans* were capable of activating murine macrophages and dendritic cells which indicated a potential role in modulating the innate immune response to the fungus [84], [85]. Contrarily, it was reported that the cargo of EVs produced by *C. albicans* biofilms includes key proteins and carbohydrates for biofilm biogenesis, making these vesicles crucial contributors to the establishment of biofilms, which are a critical component of *C. albicans* pathogenesis. Moreover, adding back EVs from wildtype biofilms to biofilms formed by strains with deletions in genes encoding important enzymes for the generation of biofilm matrix restored the loss of azole resistance in these biofilms. This goes accordingly with the fact that the biofilm matrix provides a protective layer against antifungal drugs and reveal a role in antifungal drug resistance for these vesicles [86].

EVs produced by fungi are multi-antigenic compartments that carry a number of biologically active molecules, including virulence factors, regulators and highly immunogenic components which indicates that these could activate the innate immune system and directly influence disease development [87]. Some of these elements are conserved among the different species, but others are species-specific [88]. In fact, recent studies propose that fungal EVs may represent a new alternative for the development of multivalent vaccine formulations. Various *in vitro* experiments demonstrate that fungal EVs are capable of stimulating phagocyte activity, promoting an increase in cytokine levels, modulating phagocytosis and regulating macrophage polarization. Altogether, this data indicates that fungal EVs activate the immune response during disease development, inducing pathogen killing [85], [89], [90]. Furthermore, there are several reports revealing a protective role from fungal EVs when these are exposed to the host models. For example, pretreatment of *Galleria mellonella* larvae with fungal EVs stimulates a protective response against a lethal challenge with *C. albicans*, *C. neoformans* and *Aspergillus flavus*, and more recently it was demonstrated that immunization of mice with EVs from *C. albicans* confers full protection against systemic candidiasis [87], [85], [91], [92]. However, in some models the outcome of the interaction between the host cells and the fungal EVs have also been associated with virulence and disease development, as demonstrated for *C. neoformans* and *S. brasiliensis* in murine models, respectively [93], [94]. Recently, a study revealed significant differences in the composition and roles of EVs released from two distinct strains of *C. auris* and *C. albicans* ATCC 90028. Results revealed that EVs from only one of the *C. auris* isolates potentiated the adhesion of the yeasts to epithelial cells and promoted the survival of the fungal cells modulating the host cell defense mechanisms when in culture with macrophages cells, which suggests that EVs from the same species can promote distinct changes in host cells. EVs from *C. albicans* had no effect on the adhesion factor but it had the capacity to prime the macrophages for enhanced intracellular yeast killing, following the pattern exhibited by most fungal EVs [78]. In addition, fungal EVs have been suggested to be involved in the development of antifungal resistance and a messenger compartment for transferring virulent factors [95], [86]. The multiple activities attributed to these vesicles indicate that the outcome of the interaction between fungal EVs and the host is indeed dependent on the model, species and strain that it is being investigated, which could be, more specifically, dependent on EVs characterization and composition [78].

Given the potential roles for EVs in host-pathogen interactions, specifically between host-*Candida* interactions, there is significant motivation to better understand the biology and functions of *Candida* EVs, which could open new views for understanding *Candida* pathogenesis.

1.6 Models to assay *Candida* virulence

A great amount of research on virulence can be performed with laboratory experimentation using tissue cultures. This method allows direct observation of the interaction between human cells and the pathogenic organisms and has already contributed with considerable knowledge in this field. It is intrinsically challenging to use this system to investigate the interaction of multiple cells and tissue types. Moreover, aside from the experiments performed with freshly harvested primary cells, tissue culture remains rather artificial by using standardized cell line preparations, which results in several cells that may or may not accurately reflect the biology of naturally developing cells *in vivo* [65]. Therefore, since these do not take into account the responses on the scale of an organism as a whole, cell culture systems are suitable for an initial data screening with the objective to assess fundamental data and use it to define future tests on other systems [96], [97]. With these limitations, it was concluded that infection modelling requires a whole organism where the complex host-fungus interactions can be investigated, and the disease development can be monitored right from the moment that fungal cells are introduced into the host. In order to be a good model, the specimen should be reproducible, relatively easy to set up, cost effective, and it should reproduce the major clinical symptoms that are known to occur in the human disease. With these conditions fulfilled, in-depth investigation of *Candida* pathogenesis can be performed and, consequently, inferences about *Candida*'s virulence in humans can be made [98]. Thus, to obtain a more comprehensive understanding of *Candida* species pathogenesis, it is of greatest interest to establish an effective animal model system that allows the study of *Candida*-host cells interaction.

Several large animals have been used to study *Candida* infections, such as macaques [99], piglets [100], rabbits [101] and guinea pigs [102], but nowadays the majority of *Candida* virulence research is performed with rodent models, due to advantages like ease of handling, economic factors, organism complexity, and the availability of genetically modified mouse strains that allows mimicking of human genetic conditions which offers proper predictivity [98]. In order to study *Candida* oral and vaginal infections, mucosal infection models have been performed mostly in rats and mice. Using these models, a lot of information about *Candida* virulence has been obtained through several mutant strains and clinical isolates, such as the fact that *C. albicans* pathogenesis requires yeast to hypha switching in oral infection [103]. For invasive infection models the mouse has been the most popular host of choice. There are two crucial models of *Candida* invasive infection: the intravenous infection model and the gastrointestinal colonization and dissemination model [104], being through the last one that it was demonstrated that *C. parapsilosis* had lower virulence compared to *C. albicans* but could still successfully establish persistent colonization of the gastrointestinal tract [105].

Nonetheless, several disadvantages limit the use of this model in large-scale studies, such as limited number of offspring produced, high experimental costs when comparing with other models, being time-consuming and the ethical constraints attached to it [97]. Various non-vertebrate model organisms have been developed to overcome these limitations, for instance, the fruit fly (*Drosophila melanogaster*), the wax moth (*Galleria mellonella*) and a nematode (*Caenorhabditis elegans*). These hosts have several advantages when compared with rodent models, like the simplicity, ethical expedient, inexpensive care systems, small size which enables high throughput studies designed to scan pathogen genomes for virulence-related genes or chemical libraries for antimicrobial compounds, well-developed molecular tools, and a conserved innate immunity [106]. Several studies have utilized these invertebrate models to improve the understanding of the virulence mechanism of *Candida* infection. For example, *C. elegans* model was used to compare the *in vivo* virulence of seven different *Candida* species with the *in vitro* production of proteinases and phospholipases, biofilm, and hemolytic activity [107]. In a wild-type *Drosophila melanogaster* model, a model with simple innate immunity, it was demonstrated that Pho84 gene is required for virulence of *C. albicans*, and in the *Galleria mellonella* model, it was demonstrated that a combination of molecular mechanisms, such as the presence of point mutation in the ERG11 gene, the overexpression of ERG11, and genes encoding efflux pumps, are involved in fluconazole resistance in *C. parapsilosis* [108] [109].

Despite the advantages of these invertebrate models, they still lack many structures, organ systems, and adaptive immunity that are involved in human disease pathogenesis, which limits their role in modelling human disease [110]. In this context, zebrafish has been used as a model organism alternative in numerous studies in the last years.

1.7 Zebrafish

Zebrafish represent a very interesting alternative model of increasing popularity, for studying *C. albicans*-related pathogenesis and other types of infections [111], [112], [113].

1.7.1 Species characteristics

Zebrafish, *Danio rerio* (Hamilton, 1822), is a small teleost representative of the Cyprinidae family, native to freshwater habitats in South Asia [114]. This vertebrate, illustrated in **Figure 3**, reaches 3 – 4 cm of body length as an adult and it is broadly distributed through India, Bhutan, Nepal, Bangladesh, and Pakistan, being

typically found in stagnant clear water or with a slow flow of shallow depth, approximately 30 cm, such as in streams, canals, ponds, and rice paddies [115], [116]. Zebrafish is omnivorous, and it has a varied diet that is mainly composed by zooplankton and insects but can also include phytoplankton, algae, fish scales, invertebrate's eggs and more [117], [118]. It can also tolerate and adapt to a wide range of environmental conditions, such as temperature and pH [114], [119].

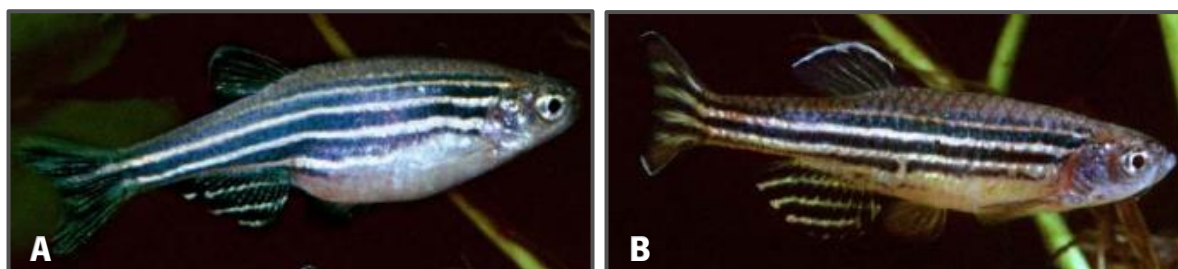


Figure 3. Zebrafish adults. (A) female and (B) male. Adapted from [147].

In the embryonic and larval phases, zebrafish are optically transparent, which allows for an easier analysis of the development during these phases (**Figure 4**). Reaching the juvenile and adult phases, the formation of blue colored bands along the trunk occurs, thus losing the transparency. Males usually appear to be more gold in color on the ventral side of their body and present a flatter abdomen than females which exhibit a more silver coloration along the trunk and a protruding belly (**Figure 3**) [120], [121]. Females have the capability to lay around 200 – 300 eggs every 2 to 3 days, which rapidly develop into larvae, *ex-utero*, within 120 h post-fertilization (hpf) [96].



Figure 4. Zebrafish embryonic development.

(A) Embryo at 8 h post-fertilization; (B) Embryo at 32 h post-fertilization;
(C) Hatched larvae at 56 h post-fertilization.

1.7.1.1 Life cycle

Zebrafish has a short generation time, typically 3 to 4 months from fertilization to adult stage, with considerably large eggs, 0.7 mm in diameter at fertilization, when compared to other fish [114]. Zebrafish life cycle is illustrated in **Figure 5**. It features a rapid embryonic development being that at 30 hpf the embryos present their first spontaneous movements demonstrating muscle activity, at 42 hpf the main organs become fully developed, and through 48 and 72 hpf hatching occurs. After hatching, the process of inflating the swim bladder occurs, which allows the larvae to start demonstrating a free-swimming behavior. Up to 7 days post fertilization (dpf), the larvae has completed most of its morphogenesis, with developments such as the protrusion of the mouth, the gut tube dropping more ventrally and the yolk extension being nearly empty, and it continues to grow rapidly. Whereas during the hatching period the embryo is usually at rest, the early larva gradually begins to swim about actively and starts moving its jaws, pectoral fins, and eyes, which produce prompt escape responses and indicate respiration, the seeking of prey and feeding [122]. The growth rate of zebrafish is rapid during the first 3 months of age, followed by a decrease over time until it ceases when reaching around 18 months of age. Zebrafish typically reach sexual maturity around 3 months of age, though zebrafish maturation is more associated with the size than with the age, being that when the specimen reaches around 23 mm it can already demonstrate some sexual traits or even be fully sexually mature, which allows for their use in breeding experiments [119], [122]. To date and to the best of our knowledge, there have been no reported studies detailing the lifespan of zebrafish in the wild. In a laboratory environment, zebrafish can live up to 5 years although after 2 years of age it is common for specimens to start evidencing muscular disorders and spinal curvatures [123].

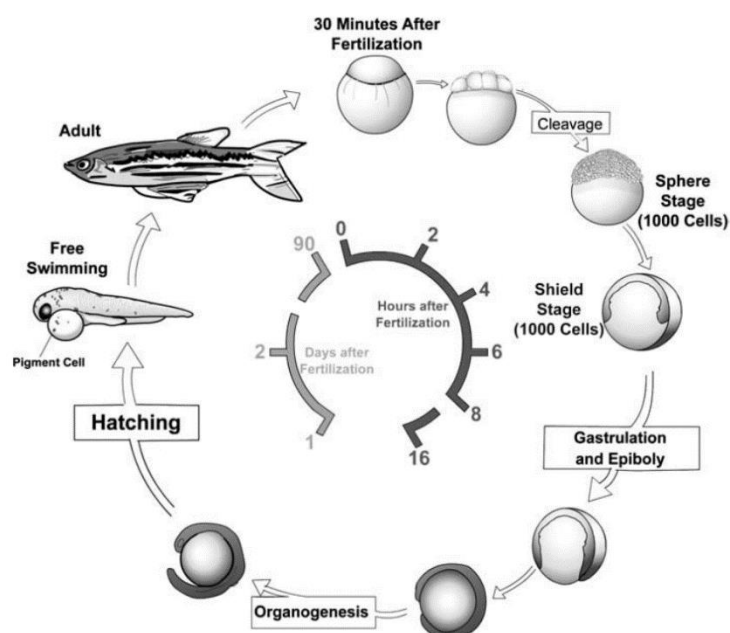


Figure 5. Life cycle of zebrafish. Retrieved from [162].

1.7.1.2 Reproduction

In nature, zebrafish exhibit an annual reproductive behavior that commences with the spawning season just before the onset of the monsoon. This phenomenon allows for the main period of specimens rapid growth to take place during the monsoon months (June - September), a period of elevated temperatures and food availability [114]. Zebrafish are asynchronous, batch spawners that breed in small groups, with females scattering clutches of eggs over the substratum for them to be fertilized by the male once they are laid [119]. Egg spawning is dependent on the presence of a male and their odors considering that females kept in isolation for extended periods of time or older females often develop a plug, consisting of necrotic clumped eggs, which clogs the oviduct and prevents any further successful spawning. Moreover, it has been revealed that females exposed to male pheromones for several days prior to spawning produce more eggs, and a lower proportion of non-viable embryos than females isolated for several days [124].

