DIPLOMARBEIT

Pathogenicity of *Candida albicans* strains with *Galleria mellonella*, a model organism for fungal infections

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Background: In the last decades previously undocumented manifestations of *Candida* infections appeared, and the incidence, especially in the last two decades, increased dramatically on account of more susceptible hosts.

Despite the use of antifungal agents, candidemia is still the most frequent life-threatening fungal disease. Denture stomatitis is recently considered to be the most common form of oral candidiasis and oropharyngeal candidiasis (OPC) is still the most frequent opportunistic infection affecting up to 90% of HIV infected individuals.

The objective of this work was to determine whether there is a difference in survival of the greater wax moth *Galleria mellonella* infected with either invasive or superficial isolates of *Candida albicans*.

Methods: For these investigations 20 different clinical *C. albicans* strains were used. They were isolated from various patients from either oral infection sites (n= 10, considered as superficial) or from systemic candidiasis (n= 10, considered as invasive), namely blood culture. Larvae of *G. mellonella* were injected with 20 µl of a *C. albicans* suspension (concentration 2,5x10^{9} cfu/ml) through the last, left proleg directly into the haemocoel. Consequently the infected larvae were kept in a petri dish covered with a kimwipe and incubated at 25°C for a maximum of seven days.

Survival assays were carried out daily. Larvae were considered dead when they displayed no movement in response to touch and were removed from the petri dish. Killing curves were plotted and statistical analyses were performed.

Results: We discovered that it is possible for the fungus to proliferate inside the haemocoel of the larvae and to colonize and kill the caterpillar. However, the results indicate that there is no statistically significant difference between superficial and invasive isolates of *C. albicans* ($p=0.6$). The killing curves of superficial and invasive isolates show similar linear curve progression.

Discussion: The insect model organism *G. mellonella* represents a suitable alternative model for the evaluation of *Candida* pathogenesis. The finding that superficial isolates have the potential to cause elevated morbidity, if systemically disseminated, requires prompt therapeutic intervention of superficial *C. albicans* infection, especially in debilitated hosts.

Hintergrund: In den letzten Jahrzehnten, vor allem in den letzten 20 Jahren, kam es vermehrt zum Auftreten von bis dahin unbekannten Manifestationen von *Candida* Infektionen und auch deren Inzidenz stieg auf Grund von immer mehr empfänglichen Patienten an. Trotz des Einsatzes von potenten Antimykotika bleibt die Candidämie die häufigste lebensbedrohliche Pilzinfektion. Neuerdings nimmt man an, dass Prothesen - Stomatitis die häufigste Form von oropharyngealer Candidiasis ist. Weiters ist die oropharyngeale Candidiasis (OPC) die häufigste opportunistische Infektion bei HIV positiven Menschen, die im Laufe der HIV Erkrankung bis zu 90% der Infizierten betrifft. Das Ziel dieser Studie war es herauszufinden, ob es einen Unterschied im Überleben der Großen Wachsmotte *Galleria mellonella* gibt, die entweder mit oberflächlichen oder systemischen klinischen Isolaten von *Candida albicans* infiziert wurde.

Methoden: In dieser Studie wurden 20 verschiedene *C. albicans* Stämme von diversen Patienten, entweder von oraler (=oberflächlicher) *Candida* Infektion (n=10) oder von positiven Blutkulturen (n=10, invasiv), isoliert. Die Larven der großen Wachsmotte wurden mit 20 µl einer *Candida* Suspension (Konzentration 2,5x10^9 cfu/ml) über den letzten linken Bauchfuß, ein direkter Zugang zum Haemocoel, beimpft. Daraufhin wurden die Larven in eine mit Filterpapier ausgelegte Petrischale transferiert und bei 25°C für maximal sieben Tage inkubiert. Täglich wurden die Larven auf ihr Überleben kontrolliert. Tote Larven, die keine Antwort auf physikalische Stimuli (Berührung mit einer Pinzette) zeigten, wurden als abgestorben bewertet und aus der Petrischale entfernt. Zur Auswertung wurden statische Analysen angewendet und Sterbekurven angefertigt.

Ergebnisse: Die Resultate zeigen, dass es *C. albicans* möglich ist sich innerhalb des Haemocoels der Larve zu vermehren, sowie die Larve zu kolonisieren und zu töten. Dennoch konnte kein statisch signifikanter Unterschied zwischen oralen und systemischen *C. albicans* Isolaten gefunden werden (p=0.6).

Diskussion: Das Insekt *G. mellonella* erweist sich als passender Modellorganismus zum Studium der Pathogenese von *Candida* Infektionen. Das Ergebnis, dass auch superfizielle Isolate das Potential zu erhöhter Morbidität besitzen, belegt die Notwendigkeit möglichst rascher therapeutischer Intervention bei oberflächlicher *C. albicans* Infektion, insbesondere in geschwächten Patientengruppen.