The spawning in domesticated zebrafish at a laboratory atmosphere is interconnected with photoperiod in order to allow reproduction throughout the year [114]. Their spawning activity usually commences within the first minute of exposure to light following darkness, continuing for about an hour. Courtship behavior in the male is triggered by female pheromones and consists of a male chasing the female rapidly, often nudging her flanks with his snout aiming to lead her to a spawning site, and once over a spawning site, the sexual mates swim side by side triggering oviposition in the female and sperm release in the male, simultaneously. This sequence of behaviors is repeated throughout the spawning period but it is the most active within the first 30 min [125].

1.7.1.3 Embryonic development

The embryonic development of zebrafish has been fully described since 1995 [122]. Seven broad periods of embryogenesis were defined to highlight the changing spectrum of major developmental processes that occur during the first 3 days after fertilization: the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods (**Figure 6**). The developmental process starts with the newly fertilized egg in the zygote period for about 40 min until the first cleavage occurs and the first cell is formed. This is followed by the cleavage period which comprises six cleavages where cell divisions occur synchronously every 15 min until the initial single cell is divided in 64 cells, around 2.25 hpf. During the blastula period, the embryo enters midblastula transition at the 512-cell stage, the yolk syncytial layer forms, and epiboly begins and reaches 30%. In the gastrula period, the primary germ layers and the embryonic axis are produced as well as the

primary structures for the head and the tail, and the epibolic process is completed. Reaching 10 hpf, the embryo enters in the segmentation period, where a variety of morphogenetic movements now occur, the somites develop, the rudiments of the primary organs become visible, the tail bud becomes more prominent, and the embryo elongates. Moreover, the first cells differentiate morphologically, and the first body movements appear. Throughout the pharyngula period, which begins at 24 hpf, the embryo head straightens out, the nervous system is being developed, and the circulatory system forms with well-delineated chambers and the heart beginning to beat just at the onset of the period. At 48 hpf, the embryo enters in the hatching period where morphogenesis of the primary organs systems is completed, the cartilage in the head and pectoral fin develops, and the embryo begins to hatch from their chorions. By day 3, the hatched larva has completed most of its morphogenesis and continues to grow rapidly. Prominent changes during the next day include the inflation of the swim bladder and the continued anterior-dorsal protrusion of the mouth. Whereas during the hatching period the embryo is usually at rest, the early larva gradually begins to exhibit free swimming, and demonstrating food-seeking and active avoidance behaviors.

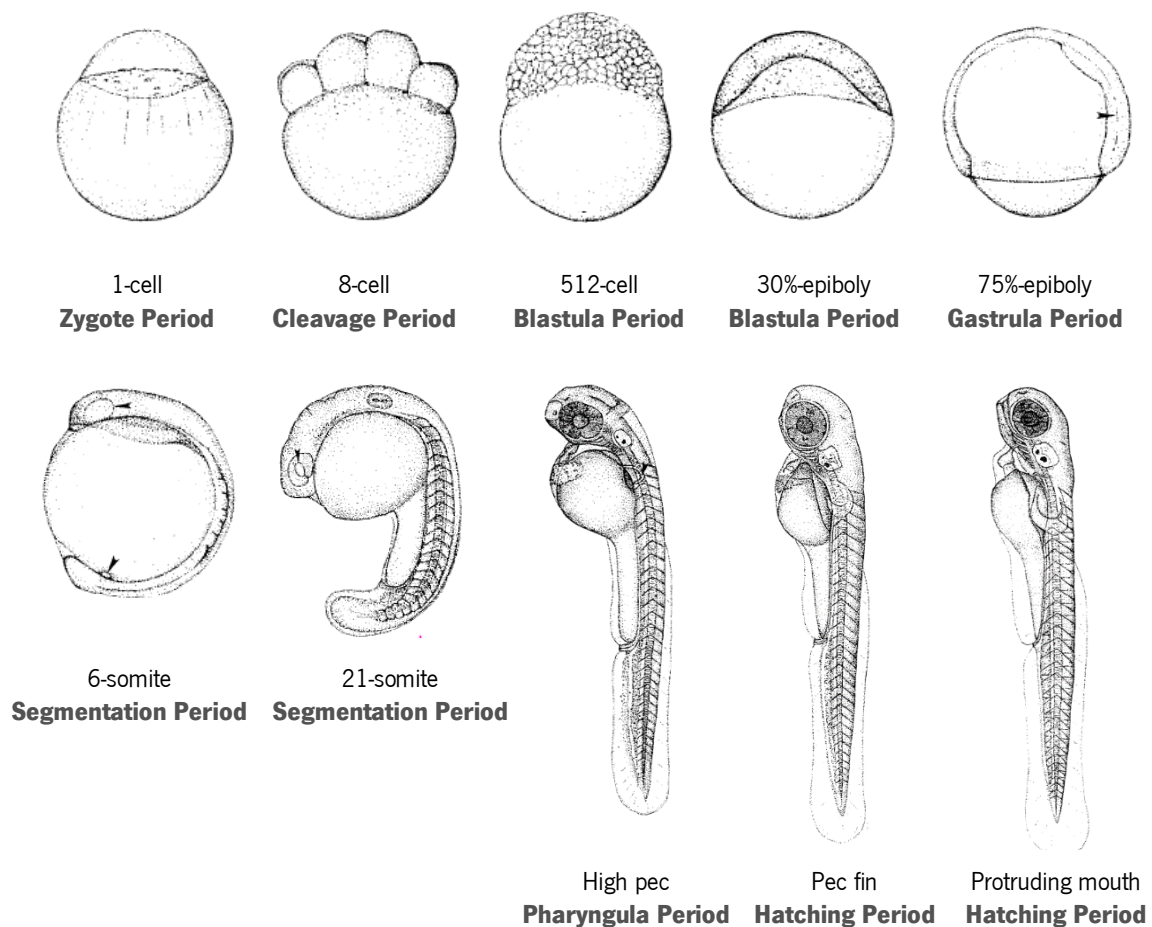


Figure 6. Stages of zebrafish embryonic development. Adapted from [122].

1.7.2 Zebrafish as a research model

In the recent years, zebrafish has been gaining acceptance as a vertebrate model host for the understanding of host-pathogen interactions, creating a bridge between simple assays based on cell culture and biological validation in traditional mammalian models such as rodents (**Figure 7**) [65]. The zebrafish has more similarities to mammals than the invertebrate models used in research in terms of anatomical structures, physiology, genetics and an immune system that comprises both innate and adaptive immune functions with a high degree of conservation with mammals, including humans [126], [127], [128]. Their embryonic development and organs functionality are similar to those of humans, and their genome, fully sequenced since 2011, reveals a high similarity with human genetics by having 70% similarity to human genes and 82% human disease-causing proteins, which is a considerable advantage in modelling human disease. Other advantages attributed to this model are the small size, ease of care and manipulation, low maintenance cost, high reproductive rate, rapid development and maturation, comprehensive molecular tools, and the fact that it allows for a more ethical *in vivo* analysis [97]. In addition to adult fish, zebrafish embryos also offer unique advantages as a host for the study of infections, such as a rapid and external development [129] and its optical transparency, which enables real-time visualization to track the progress of the pathogens and the responsive immune cells simultaneously [111]. Importantly, the use of zebrafish in research goes accordingly with the 3Rs of animal research which stand for Replace, Reduce, and Refine whose main goal is to alter traditional animal testing practices so that animal experimentation is reduced to a minimum whenever possible, attempting as well to lower animal distress and suffering during research to a minimum [130]. Studies have confirmed that fish have a less developed nervous system comparing to other mammalian models and that, although they are neurologically equipped with nociceptors (sensory receptors that send signals that cause pain perception) and unconscious emotional responses, they do not suffer pain and awareness feelings [131]. Moreover, in line with the European Commission Directive of 2010, (Directive 2010/63/EU), experiments with the earliest stages of zebrafish, up to 5 dpf, are not regulated as animal studies since these have not yet achieved free living. Therefore, although there are many promising advances in alternative research models, zebrafish offer a unique opportunity to make use of a living organism but still follow the principles of the 3Rs, reducing the number of animals needed to perform testing as well as the time and resources compared to other animal models.

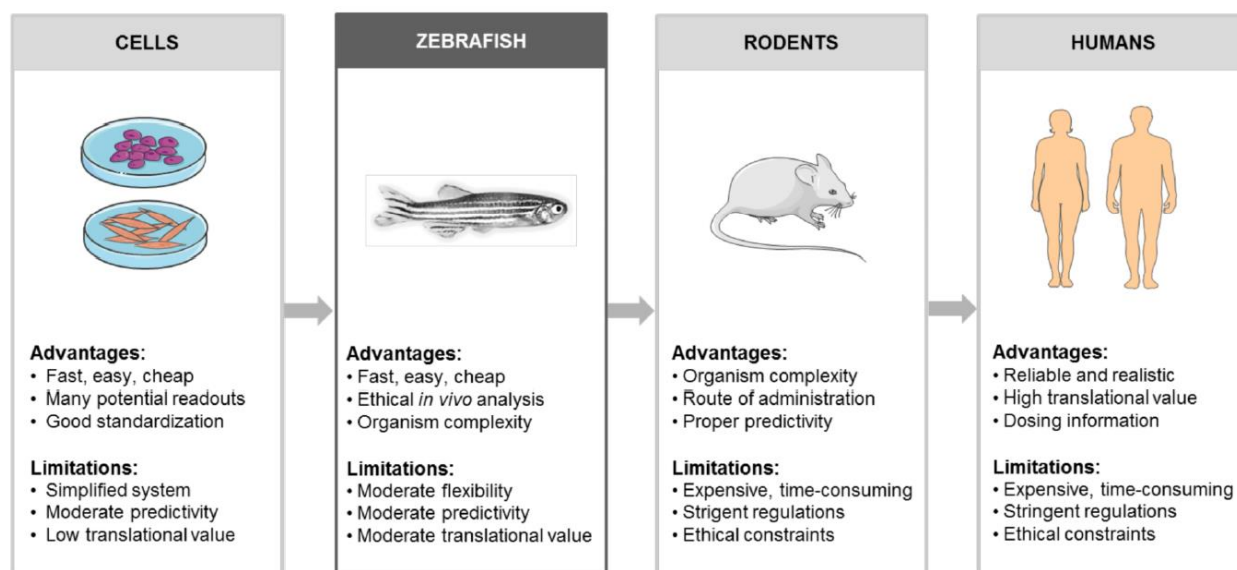


Figure 7. Sequence of the regular flow followed at research trials, from pre-clinical to clinical testing. Retrieved from [97].

Like any vertebrate model organism with defined structures and organs, the zebrafish model offers several routes and sites for infection. Bloodstream infections, performed via the tail vein or the duct of Cuvier, have been the most commonly used due to the ease of injection, and the clear parallel this route offers to other animal models. Infection can also occur through injection into the embryonic zebrafish yolk sac which can lead to systemic infection, microinjection into the hindbrain ventricle at 36-48 hpf, injection into the swim bladder, eye, peritoneal cavity, and into spinal cord tissues, although the latter can be more technically difficult [65]. Beyond these localizations for infection, a zebrafish egg bath infection model was also established as a simple noninvasive model for the study of *C. albicans* pathogenesis [111].

Several studies have already been performed with the adult zebrafish or the zebrafish embryo as a model in different knowledge areas, some of them demonstrating that zebrafish embryo model offers a reasonable alternative to the use of adult zebrafish. For example, a toxicity study confirmed that fish embryo toxicity tests (FET) with zebrafish are neither better nor worse than acute fish toxicity tests, which provided a strong scientific support for the FET as a substitute for the acute fish toxicity test [132]. Nowadays, the OECD (Organization for Economic Co-operation and Development) recommends FET as an alternative and credible method for assessing aquatic toxicity [133], [134]. An infection study with *C. albicans* using adult zebrafish as a model demonstrated that key regulators of the filamentous growth regulatory pathway are required for invasion, which was also demonstrated for the embryo model [112], [113]. Another study established an infection model in zebrafish larvae to study the pathogenesis of *Aeromonas hydrophila* in order to reduce the research using murine and adult fish models and consequently, their sacrifice [135].

1.7.3 Modeling human *Candida* infections in zebrafish

Considering all the advantages that have been described for the zebrafish embryo, it is clear that it is a good model to use for *Candida* pathogenesis investigation. Furthermore, candidiasis is highly controlled by innate immunity [136], and with the use of the embryonic stage, zebrafish provides an appropriate system to study the innate immune responses *in vivo*, in a situation where no adaptive response has yet been developed, considering that early lymphocytes first appear in 4-day-old larvae and several weeks are required for a full adaptive immunity to be developed [137]. The innate immune system of the zebrafish is mainly comprised by the complement system, toll-like receptors, macrophages and neutrophils, and it is detectable as well as active at day 1 of embryogenesis [138], [139]. Primitive macrophages appear around 24 hpf, just before the beginning of blood circulation and any other type of leukocyte appearing in the embryo, while neutrophils can appear as soon as 33 hpf [139], [140]. Neutrophils are the most abundant type of circulating leukocyte in humans as in zebrafish and are commonly the first responders recruited to sites of tissue injury or infection in the host, playing a critical part in the immune response of the host [138]. These are considered highly motile, since they are strongly attracted to bacteria and able to circulate within the tissues and quickly cross epithelia, in order to reach the microorganisms [140]. Macrophages are subsequently recruited to inflamed tissues, and these have the ability to phagocytize apoptotic bodies, engulf and destroy large amounts of bacteria present in the blood, and sense the presence of bacteria in body cavities isolated from the blood, migrating into them to eliminate the microorganisms [139]. When comparing the phagocytic capacity of these two leukocyte types, it is possible to conclude that only a small fraction of neutrophils phagocyte microbes, and to a much lesser extent relative to the primitive macrophages. This conclusion, along with the fact that neutrophils are also attracted to several stressed and malformed tissues, suggests that the main function of these leukocytes may go beyond phagocytosis in antimicrobial defense [140]. Several inflammatory genes are already known to be involved in the immune system response of zebrafish to an infection, such IL-1 β , TNF- α , and iNOS.

Several studies with zebrafish as a model have been performed throughout the years regarding *Candida* pathogenesis. One study with major importance for this field was accomplished by Chao *et al.* (2010), where an adult zebrafish model was developed for the study of *C. albicans* pathogenesis. They showed that *C. albicans* could shift between the yeast and hyphae form, colonize and invade adult zebrafish at various anatomical sites and kill the fish in a dose-dependent manner. They also verified that *C. albicans* hypha-associated genes were highly expressed during the early phase of zebrafish infection, and the genes of the immune response of zebrafish were upregulated from 2 to 15 h post infection (hpi). Together, these data indicated that the expression of *C. albicans* hypha-associated genes correlated positively with the advance of

invasion in the infected zebrafish and that the immune responses were indeed activated to defend against the pathogen [113]. These results were similar to others reported in previous mouse model studies [141], [142], [143]. It was also demonstrated that zebrafish embryos enabled real time visualization of the interaction between the fungal and the host immune cells. These results validated the conclusion that adult zebrafish are a useful and effective model host to study this opportunistic fungal pathogen. Since then, several experiences were performed for the study of *Candida* pathogenesis with the use of the zebrafish model in distinct stages: adult, larvae or embryo. For example, Archambault *et al.* (2019), performed a study with zebrafish in the larvae stage as host, where it was observed that *C. albicans* grows as both yeast and epithelium-damaging filaments, and *C. parapsilosis* remains in yeast morphology during infection [144]. It was also demonstrated that mucosal infection in larvae zebrafish by *C. albicans* caused significant mortality and induced the production of higher levels of inflammatory cytokines, when compared to infection by *C. parapsilosis*, which is consistent with what was seen in other infection models, like mice and *in vitro* challenged epithelial cells [136], [145], [146].

Although some studies already used the zebrafish embryo as host to assess *Candida* pathogenesis, it is still necessary to verify if it is, in fact, an effective model system for the understanding of *Candida* yeasts particularly in specific aspects such as exosome-mediated infection.

1.7.4 Fish embryo test (FET)

In 2006, the OECD designed FET as an alternative for the acute fish toxicity test. Nowadays, FET using zebrafish has been optimized, standardized, and validated during an OECD validation study and adopted as a test to assess toxicity of embryonic forms of fish [147], [134]. Given its excellent correlation with the acute fish toxicity test and the fact that non-feeding developmental stages of fish are not categorized as protected stages according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes, the FET is ready for use not only for range-finding but also as a true alternative for the acute fish toxicity test, as required for a multitude of national and international regulations. If, for ethical reasons, not universally accepted as a full alternative, the FET represents at least a refinement in the sense of the 3Rs principle [148].

Europe Council guidelines concerning experimental animal protection allow zebrafish embryos to be used up to the moment of free living, i.e., up to 120 hpf, since by reaching this developmental age, the larvae have consumed all the energetic reserve of yolk, and from that moment on needs to seek for food. Therefore, for the present tests, it will not be required a specific license given that these will be performed just up to 80 hpf.

1.8 Outline and aims of the thesis

Over the years, the increasing occurrence of *Candida* infections, and the rising of drug-resistant species have substantially contributed to greater percentages of morbidity and mortality, particularly amongst immunocompromised patients [1], [2]. Accordingly, *Candida* infections have become a considerable weight in public health, which leads to a necessity of better understanding their pathogenesis to improve therapeutic options and facilitate the development of new and enhanced antifungal drugs [64]. Over the past decade, more prominence has been placed on treatments aiming to target specifically the host-pathogen interaction which, in theory, would allow the new drugs to have minimal impact on the survival of commensal organisms, being less prone to eliciting resistance in the microbiome [65]. Therefore, an improved understanding of the interactions between host-*Candida* species may lead to new targets for the antifungal drugs and consequently assist in the control of these infections. More recently, EVs have gained much interest in research as a ubiquitous mechanism for transferring information between cells and organisms across all three domains of life [66]. In addition to their influence on normal physiology, these particles have been pinpointed as mediators of a previously unrecognized mechanism to package and protect cargo for uptake and integration into other cells, namely pathogen secretion products, thus emerging as a functionally diverse type of host-pathogen interaction. Considering the potential roles reported for EVs in host-*Candida* interactions, improving existing knowledge of the biology of *Candida* EVs and their functions in an infection situation could open new views for understanding *Candida* pathogenesis and help elucidate possible therapeutic targets and develop potential vaccines or immunotherapy against this infection. Taking all of this into account, the present work aimed at isolating exosomes of *C. albicans* SC5314 and *C. albicans* 124A with a high degree of purity and free of cellular contamination for further characterization, cytotoxicity evaluation, phagocytosis analysis and cytokine production when in culture with murine macrophage-like cell line J774A.1. Furthermore, this work aimed to establish an effective zebrafish embryo model to study host-*Candida* interactions, which is relevant for this thesis by allowing further studies on the role of exosomes in *Candida* pathogenesis in the scale of a whole organism, but also would contribute for other future projects that intend to study *Candida* infections.

Chapter 2. Materials and Methods

2. Materials and Methods

2.1 Strains, Media, and Growth Conditions

The *C. albicans* yeast strains used in this work and the media in which they were grown are listed in **Table 4**. Yeast strains were maintained in YPD plates (1% yeast extract (w/v), 1% peptone (w/v), 2% glucose (w/v) and 2% agar (w/v)). When yeast pre-inoculums or inoculums were needed, strains were grown in yeast extract peptone dextrose (YEPD) liquid medium (1% yeast extract (w/v), 1% peptone (w/v) and 2% glucose (w/v)) or in minimal medium (MM) (2% glucose (w/v) and 0.67% yeast nitrogen base without amino acids (w/v)), the two media tested with the purpose of optimizing the experimental conditions of this work. Firstly, pre-cultures were prepared by transferring a portion of cell biomass of each strain into 10 mL of specific media overnight at 37 °C with the agitation of 150 rpm. The following morning, cell cultures were prepared by diluting the pre-cultures in 100 mL of specific media in a 250 mL erlenmeyer flask, to an optical density (OD) at 640 nm (OD₆₄₀) of approximately 0.1 and incubated with the agitation of 150 rpm at 37 °C for 48 h.

Table 4. Strains and media used in this study.

Strain	Information	Media
<i>Candida albicans</i> SC5314	Clinical Isolate – Systemic Infection [149]	YEPD / MM
<i>Candida albicans</i> 124A	Clinical Isolate – Systemic Infection [150]	

2.2 Obtaining yeast-free supernatant

After incubation, cell cultures were centrifuged for 5 min at 4000xg and 4 °C, the supernatants filtered through sterile nitrocellulose membrane filters (0.45 µm pore size), and the resulting filtrates concentrated. In order to optimize the experimental conditions, two different concentration mechanisms were tested:

- Amicon® Stirred Cell - a pressure-based sample concentration device with 1,000 Dalton molecular weight cutoff filters
- Amicon® Ultra 15 mL centrifugal device with Ultracel® 3,000 Dalton molecular weight cutoff filters

Concentrated samples were further subjected to a filtration through pore size of 0.2 µm sterile nitrocellulose membrane filters to ensure sterility and absence of other cellular elements but exosomes in the samples. The media used to grow the yeast cultures were subjected to the same procedure that *C. albicans*-free supernatants were as an experimental control.

2.3 Exosomes analysis

Samples size and polydispersity were determined by Dynamic Light Scattering (DLS) and particles surface charge measured by Electrophoretic Light Scattering (ELS). Exosomes production and purification was assessed by western blot protocol, and proteins quantified by Pierce™ BCA Protein Assay Kit. The morphology of the exosomes was assessed by Scanning Transmission Electron Microscopy (STEM).

2.3.1 Sample size and polydispersity determination by dynamic light scattering

The size and polydispersity of samples were measured using a Zetasizer Nano ZS particle analyzer and analyzed with the Zetasizer Software (Malvern Instruments) according to the manufacturer's protocol. This technique involves the measurement of the scattered light from small particles, measuring their diffusivity when undergoing Brownian motion, which can provide information about the particle's size and their state of motion. The Brownian motion of the particles causes the scattering light to fluctuate randomly. Small particles move faster than large ones which makes this random fluctuation of the scattering light also faster [151], [152]. The measured particles will have a certain level of heterogeneity. To determine the uniformity degree of the samples in terms of the particle's size, the polydispersity index (PDI) is used. Samples are considered monodisperse if the PDI is below 0.1 and polydisperse for higher values, however, many factors may affect this parameter such as the particles' heterogeneity, aggregation or contamination. High polydispersity values can lead to unreliable results, far from the real size of the particles [151]. In summary, the analysis of the size and PDI was carried out by placing 1000 µl of the solutions in a polystyrene cuvette, setting an attenuator between 6 and 9, and a count rate always superior to 150 counts.

2.3.2 Surface charge measurement by electrophoretic light scattering

The ELS method is based on electrophoretic principles: when a solution is inserted into a cell with two electrodes and an electrical field is applied into those, the existing particles that have a net charge will migrate towards the oppositely charged electrode. This migration has a specific velocity, which is known as the mobility of the particles, which is related to their zeta potential. With this technique, it is possible to determine the particle charge in the solution. This approach measures both the outer layer and the solvation layer of the particle and the charge between these two layers is called zeta potential (**Figure 8**). Such characteristic leads to a wide range of implications, with the most significant one being the possibility of the real charge of particles being mitigated by the solvent. For this measurement, an aliquot of 800 μl of the solutions prepared for the DLS analysis was placed in the zeta cuvette.

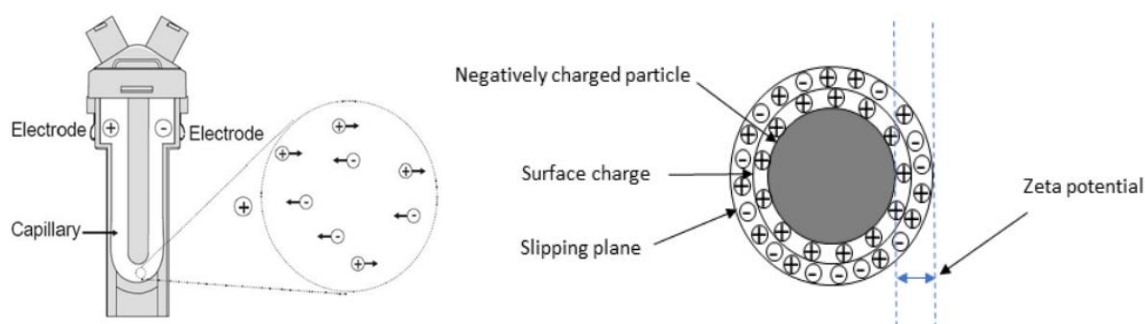


Figure 8. Schematic representation of a dip cell. Retrieved from [163].

2.3.3 Scanning transmission electron microscopy analysis

The morphology of the exosomes was assessed by STEM analysis, a hybrid electron microscopy technique used for imaging and morphological characterization with atomic-scale resolution. In summary, after the concentration of the *C. albicans* SC5314-free supernatant, *C. albicans* 1244-free supernatant and the YEPD medium using the Amicon® Ultra-15 Centrifugal Filter Unit and subsequent filtration, a 200-mesh copper grid coated with carbon was immersed in each sample. After 1 min of immersion, the samples were left to dry, and were posteriorly analyzed in an ultra-high-resolution scanning electron microscope (NanoSEM FEI Nova 200 (FEG/SEM)). The microscope was operated at 15 kV.

2.3.4 Protein concentration determination

Protein was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

2.3.5 Protein gel electrophoresis and Western blot techniques

Firstly, samples were subjected to 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), where a broad range SDS-PAGE molecular weight marker (MWM) (11-245 kDa) was used (NZYTech). In order to optimize the experimental conditions, two different protein concentrations were tested:

- Gel loaded with 8 µg of protein directly from samples
- Gel loaded with 20 µg of protein, whose samples were submitted to trichloroacetic acid (TCA) protein precipitation (PanReac AppliChem, ITW Reagents)

Control gels were stained with Coomassie Brilliant Blue G250 and Silver Nitrate and remaining gels were transferred to nitrocellulose blotting membranes (GE Healthcare Whatman™ Protran™). The membranes were blocked with 5% non-fat dry milk (w/v) in Tris-saline buffer containing 0.05% Tween (v/v) for 2 h at room temperature. Afterwards, membranes were incubated overnight at 4 °C with a pool of antisera (1:1000) from immunized rabbits and bound antibodies were detected with Goat anti-Rabbit IgG HRP conjugate (1:20000). In this work, four different exosome antibodies were used (EXOAB products): anti-CD9, anti-CD63, anti-CD81 and anti-Hsp70. Detection was fulfilled by the enhanced chemiluminescent method using Immobilon® ECL Ultra Western HRP Substrate and a ChemiDoc™ MP imaging system.

2.4 Macrophage studies

The *in vitro* studies were performed using the murine macrophage cell line J774A.1 that was cultured and maintained in complete medium, Dulbecco's Modified Eagle Medium (DMEM) composed by high glucose, L-glutamine, and 25 mM HEPES supplemented with 10% FBS and 1 mM sodium pyruvate (ThermoFisher Scientific) at 37 °C in a 5% CO₂ atmosphere. After confluent growth, macrophage cells were recovered, washed, and resuspended in complete medium to the desired final cell concentration.

2.4.1 Cytotoxicity analysis

Macrophages were plated onto 96-well tissue culture plates (Orange Scientific) at 1×10^5 cells/well and incubated to adhere overnight at 37 °C in a humidified atmosphere of 5% CO₂. Different stimuli were added to the cells: the concentrated samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit concentration system, specifically *C. albicans* SC5314-free supernatant and *C. albicans* 124A-free supernatant whose growth was performed in minimal medium, alongside with minimal medium used as an experimental control, and lipopolysaccharide (LPS) used as a positive control since it induces host inflammatory responses and cell injury.

Cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) assay after 24 h of incubation, according to the manufacturer's instructions. The MTT assay is a colorimetric assay to assess cell metabolic activity, where MTT is a water-soluble tetrazolium salt that can be modified into an insoluble formazan that has a purple color, by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. Enzymatic activity was quantified using a solution of DMSO:ethanol (1:1) to solubilize MTT formazan and measuring absorbance at 570 nm. As a control of viability (100%) untreated cells were used while for control of cytotoxicity (100%) cells treated with Tris base were utilized.

2.4.2 Phagocytosis analysis

2.4.2.1 Preparation of yeast cells

In this protocol, wild type strain of *Candida albicans* SC5314 was used. Firstly, a pre-inoculum was prepared and incubated at 26 °C in YEPD medium with the agitation of 160 rpm for 24 h. The following day, cell culture was prepared by diluting the pre-culture to an OD₆₄₀ of approximately 0.1 and grown until OD₆₄₀ reached approximately 0.5, reaching the exponential phase. Once the cell culture reached the desired OD, cells were collected by centrifugation at 5000 rpm for 3 min at 4 °C and washed in sterile 1x phosphate buffered saline (PBS) buffer. To fix cell wall PAMPs, yeast cells were incubated for 15 min in formol/ethanol (1:9), washed two times in sterile 1x PBS buffer for removal of formol/ethanol solution and resuspended in sterile 1x PBS buffer. To proceed with the assay, yeast cells were incubated for 10 min with 1 µM SYTOX Green at room temperature (RT) in the dark. After incubation, cells were collected by centrifugation at 5000 rpm for 3 min at 4 °C and resuspended in complete DMEM. Serial dilutions were performed to count the

cells on a hemocytometer and a final working solution was achieved by diluting the solution with complete DMEM to a final concentration of 5×10^6 cells/mL.

2.4.2.2 Preparation of J774A.1 macrophage cell line

Macrophages were cultured in complete DMEM at 37 °C in a 5% CO₂ atmosphere. After confluent growth was achieved, macrophage cells were recovered, washed, and resuspended in complete DMEM to a final concentration of 1×10^6 cells/mL. For fluorescence microscopy assays, 500 µl of the resulting cell suspension was transferred to 24-well tissue culture plates containing clean sterile glass coverslips (Ø13 mm). Afterwards, different stimuli were added to the cells: the concentrated samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit concentration system, specifically *C. albicans* SC5314-free supernatant and *C. albicans* 124A-free supernatant whose growth was performed in minimal medium, alongside with minimal medium used as an experimental control, and LPS used as a positive control. Finally, cells were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere, to allow macrophage adherence and incubation with the stimuli. In the following day, phagocytic cells were promptly used for phagocytic assays. Duplicates were done for each plate.

2.4.2.3 Phagocytosis of *Candida* cells

Macrophages were incubated with the labeled yeast suspension at a multiplicity of infection (MOI) of 1M:5Y for 45 min, at 37 °C and 5% CO₂. After incubation, samples were kept on ice to stop phagocytosis and incubation with propidium iodide (PI) at a final concentration of 0.001 µg/mL for 5 min at RT was conducted. Cells were analyzed by fluorescence microscopy (Leica DM5000B), and subsequential microscopy images were analyzed using ImageJ cell counter software.

2.4.3 Cytokine production

Macrophages were plated onto 96-well tissue culture plates (Orange Scientific) at 1×10^5 cells/well and incubated with different stimuli for 24 h: the concentrated samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit concentration system, specifically *C. albicans* SC5314-free supernatant and *C. albicans*

1244free supernatant whose growth was performed in minimal medium, alongside with minimal medium used as an experimental control, and LPS used as a positive control. The following day, macrophages were incubated with labeled yeast suspension at a MOI of 1M:5Y for 45 min. The release of pro-inflammatory cytokines TNF- α and IL-10 was quantified after the 24 h of incubation with the stimuli and after the 45 min incubation with the yeast suspension. The Mouse TNF- α Elisa Kit (Sensitivity: 8 pg/mL) and the Mouse IL-10 Elisa Kit (Sensitivity: 32 pg/mL) (Thermo Fisher Scientific) were used according to the manufacturer's instructions. Macrophages alone were used as a negative control.

2.5 Zebrafish studies

2.5.1 Zebrafish maintenance and husbandry

For the animal well-being, various maintenance activities were executed daily such as feeding, monitoring of freshwater physicochemical properties (pH, chlorine, nitrate and nitrite) and aquaria sanitation. The aquaria set, illustrated in **Figure 9**, was supplied by a UV lamp sterilized filtering and an activated carbon filter.

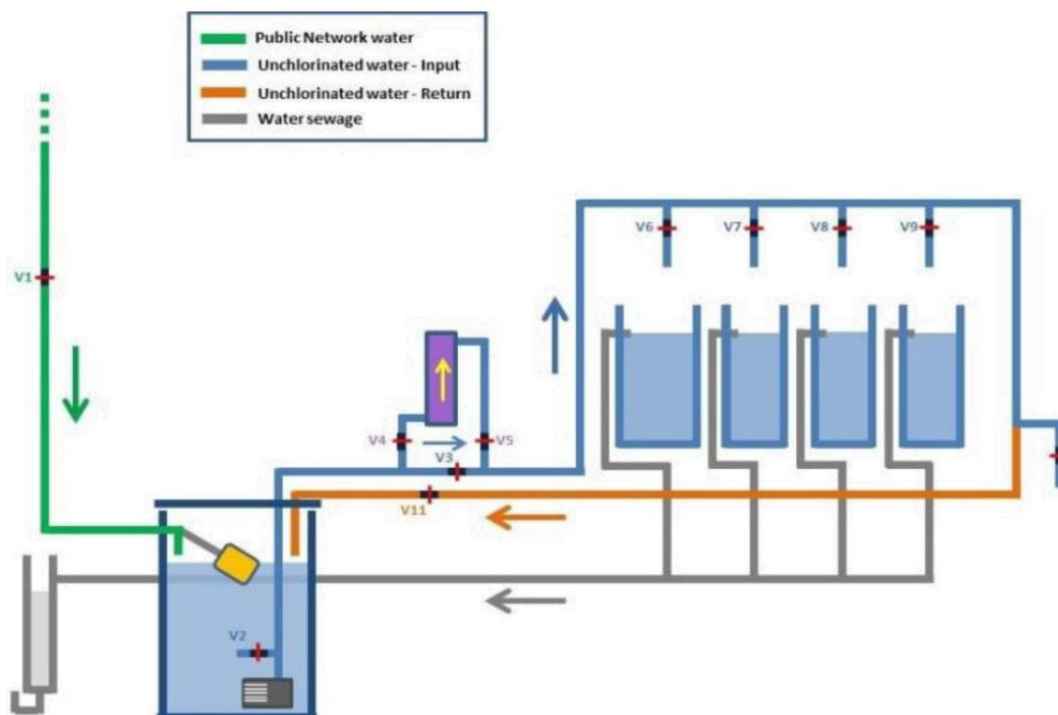


Figure 9. Freshwater aquaria system layout.

2.5.2 Parental zebrafish feeding

To achieve reproductive fitness with a high rate of viable egg spawning, the zebrafish breeding stock requires a good nutrition. Every day, animals were fed three times with two different types of food: solid food as commercial Nutrafin Max flakes, or live food as nauplii of brine shrimp *Artemia sp.* cultured in laboratory. Live food is of particular importance due to the inherent higher nutritional value and to the fact that it acts as a stimulus factor for fish to “hunt” it, which incentive their predatory instinct. For live food preparation, *Artemia* was incubated in artificial salt water at 35 ppm, 28.0 ± 1.0 °C in a tapered cylindrical container at the bottom, where dry cysts were left to hatch for 24 to 48 h. When hatched, 24 h nauplii were used to feed developing larvae and 48 h nauplii were used to feed zebrafish adults, which usually corresponded to the first meal of the day or to the last on pre-spawning days. At the weekend, parental fish were just fed with a solid flakes by an automatic feeder, twice a day.

2.5.3 Stock aquarium maintenance

The stock aquarium is the aquarium used to house the zebrafish adults exploited as breeding specimens (F0, parental), which had a total capacity of 100 L (**Figure 10**). To maintain the optimal freshwater conditions for zebrafish, several parameters needed to be daily verified and adjusted if necessary: temperature around 25.0 ± 2.0 °C; pH of 7.40 ± 0.5 ; chlorine at zero level, the nitrates and nitrites at values close to zero. Moreover, the tank was renewed, and filtered by activated carbon filtering, 50% daily automatically by peristaltic pumps, under an open circulation system. Optimal environment conditions were also ensured by water aeration through air stones placed at the aquarium corners and by removal of fecal waste twice a day manually, using hand nets.



Figure 10. Stock aquarium.

2.5.4 Breeding & Hatching aquaria

The breeding system is composed of an aquarium with capacity of about 30 L, that it is equipped with a spawning cage to proceed with the breeding protocol (**Figure 11A**). This cage features a net at the bottom which allows for the eggs to deposit on the base of the aquarium, where the breeding fish have no direct contact, and as a result cannibalism behavior by the parental animals is avoided. To mimic the zebrafish natural habitat and induce spawning, marbles were added to the bottom of the cage [153]. Zebrafish females tend to rub their genital papilla on the marbles, which serves as substrate supporting the releasing of oocytes to the freshwater, a behavior that males capture as a signal to release their sperm, and consequently external fertilization occurs [114]. In order to secure a stable environment for reproduction, the breeding system was always assembled in the mornings, and used in late afternoons to allow time for the water to reach the optimal conditions.

When successful reproduction occurs, with about 1000 embryos spawned, the remainder embryos are incubated in a 5 L aquarium, under the same physical-chemical conditions of the stock aquarium. This small tank is placed inside a 30 L aquarium that serves as a water bath for fish development, a complex that is designated as hatching aquarium (**Figure 11B**). Throughout the embryos development, dead embryos were verified and removed daily to prevent microbiological cross-contamination, and the freshwater was renewed daily to ensure optimal oxygenation.

For the FET protocol, a 30 L aquarium was adapted in order to allow incubation of microplates containing the developing embryos exposed during the assay with optimal conditions. This system, designated as “microplates aquarium” (**Figure 11C**), consists of a foam support platform that permits the microplates to be partially immersed in acclimated freshwater at 28 °C, which is an essential requirement of this protocol that was ensured from the beginning (2 hpf) to the end (80 hpf) of the exposure.

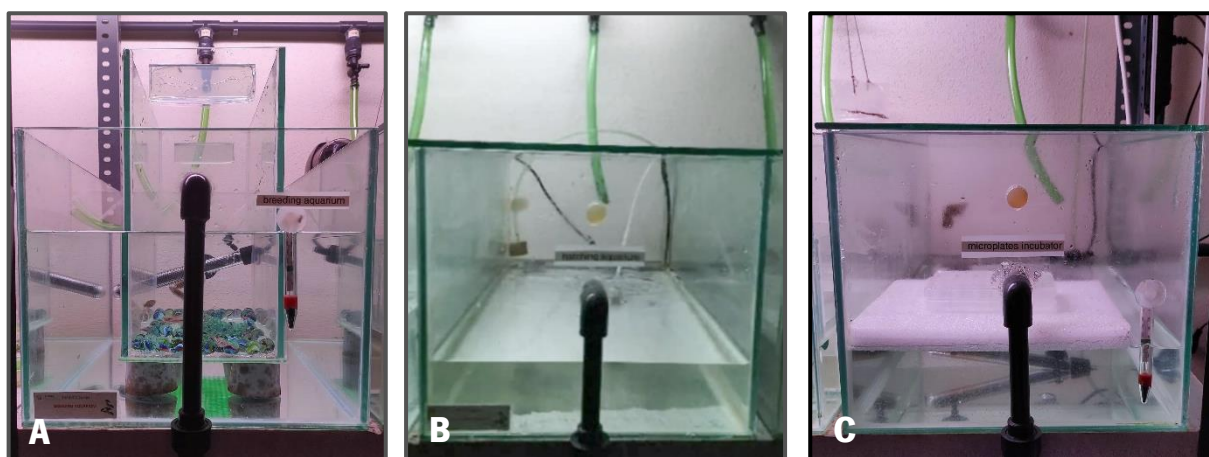


Figure 11. Breeding and Hatching aquaria.

(A) Breeding system assembled; (B) Hatching aquarium; (C) Aquarium for microplates incubation.

2.5.5 Photoperiod

Zebrafish is a photoperiodic animal, so the periods of light and dark should be regular and constant without interference or disturbance, especially on pre-breeding days [114]. When fish are isolated for the breeding protocol, the dark period serves to accommodate the animals in the new environment which is at the breeding aquarium, and when the lights turn on, the zebrafish begins its mating behavior that ends with a spawning event. At the NANODANIO laboratory, a timer was set to enable 12 h of light and 12 h of darkness.

2.5.6 Infection of zebrafish embryos with *Candida* cells

2.5.6.1 Preparation of yeast cells

For this protocol, wild type strain of *C. albicans* 124A was used. Firstly, a pre-inoculum was prepared and incubated at 30 °C in YEPD medium with the agitation of 200 rpm overnight. The following day, cell culture was prepared by diluting the pre-culture to an OD₆₄₀ of approximately 0.1 and grown until OD₆₄₀ reached approximately 0.5, reaching the exponential phase. Once the cell culture reached the desired OD, cells were collected by centrifugation at 5000 rpm for 3 min at 20 °C, washed two times in sterile deionized H_2O , and resuspended in 5 mL of sterile deionized water. Subsequently, cells were counted on a hemocytometer to allow the making of an initial working solution with 1×10^8 cells/mL, by diluting the cell solution with sterile deionized water. Using this initial solution, serial dilutions were performed to obtain the remainder of the working solutions with different *Candida* cells concentrations: from 1×10^7 cells/mL to 1×10^4 cells/mL.

2.5.6.2 FET protocol

The FET protocol begins with the parental fish isolation at the breeding cage at the end of the day, using a sex ratio of 3 females and 5 males, and can be done once or twice during the week. The next day, 1 h after the onset of the light period, zebrafish embryos were collected and washed, and viable embryos separated from the dead with the naked eye, as viable embryos are transparent, while unviable eggs are opaque and white. For this assay, the FET protocol was adjusted (**Figure 12**). At 2 hpf, the embryos were transferred to a 24-well plate with a final volume of 2 mL per well, with HEPES E3 buffer as the chosen medium since it was previously demonstrated that it induces enhanced zebrafish embryos survival and, as a saline solution, it offers the best compromise between ensuring the optimum pH for zebrafish embryos, i.e. pH 7.4, and avoiding sublethal effects throughout development. Embryos co-incubation with *Candida* cells was performed

at 6 hpf, by removing the original medium and adding 1900 μL of HEPES E3 buffer along with 100 μL of the specific *Candida* working solution to the wells, which results in incubations with 1×10^7 to 1×10^3 *Candida* cells. As experimental control, zebrafish embryos were incubated with 1900 μL of HEPES E3 buffer along with 100 μL of deionized water, since it is the solvent used in the yeast cells solutions. The test conditions and the defined control had 2 replicates, each well representing one replicate with 10 embryos, and survival analysis in each condition was performed at 8, 32, 56 and 80 hpf. For FET to be reliable, the mortality rate from the defined control must be below 10%.

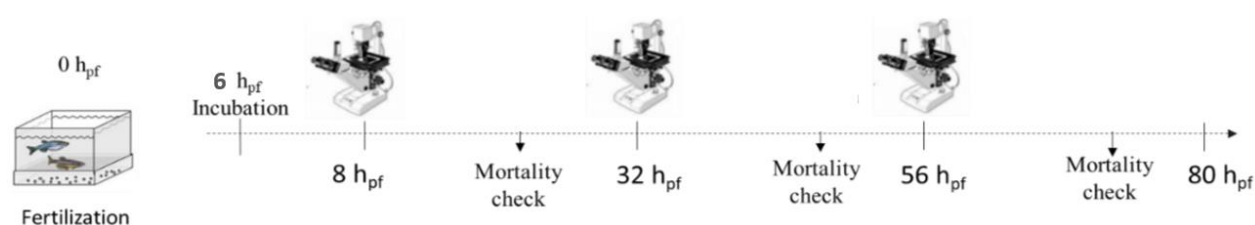


Figure 12. Scheme of FET to evaluate the effect of zebrafish embryos co-incubation with *Candida* cells on their survival rate. Adapted from [164].

Chapter 3. Results and Discussion

3. Results and Discussion

The first aim of this work was to obtain exosomes of *C. albicans* SC5314 and *C. albicans* 124A with a high degree of purity and free of cellular contamination for further characterization. For this purpose, *C. albicans* supernatants needed to go through a series of filtrations and further concentration to ensure sterility. In order to optimize the experimental conditions for the desired isolation and characterization, two different concentration mechanisms were tested, as well as two different media in which yeast needed to grow.

3.1 Exosomes isolation and Comparison between concentration systems

With the purpose of isolating *C. albicans* exosomes, *C. albicans* cell cultures were centrifuged, the supernatants filtered through pore size of 0.45 µm sterile nitrocellulose membrane filters and the resulting filtrates concentrated. After concentration, samples were subjected to a last filtration through pore size of 0.2 µm sterile nitrocellulose membrane filters to ensure sterility and absence of other cellular elements except exosomes in the samples.

Aiming to optimize the concentration phase of the isolation of *C. albicans* exosomes, two different concentration techniques were tested: Amicon® Stirred Cell, a pressure-based sample concentration device, and Amicon® Ultra 15 mL centrifugal device. For this comparison, incubation was proceeded in YEPD medium with the *C. albicans* SC5314 strain, and the remaining process was accordingly with what was previously described. With the Amicon® Ultra-15 Centrifugal Filter Unit, the *C. albicans* SC5314 – free supernatant was concentrated by 47% with 15 min centrifugation, whereas using the Amicon® Stirred Cell, 75% concentration was achieved within 5 h (**Table 5**).

Table 5. Concentrations achieved in the two different concentration systems.

Concentration system	Concentration (%)	Time (h)	Membrane NMWL*
Amicon® Ultra-15	47	1/4	3 kDa
Centrifugal Filter Unit			
Amicon® Stirred Cell	75	5	1 kDa

*NMWL - Nominal Molecular Weight Limit

Concentration carried out with each device results in two different samples, the concentrated and the residue sample. Size, PDI and zeta potential measurements of all four samples, two of each device, were then carried to assess physicochemical properties of the samples, in order to verify which concentration device was better suited for this protocol. **Figure 13** demonstrates the representative mean size, PDI and ζ -potential and **Figure 14** demonstrates the DLS size distributions as a graphical illustration of the populations of all four samples.

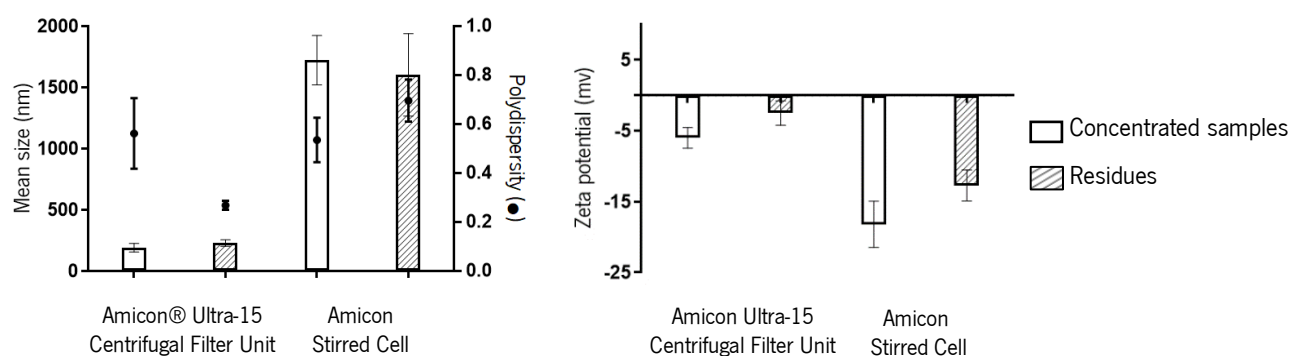
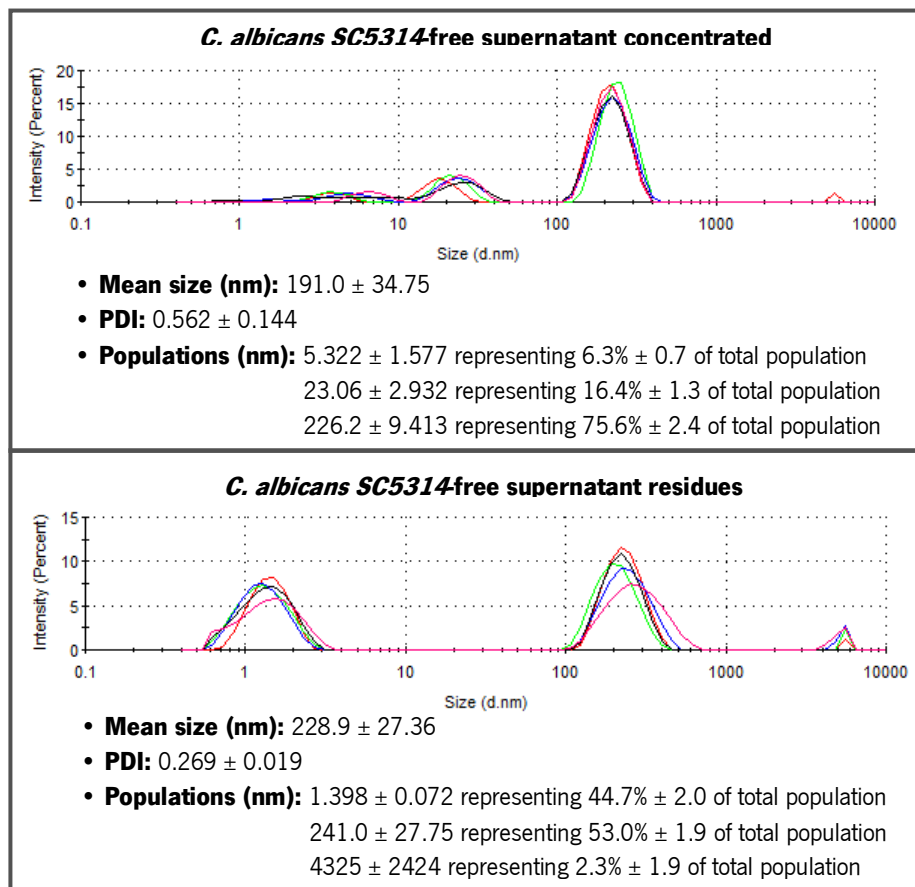


Figure 13. Representative mean size, PDI and ζ -potential of samples resulting from the two concentration systems tested: Amicon® Ultra-15 Centrifugal Filter Unit and Amicon® Stirred Cell, where *C. albicans* SC5314-free supernatant whose growth was performed in YEPD medium was used.

Results obtained illustrate significant differences in the evaluated parameters between the samples of each concentration device. The analysis of the samples resulting from the concentration with the Amicon® Stirred Cell mechanism revealed high particle sizes, above 1500 nm, which are distributed by one population in the sample as displayed in **Figure 13** and **Figure 14**, respectively. These results indicate that sample concentration with this device may cause the agglomeration and even coalescence of the exosomes present in the supernatant, leading to bigger sizes, higher polydispersity index and higher values for the ζ -potential. With this technique, analysis of the exosomes populations is unfeasible and consequently this method is not a viable option for this protocol. Contrastingly, samples from the Amicon® Ultra-15 Centrifugal Filter Unit revealed populations with mean size of approximately 200 nm, which corresponds to the size range reported for exosomes. This analysis suggests the isolation of exosomes in the concentrated sample, although also demonstrates that some exosomes can pass through the membrane into the residues samples since a population is detected in the residues sample with about the same size as in concentrated sample. With the overall results, the Amicon® Ultra-15 Centrifugal Filter Unit was considered the best suited concentration device for this protocol, and all further work was carried out with this method.

Amicon® Ultra-15 Centrifugal Filter Unit



Amicon® Stirred Cell

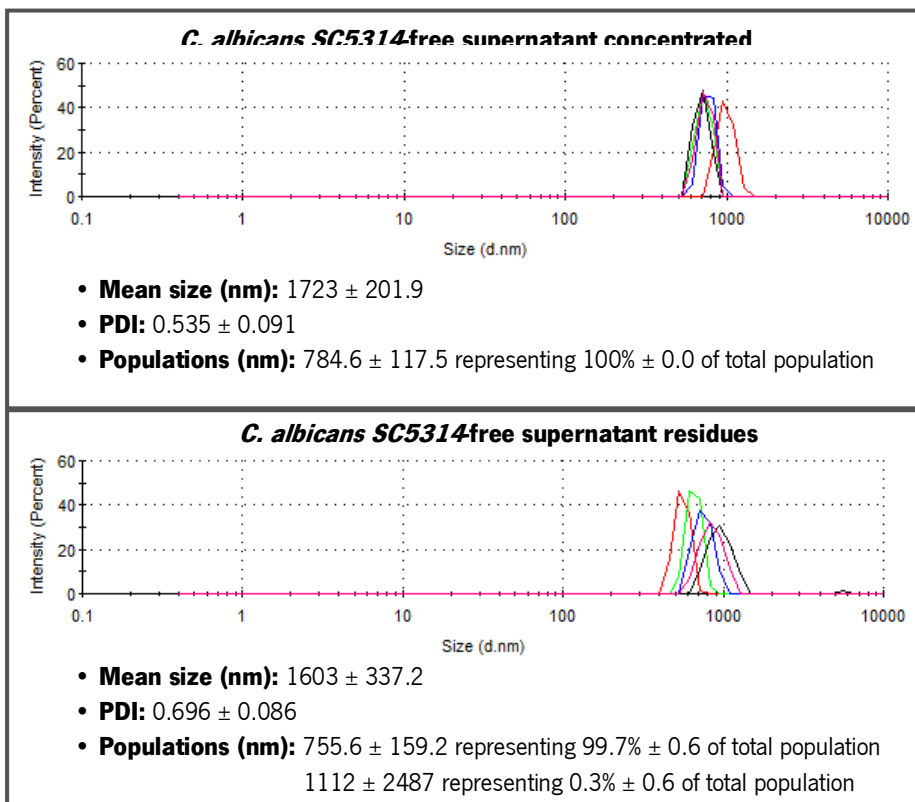


Figure 14. DLS size distributions of samples resulting from the two concentration systems tested: Amicon® Ultra-15 Centrifugal Filter Unit and Amicon® Stirred Cell, where *C. albicans* SC5314-free supernatant whose growth was performed in YEPD medium was used. The mean size, PDI and populations size are indicated.

3.2 Characterization of *C. albicans* exosomes

Optimization of the protocol for the characterization of *C. albicans* exosomes also included the testing of two different media which can support yeast strains growth: YEPD medium and minimal medium. For this comparison, incubation was proceeded in each media with *C. albicans* SC5314 and *C. albicans* 124A strains, and the remaining process to isolate their exosomes was performed as previously described, using the Amicon® Ultra-15 Centrifugal Filter Unit for the concentration phase as selected. The specific media in which the yeast strains were grown was subjected to the same procedure as the *C. albicans* supernatants as an experimental control. In order to achieve a higher concentration rate, two more 15 min centrifugations were added to the existing concentration protocol, resulting in a total concentration of 67% of *C. albicans*-free supernatants and media tested (Table 6).

Table 6. Concentration achieved in the Amicon® Ultra-15 Centrifugal Filter Unit.

Sample	Concentration (%)	Time (h)	Membrane NMWL
<i>C.albicans</i>-free supernatants/ Media	67	3/4	3 kDa

The resulting samples from the Amicon® Ultra-15 Centrifugal Filter Unit, both concentrated and residual samples from the two *C. albicans* strains used in each media and the specific media used to grow them, were then subjected to DLS analysis to determine the size and distribution of particles (PDI) and ELS analysis to determine the surface charge of the particles (zeta potential), in order to address possible differences between samples which grown in different media. Every sample was evaluated in the same conditions, only varying the medium in which growth was carried out.

Figure 15 and **Figure 17** demonstrate the representative mean size, PDI and ζ -potential of samples whose growth was performed in YEPD medium and minimal medium, respectively while **Figure 16** and **Figure 18** demonstrate the corresponding DLS size distributions. The DLS analysis of the *C. albicans*-free supernatants whose growth was performed in YEPD medium revealed a mean particle size of approximately 100 nm of and 200 nm for *C. albicans* SC5314 and *C. albicans* 124A supernatants, respectively. However, high polydispersity values are associated with these results for both samples, signifying a broad particle distribution, which consequently reduces the importance of the average size value reported alongside with PDI and indicates that a distribution analysis should be more representative.

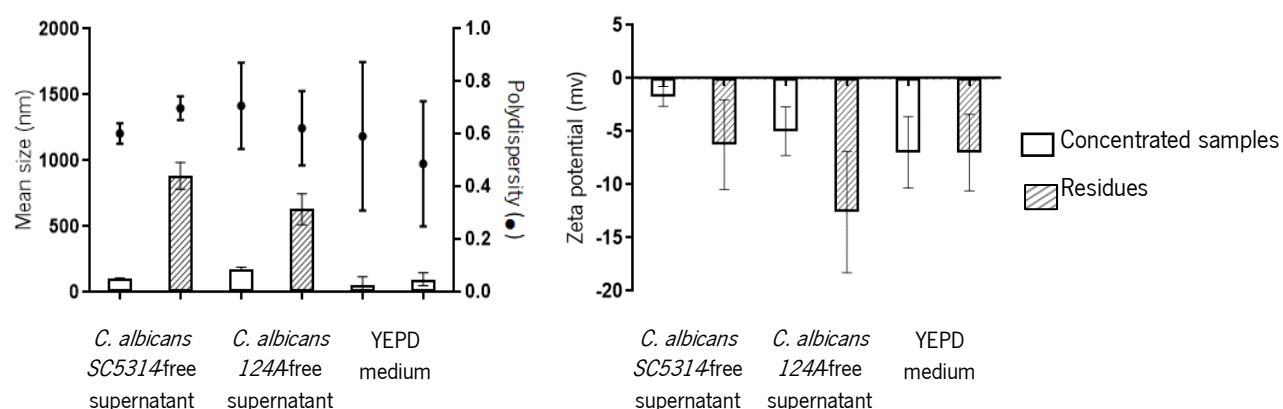


Figure 15. Representative mean size, PDI and ζ -potential of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where *C. albicans* SC5314 and *C. albicans* 124A-free supernatants whose growth was performed in YEPD medium were used. YEPD medium was used as control.

Analysis of the DLS size distribution in **Figure 16** clearly detected three separate populations across all the samples, being that the population with the highest concentration of particles in both *C. albicans*-free concentrated supernatants should represent the exosomes that we wanted to isolate. The presence of the other populations appears to correspond to residues in the samples for the smallest particles and to some level of particle aggregation in the populations of greater sizes which justifies the high polydispersity values noted in **Figure 15**. These populations were not taken into account for the analysis due to their low number in the samples. In more depth, the ζ -potential measurement and the DLS size distribution, represented in **Figure 15** and **Figure 16** respectively, suggested negatively charged (-1.7 ± 0.93 mV) exosomes with a mean size of 225.1 ± 19.02 for *C. albicans* SC5314 and negatively charged (-5 ± 2.3 mV) exosomes with a mean size of 443.5 ± 26.06 for *C. albicans* 124A.

Both *C. albicans*-free supernatants concentrated samples whose growth was performed in YEPD medium were also subjected to STEM-analysis in order to assess the morphology of the exosomes structures. This analysis failed to demonstrate exosomes structures in both of concentrated samples, which did not allow to compare results with what was already observed by the DLS assays.

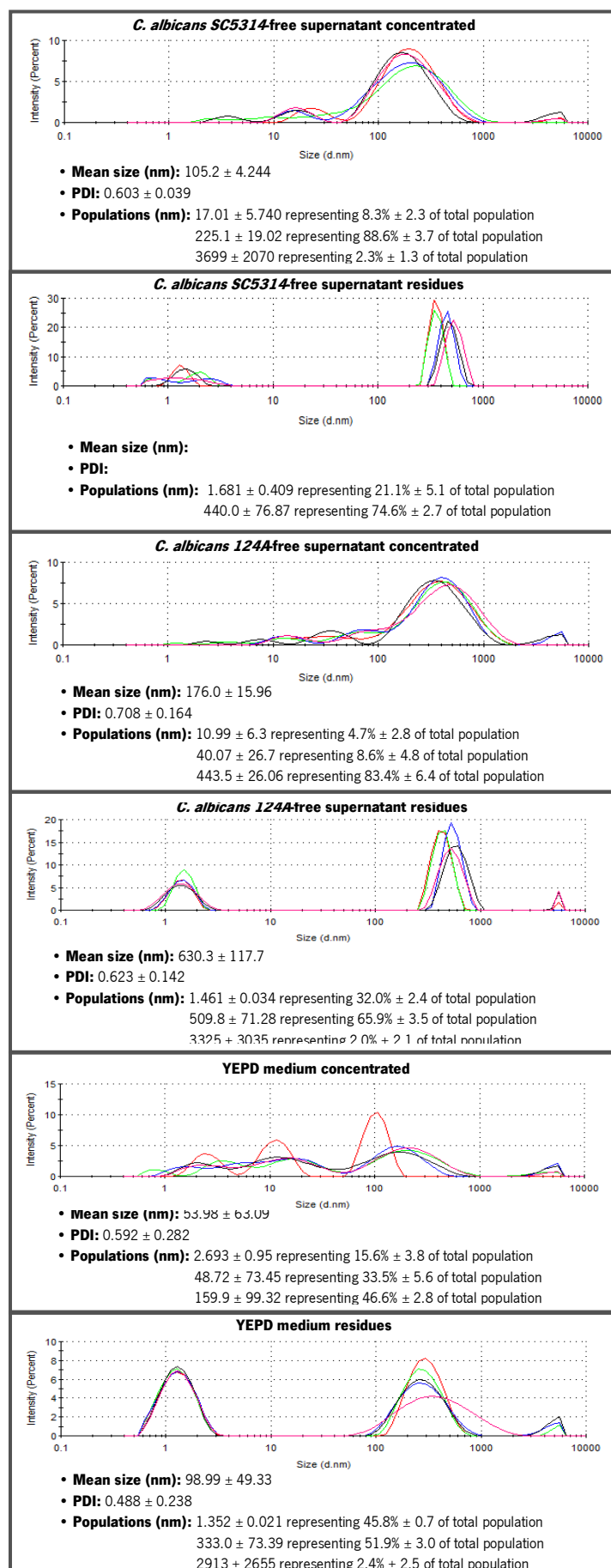


Figure 16. DLS size distributions of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where *C. albicans* SC5314 and *C. albicans* 124A-free supernatant whose growth was performed in YEPD medium were used. YEPD medium was used as control. The mean size, PDI and populations size are indicated.

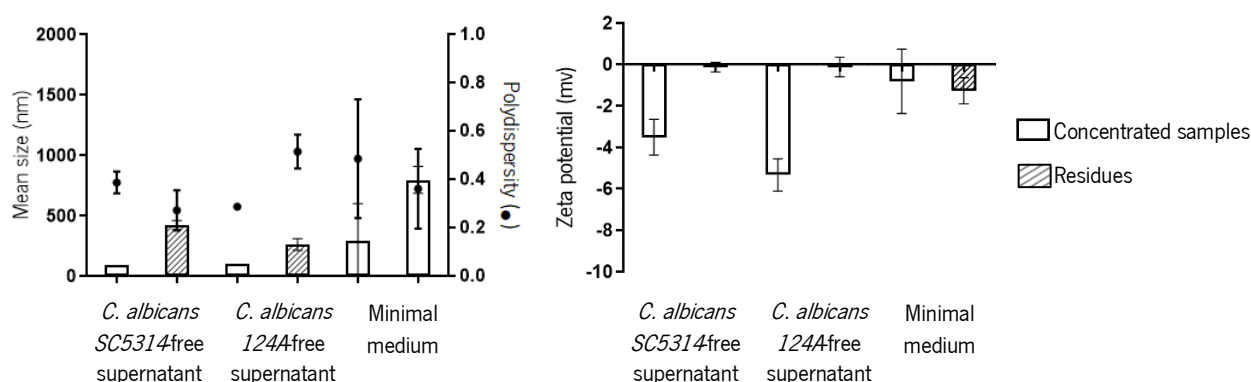


Figure 17. Representative mean size, PDI and ζ -potential of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where *C. albicans* SC5314 and *C. albicans* 124A-free supernatant whose growth was performed in minimal medium were used. Minimal medium was used as control.

When comparing the DLS analysis of samples whose growth was conducted in YEPD medium and samples whose growth was conducted in minimal medium, differences can be observed. The DLS analysis of the *C. albicans*-free supernatants whose growth was supported by minimal medium displayed a mean particle size of approximately 100 nm for both *C. albicans* strains and lower values for the polydispersity parameter (**Figure 17**). This indicates that distribution of the particles is less broad than it was when in YEPD medium, nonetheless, as previously noted, a distribution analysis should be more accurate than the mean size values since it is possible to analyze the different populations present in the samples and more accurately characterize the exosomes populations. In **Figure 18**, different size populations are clearly detected, being that the one with the highest concentration of particles in both *C. albicans*-free supernatants concentrated should illustrate the exosomes present in the samples in similarity with the DLS analysis in **Figure 16**. Nonetheless, the other populations in these samples have a much lower presence and, therefore, a reduced interference with the exosomes population and the overall analysis. Accordingly, this can also be perceived through the DLS analysis of both media, in which YEPD medium has more different and dispersed particles populations when comparing to the minimal medium. Overall, the results indicate that growth performed in minimal medium induces an optimized analysis due to less particles populations inherited from the medium, therefore concentrated samples whose growth was carried out in minimal medium were the ones chosen to perform all of the following assays. The ζ -potential measurement of these samples, represented in **Figure 17**, indicates negatively charged exosomes for both of *C. albicans* strains in agreement with the previous ELS analysis. Particularly, the overall analysis indicates negatively charged (-3.5 ± 0.87 mV) exosomes with a mean size of 157.1 ± 7.786 for *C. albicans* SC5314 and negatively charged (-5.3 ± 0.78) exosomes with a mean size of 147.6 ± 9.915 for *C. albicans* 124A.

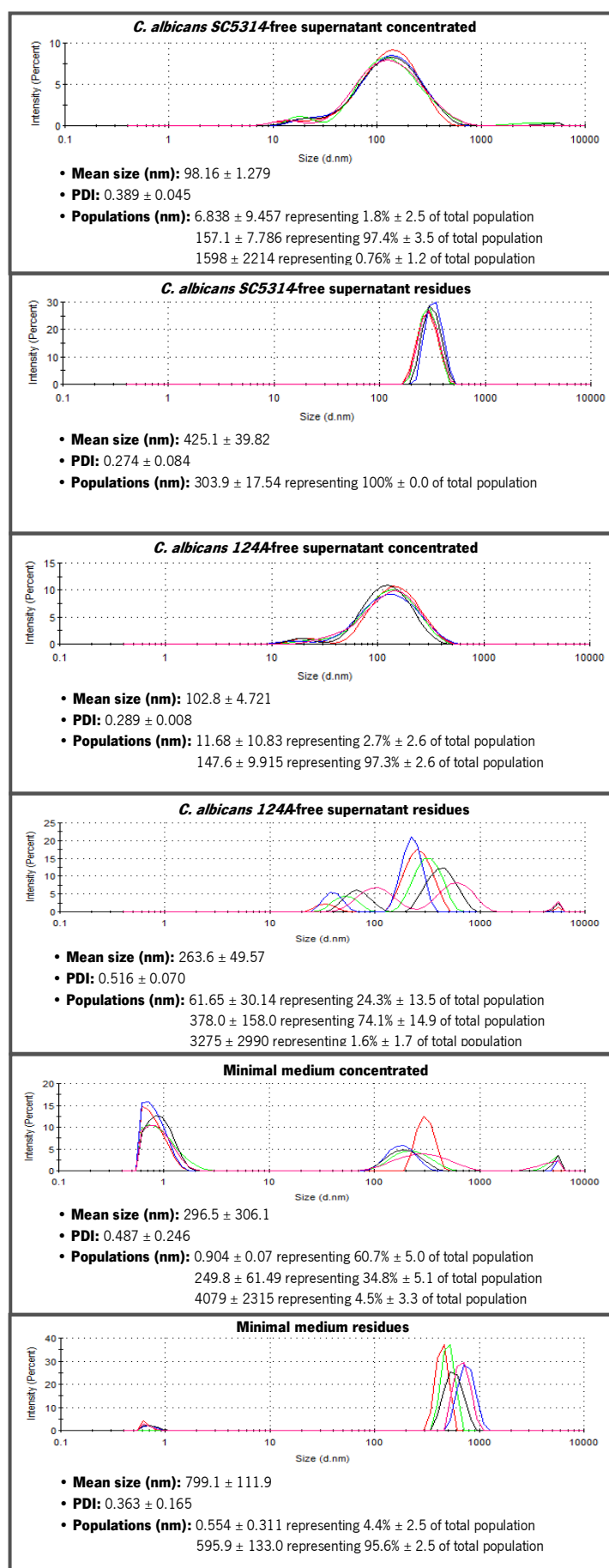


Figure 18. DLS size distributions of samples resulting from the Amicon® Ultra-15 Centrifugal Filter Unit, where *C. albicans* SC5314 and *C. albicans* 124A-free supernatant whose growth was performed in minimal medium were used. Minimal medium was used as control. The mean size, PDI and populations size are indicated.

These results are in agreement with what was previously reported for *C. albicans* SC5314 exosomes in [82], where most vesicles had sizes between 50 and 100 nm, and a smaller percentage had larger sizes reaching more than 200 nm. Slight differences in the distribution of EVs particle size between different strains have been reported, as in [83], but for these specific *C. albicans* strains no significant differences were noted.

3.3 Analysis of exosomes isolation by Western blotting

In order to further validate exosomes isolation, immunoblotting assays were performed using antibodies recognizing four different molecular markers of exosomes: anti-CD9, anti-CD63, anti-CD81 and anti-Hsp70. For this analysis, *C. albicans* SC5314-free supernatant and *C. albicans* 1244-free supernatant concentrated samples whose growth was performed in minimal medium were used to evaluate the exosomes isolation, alongside with minimal medium used as control and the residues of both supernatants samples to verify if particles loss occurred during the process. As mentioned before, two different protein concentrations were tested for the purpose of optimizing the protocol: for each antibody one gel was loaded with 8 μ g of protein directly from the samples and other gel was loaded with 20 μ g of protein, whose samples were submitted to TCA protein precipitation. In **Figure 19** is represented the western blot analysis using the anti-Hsp70 exosome antibody for both protein concentrations tested.

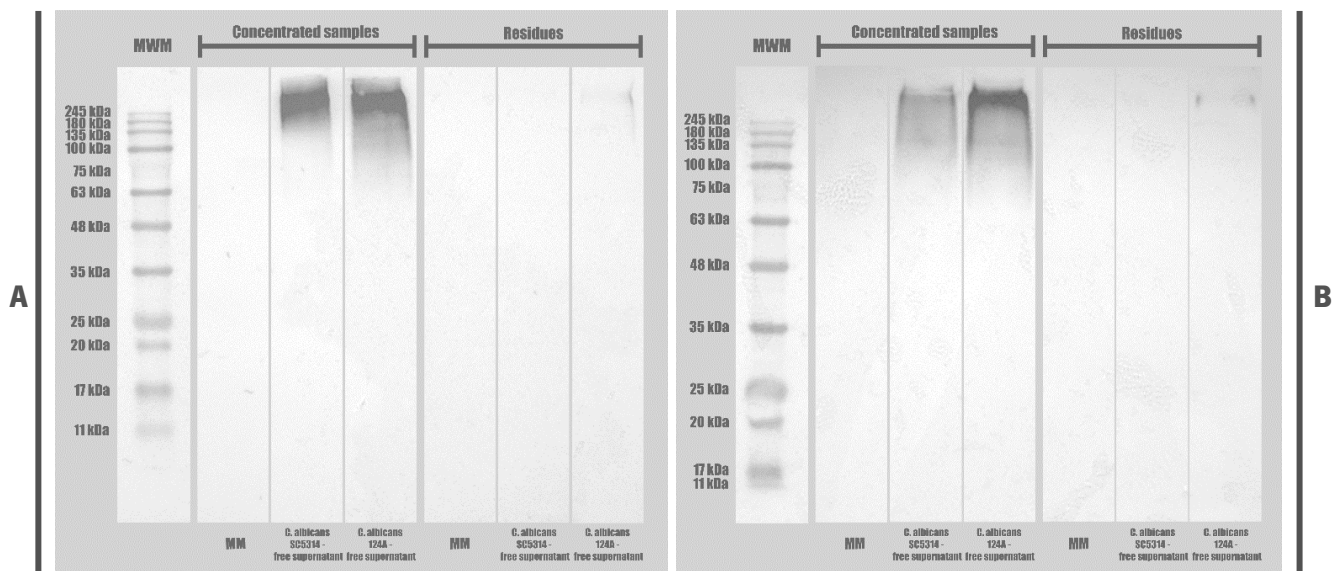


Figure 19. Western blot analysis of *C. albicans*-free supernatants concentrated samples alongside with their residues using anti-Hsp70 exosome antibody. Minimal medium (MM) was used as control.

(A) gel loaded with 8 μ g of protein directly from the samples;

(B) gel loaded with 20 μ g of protein, whose samples were submitted to TCA protein precipitation.

The results show the presence of the vesicle marker Hsp70 antigen epitope in both of *C. albicans*-free supernatants concentrated samples, while reaction is negative in the control, minimal medium, and a minimal presence of this vesicle was detected in the residues samples which implicates a minimal loss during the experimental process. The positive result in the concentrated samples indicates the presence of *C. albicans* exosomes, which goes in agreement with previous reports that also detected this protein in *C. albicans* vesicles, and further validates the isolation protocol [76]. The protein band for the heat shock protein Hsp70 is reported to be located at a molecular weight of circa 55 kDa – 70 kDa, however our results demonstrate detection starting at a molecular weight above 245 kDa until circa 70 kDa which can possibly be explained by protein glycosylation that causes detections in higher molecular weight than expected. In terms of protein quantities, no significant differences were noted in this assay.

The western blot assays using the anti-CD9, anti-CD81, and anti-CD63 antibodies revealed the absence of these proteins on the exosomes. These tetraspanins are considered to be biomarkers of extracellular vesicles by several authors, but unfortunately, proteomic analyses of fungal EVs have revealed that many of the markers that have been so useful for the isolation and study of mammalian EVs are not present in fungal EVs [154], [155], [156]. In *C. albicans* research, two promising putative markers have been suggested for *C. albicans* EVs, the plasma membrane proteins Sur7 and Evp1, which are indicated to have potential of being fungal equivalents of the tetraspanins markers due to their topological similarity to CD9, CD81 and CD63, but antibody-based validation is required and commercial antibodies for these are not yet available [83]. Our results are in accordance with this hypothesis, nonetheless there are reports revealing the detection of these specific tetraspanins in the fractions of *Candida* exosomes demonstrating inconsistency among the literature, which can be due to different experimental conditions, species and strains specificity used that could lead to different EVs composition and characterization [157].

3.4 The effect of exosomes in cytotoxicity, uptake and cytokine production by macrophages

One of the main concerns before studying the interaction of these EVs with any cell line is the evaluation of its cytotoxicity. In this sense, the effect of the *C. albicans* SC5314 and *C. albicans* 124A exosomes on the metabolic activity of J774A.1 cells was evaluated using the MTT assay after 24 h of co-incubation, alongside with minimal medium used as an experimental control, and LPS used as a positive control since it is a highly conserved cell wall component of gram-negative bacteria known to activate immune cells [158]. As represented in **Figure 20**, a slight decrease in cell metabolic activity is observed with the *C. albicans*-free

supernatants, particularly for the *C. albicans* SC5314 strain. Nonetheless, both exosomes samples were well tolerated by the macrophage cells, showing viability values above 80% and 90% for *C. albicans* SC5314free supernatant and *C. albicans* 124Afree supernatant, respectively, which indicates that both of *C. albicans* exosomes are not cytotoxic to these cells.

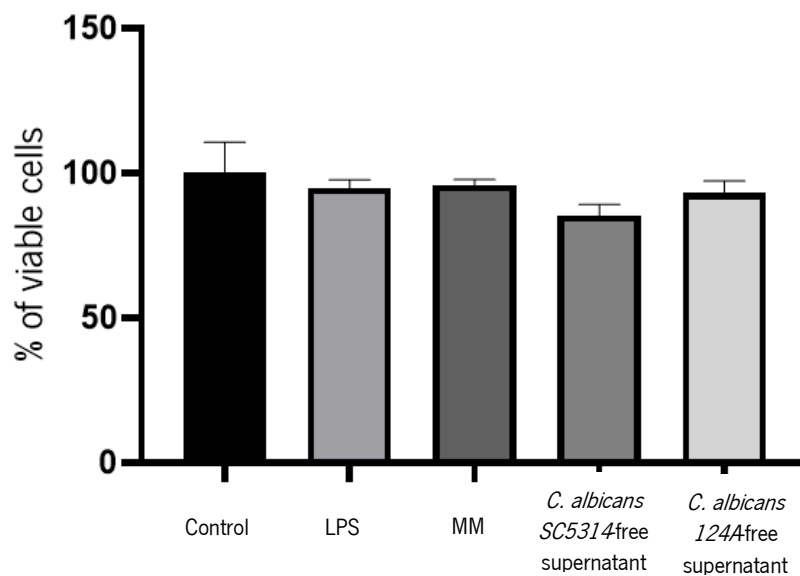


Figure 20. Viability of J774A.1 cells after 24 h incubation with *C. albicans* SC5314 and *C. albicans* 124A free supernatants. Minimal medium (MM) was used as experimental control and LPS as positive control. Viability was assessed using the MTT assay after 24 h incubation.

In order to elucidate the effect of exosomes in the cellular uptake of *C. albicans*, J774A.1 cells were firstly incubated with both of the exosomes samples for 24 h at 37 °C in a 5% CO₂ atmosphere. On the following day, the activated phagocytic cells were incubated with formol-killed *C. albicans* cells previously stained with Sytox Green for 45 min and then contra-stained with PI, according to our previous developed phagocytosis assay [159]. This specific protocol takes advantage of PI quenching effect of the non-internalized yeast cells, since PI is not permeant to live cells, and of the integrity of the Sytox Green fluorescence of *C. albicans* cells inside live macrophages, since Sytox Green is a high affinity nuclear stain that penetrates cells and intercalates with nucleic acids. Therefore, this protocol enabled the differentiation of the non-labeled phagocytes, representing phagocytes with no interaction with yeast cells, the Sytox Green labeled phagocytes, representing phagocytes that have internalized yeast, and the dead phagocytes that become stained with PI.

For this phagocytosis analysis, fluorescence microscopy images were taken, and subsequently analyzed using ImageJ cell counter software to assess the percentage of macrophages with and without internalized yeast cells, and dead macrophages (**Figure 21**). Results revealed lower percentages of macrophages with

internalized yeast cells in both test conditions where macrophages were previously incubated with *C. albicans* SC5314 and *C. albicans* 124A exosomes when compared with the control condition, which suggests that the presence of *C. albicans* exosomes affects the phagocytic capacity of macrophages to some extent. The percentage of macrophage phagocytosis was lower for the macrophages previously incubated with *C. albicans* SC5314 exosomes than with *C. albicans* 124A exosomes, nonetheless without a considerable difference. This difference in macrophage phagocytosis between the control and the test conditions is illustrated in **Figure 22**, which consists of fluorescence microscopy images taken of the control (**Figure 22A**) and of the test condition where macrophages were previously incubated with *C. albicans* SC5314 exosomes (**Figure 22B**).

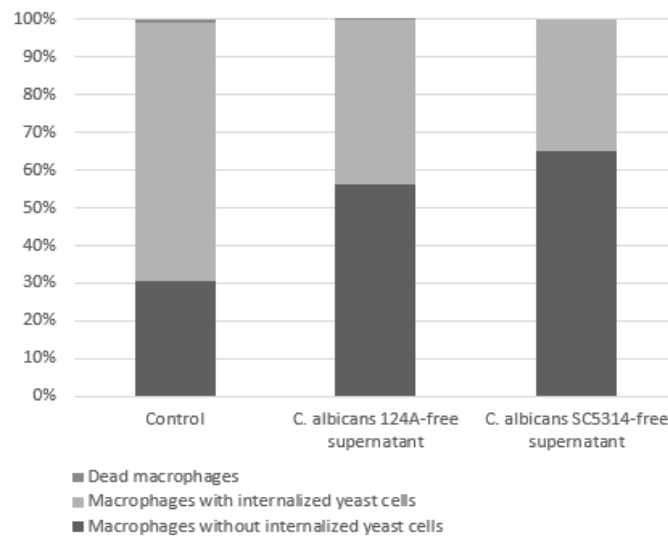


Figure 21. Quantification of macrophage phagocytosis of yeast cells evaluated by fluorescence microscopy, where macrophages from test conditions were previously incubated with *C. albicans* SC5314 and *C. albicans* 124A-free supernatants for 24 h. (■) Macrophages without internalized yeast cells; (■) Macrophages with internalized yeast cells; (■) Dead macrophages.

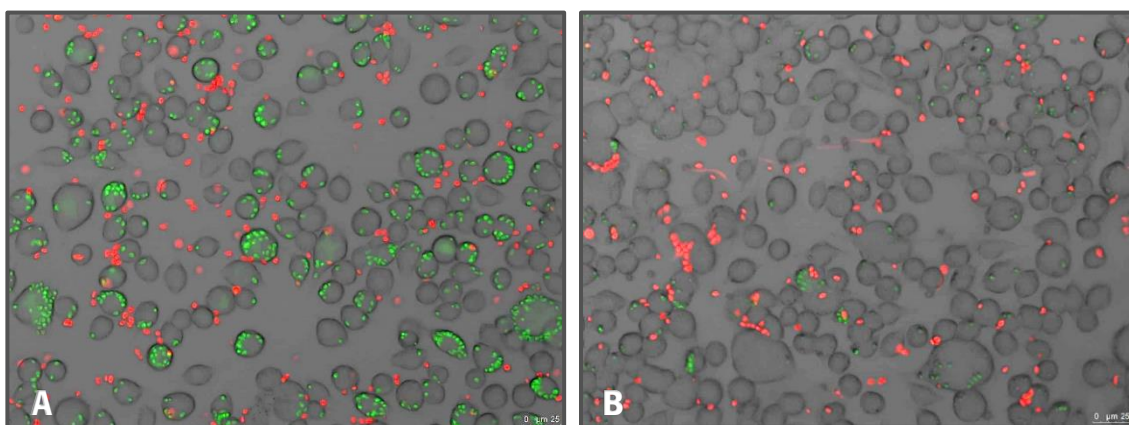


Figure 22. Illustration of macrophage phagocytosis of yeast cells by fluorescence microscopy in the control (A) and the test condition where macrophages were previously incubated with *C. albicans* SC5314-free supernatant for 24 h (B). (●) Internalized yeast cells; (●) Non-internalized yeast cells.

After the phagocytosis analysis, it was important to evaluate the effects that the presence of *C. albicans* exosomes could have in the macrophages activation. Accordingly, secretion of pro-inflammatory cytokines, namely TNF- α and IL-10, by the macrophages was quantified by ELISA after 24 h co-incubation with both *C. albicans* exosomes samples and after 45 min incubation with yeast cells, which corresponds to before and after infection, respectively (**Figure 23**). The inflammatory cytokine TNF- α is associated with the control of candidiasis, whereas IL-10 is described to facilitate an immuno-suppressive response favoring *C. albicans* dissemination [160].

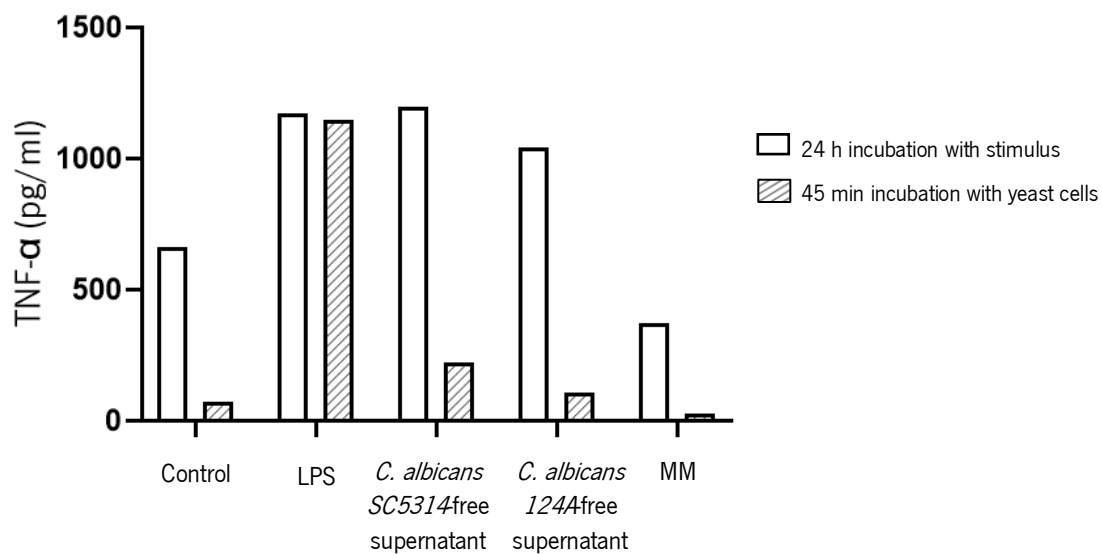


Figure 23. Production of TNF- α by J774A.1 cells after 24 h incubation with *C. albicans* SC5314 and *C. albicans* 1244free supernatants, and after 45 min incubation with *C. albicans* SC5314 yeast cells. Minimal medium (MM) was used as experimental control and LPS as positive control.

The results obtained from the samples before infection indicate that the presence of both of *C. albicans* exosomes samples induced the production of the pro-inflammatory cytokine TNF- α in J774A.1 cells with considerably higher levels than the control, suggesting effective activation of macrophages by these vesicles. After infection, a reduction in TNF- α production is observed in all samples, except for LPS as expected, since it is known to induce host inflammatory responses. Nonetheless, TNF- α secretion levels by the macrophages that were pre-incubated with *C. albicans* exosomes remained higher than in the control but correlated inversely with the percentage of phagocytosis. Regarding the production of the pro-inflammatory cytokine IL-10, no significative levels were secreted by J774A.1 cells to allow detection. Altogether, this data suggests

that *C. albicans* exosomes stimulate the host immune system inducing TNF- α secretion before infection while simultaneously reducing the phagocytic capacity of the macrophages. The presence of *C. albicans* exosomes in an infection situation seems to result in a balance within the inflammatory response and the survival of the pathogen, which supports the commensalism interactions between the yeast and the host.

These results are in line with a previous report that demonstrated that *C. albicans* EVs are immunologically active and can potentially interfere with the host response in the setting of invasive candidiasis, where higher levels of TNF- α secreted by macrophages were also detected. Contrarily to our results, this report also detected high levels of IL-10 [85]. Another report demonstrated that *C. albicans* EVs followed the pattern exhibited by most fungal EVs, as they induced killing and did not induce the production of TNF- α and IL-10 pro-inflammatory cytokines, which is discordantly with what our results indicate [78]. Nonetheless, different macrophages cell lines and strains of *C. albicans* were used in these studies, reinforcing the possibility that the biological activities of EVs could be model and strain specific, which is a hypothesis that has been suggested in earlier studies [77], [78].

3.5 The effect of zebrafish embryos co-incubation with *Candida* cells on their survival rate

After the characterization of *C. albicans* exosomes and the study of their role in an infection environment using a cell culture model, it is important to evaluate their role in the context of a whole organism where the complex host-fungus interactions can be investigated, and the disease development can be monitored right from the moment that fungal cells are introduced into the host. In this context, *Danio rerio* early life was the selected model for this analysis given the high similarity to mammals, as genotypic and embryonic development, at these early stages, and all the advantages that have been attributed to their use in research. To proceed with the exosomes studies, first it was necessary to establish the zebrafish embryo as an effective and feasible model to study host-*Candida* interactions.

Therefore, the survival rates of infected zebrafish embryos with different doses of *C. albicans* cells were determined to evaluate the feasibility of using zebrafish early stages as a model host for *C. albicans* infections. Embryos co-incubation with *Candida* cells was performed at 6 hpf with different doses that ranged from 1×10^7 to 1×10^3 *Candida* cells. All of the conditions had 2 replicates, each well representing one replicate with 10 embryos, and survival analysis in each condition was performed at 8, 32, 56 and 80 hpf. In **Figure 24** is represented the survival rates of zebrafish embryos co-incubated with *Candida* cells in three independent experiments.

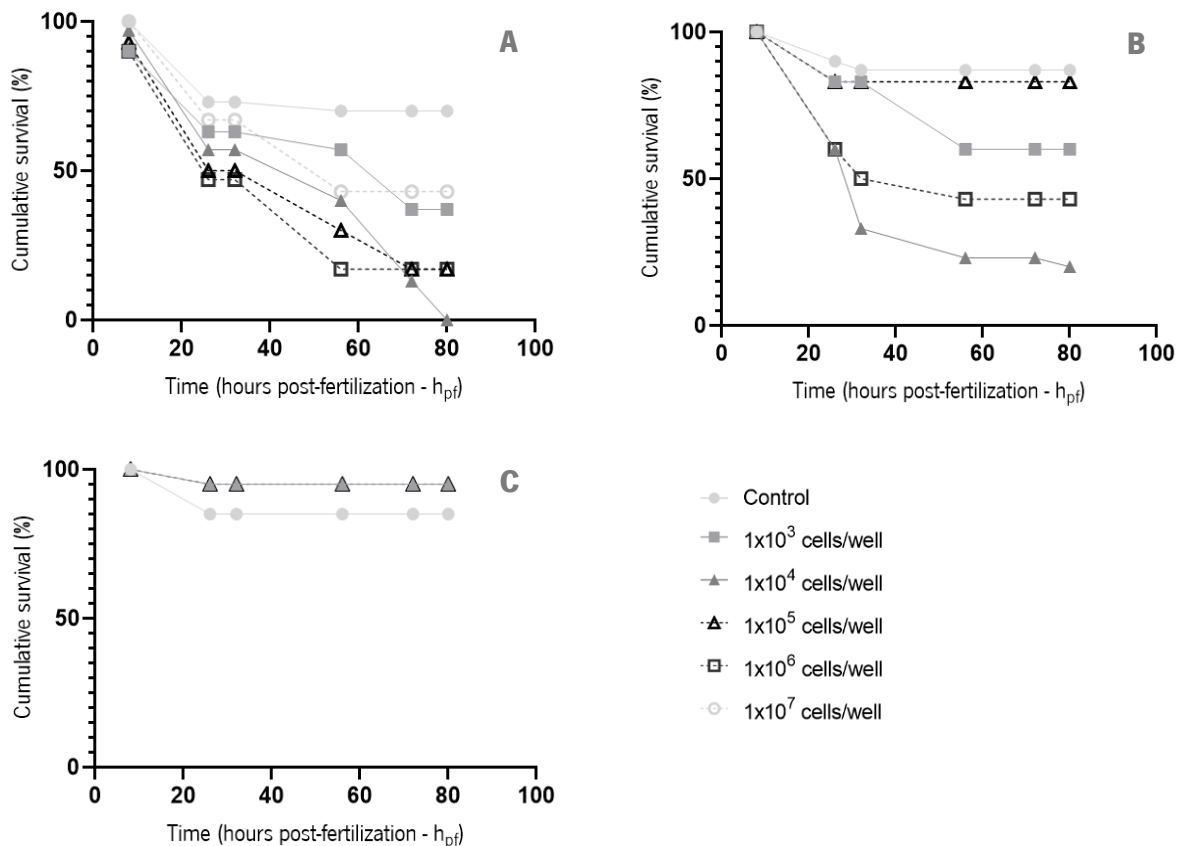


Figure 24. The survival rates of zebrafish embryo infected with different doses of *C. albicans* SC5314 along 80 hpf in three independent replicates (A, B and C).

As shown in **Figure 24**, the survival rates with the different *Candida* cells doses significantly differ in between replicates which indicated a problem in reproducibility for this assay. This inconsistency suggests that the inoculation doses were not consistent between replicates, which can be explained by some difficulties in the execution of the serial dilutions, and that a control with colony forming units (CFUs) should be performed to confirm the number of viable cells present in the samples. Nevertheless, the survival rate in the control was noticeably higher than for the *Candida* cells exposed specimens, independently of the test concentration, which indicates that infection by *Candida* cells does affect the survival of zebrafish embryos.

Unfortunately, it was not possible to preform further assays to solve the variability noted and to proceed with the establishment of the model due to restrictions of time and lack of specimens to perform the assays. Regardless of the difficulties, these preliminary results allowed the conclusion that infection by *Candida* cells by co-incubation in the water does affect embryo survival and possibly in a dose-dependent manner, as well as the identification of variables that are necessary to control, such as always doing controls using CFUs to verify the *Candida* cells concentration in the solutions, in order to implement the zebrafish embryo as a model for the study of host-*Candida* interactions.

Chapter 4. Final Remarks and Future Perspectives

4. Final remarks and future perspectives

Isolation and characterization of *C. albicans* SC5314 and *C. albicans* 124A exosomes was achieved using the Amicon® Ultra-15 Centrifugal Filter Unit concentration device and minimal medium to grow the yeasts. The physico-chemical properties of the isolated exosomes were studied using DLS and ELS which did not reveal significant differences between the two strains, with measurements indicating negatively charged exosomes with a mean size around 150 nm for both of *C. albicans* strains.

Macrophages studies were performed to assess the role of *C. albicans* exosomes in an infection situation. Neither of the exosomes samples were proven to be cytotoxic to the J774A.1 cells. Regarding the uptake and cytokine production by the macrophages infected with yeast cells when previously co-incubated with the exosomes, the results suggests that exosomes stimulate the host immune system inducing TNF- α secretion while simultaneously reducing the phagocytic capacity of the macrophages and consequently inhibiting the killing of the yeast cells. The presence of *C. albicans* exosomes in an infection situation seems to result in a balance within the inflammatory response and the survival of the pathogen, which supports the commensalism interactions that can occur between the yeast and the host.

Evaluation of the roles of *C. albicans* exosomes in an infection environment in the context of a whole organism is also crucial for a more comprehensive understanding of these vesicles. In this context, zebrafish embryo was the selected model for this analysis given the high similarity to mammals and all the advantages attributed to their use in research namely: small size, external and rapid development, optical transparency, low cost of maintenance and ethical *in vivo* analysis. To proceed with the exosomes studies, it was necessary to establish the zebrafish embryo as an effective and feasible model to study host-*Candida* interactions. Therefore, the survival rates of infected zebrafish embryos with different doses of *C. albicans* cells were determined to evaluate the feasibility of using zebrafish early stages as a model host for *C. albicans* infections. Preliminary results allowed the conclusion that infection by *Candida* cells does affect embryo survival, as well as the identification of variables that are necessary to control in order to implement the zebrafish embryo as a model for the study of host-*Candida* interactions.

Ideally, in the future, repetitions will be performed to confer greater statistical strength of the results obtained. Further assays are needed to reduce the variability regarding the survival of zebrafish embryos co-incubated with *Candida* cells by performing CFUs controls in the previous protocol or by conducting the infection by microinjection, which is a more invasive model but it offers a direct route of administration. Once the zebrafish embryo is established as a viable model to study *Candida* infections, *in vivo* tests to assess the roles of these exosomes can be performed. Ultimately, the expectation is that the better understanding of the biology and function of *Candida* EVs, specifically exosomes, could open new horizons for the understanding

of *Candida* pathogenesis and help elucidate possible therapeutic targets and design new therapies against this infection.

Chapter 5. References

5. References

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Chapter 6. Supplementary data

6. Supplementary data

Table 7. Count rates and attenuators of samples obtained from the two concentration systems.

Concentration system	Sample	Count Rate (kcps)	Attenuator
Amicon® Ultra-15	Concentrated	365.56	10
Centrifugal Filter Unit	Residues	178.42	11
Amicon® Stirred Cell	Concentrated	233.4	8
	Residues	206.8	10

Table 8. Count rates and attenuators of samples obtained from the Amicon® Ultra-15 Centrifugal Filter Unit which growth was performed in YEPD medium.

Sample		Count Rate (kcps)	Attenuator
<i>C. albicans SC5314</i>	Concentrated	189.5	9
free supernatant	Residues	190.2	11
<i>C. albicans 124A</i>	Concentrated	518.06	9
free supernatant	Residues	294.34	11
YEPD medium	Concentrated	218	10
	Residues	302.58	11

Table 9. Count rates and attenuators of samples obtained from the Amicon® Ultra-15 Centrifugal Filter Unit which growth was performed in minimal medium.

Sample		Count Rate (kcps)	Attenuator
<i>C. albicans SC5314</i>	Concentrated	377.86	10
free supernatant	Residues	251.52	11
<i>C. albicans 124A</i>	Concentrated	181.36	9
free supernatant	Residues	251.34	11
Minimal Medium	Concentrated	76.08	11
	Residues	275.74	11