Abbreviations

ALS	agglutinin-like sequence
AMB	amphotericin B
AMP	antimicrobial peptide
APC	antigen presenting cell
cfu	colony forming unit
CMC	chronic mucocutaneous candidiasis
EDTA	ethylenediaminetetraacetic acid
ERG	ETS related gene
FACS	fluorescence-activated cell sorting
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
Hsf1	heat shock factor 1
HSP	heat shock proteins
IL	interleukin
IPS	insect physiological saline
KCl	potassium chloride
MALDI-TOF	matrix assisted laser desorption ionisation-time of flight
MAPK	mitogen-activated protein kinases
NaCl	sodium chloride
OPC	oropharyngeal candidiasis
PAMP	pathogen-associated molecular patterns
ProPO	prophenoloxidase
ProPO-AS	prophenoloxidase activating system
PRR	pattern recognition receptors
RNS	reactive nitrogen species
ROS	reactive oxygen species
RVVC	recurrent vulvovaginal candidiasis
SAB	sabouraud glucose agar
sp./ spp.	species/ species (plural)
Th	T-helper cell
TLR	toll-like receptor

TrisHCl	tris(hydroxymethyl)aminomethane hydrogen chloride
VVC	vulvovaginal candidiasis

1. Introduction

1.1. Candida

1.1.1. Biologic taxonomy

Fungi form a distinct group (kingdom) of eucaryotic (higher) organisms like plants and animals. (Dismukes et al. 2003, p. 3) The characteristic features of eucaryota are cells containing a real nucleus and a cell membrane. However, genetic studies support that fungi are related phylogenetically closer to the kingdom of animals rather than to the plants. (Guarro et al. 1999)

The kingdom of fungi is specified as chlorophyll-free organisms having a rigid cell wall consisting of chitin within hyphal growth forms, and glucane and mannan within budding fungi (yeasts). Missing photosynthesis, fungi feed on external digestion by excreting lytic enzymes and following absorption of nutrients, they are heterotrophic. Additionally the cell structure is kept simpler comparing with other eucaryotes, where cells unite to tissues and organs. In fungi cells are forming a chain of tubular, filament-like elements or single uni-cellular cells. Some pathogens (dimorphic fungi) are dimorphic, able to change their appearance from multicellular mycelia to single-celled yeast cells.

The majority of multicellular fungi in the vegetative stage are composed of filamentous cells, a web of branching hyphae building a real mycelium. During the vegetative (growth) period hyphae lengthen. Aseptate hyphae are characteristic for primitive fungi (zygomycota), in comparison to septate ones, sectioned by cross-walls, found in more advanced groups (asco- and basidiomycota). (Dismukes et al. 2003, p. 3)

In contrast yeasts proliferate by budding out similar cells at various parts from the surface of a mother cell. Those increase in size and separate in order to complete mitosis.
In special circumstances, single cells or hyphae form pseudohyphae, a continuous

elongation of parent cell without budding, resulting in an attached chain of elongated cells with constrictions at cell junctions resembling true hyphae. The pseudohypha is considered to be an intermediate growth form of yeast and hyphal morphologies.

Some yeasts are even able to form true hyphae from yeast cells or as branches of existing hyphae, outgrowing a germ tube by apical growth and developing septa following the growing tip of hyphae. Repeated budding takes place behind the newly formed cross-wall merely at the perpendicular axis of the main hyphae. (Calderone 2002, p. 14–15)

From the biologic point of view, fungi were until recently divided into fungi perfecti (teleomorph) and imperfecti (anamorph) according to the kind of reproduction. In the perfect stage they reproduce by forming sexual spores undergoing meiosis either endogenous in structures such as ascus (a round or oval configured sacklike cell, which releases ascospores) or exogenous basidiospores as in the case of basidiomycetes.

In comparison the imperfect stage, where until now only asexual spores (conidia) sprouting by mitosis and creating an exact copy of the parent cell have been discovered, generate arthrospores (spore formed by breaking of hypha or by fission of a single cell), blastospores (spores formed by budding and denoted blastoconidia) or sporangiospores (spores within a sacklike structure called sporangium).

The vast majority of human pathogenic fungi belong to the group of fungi imperfecti proliferating in vegetative stage. (Seebacher 1990, p. 13–14, 24, 257-259)

Molecular techniques have shown that the separation into fungi perfecti and imperfecti does not make a sense, because many fungi can reproduce both sexually and asexually (e.g. anamorph *Candida krusei*, teleomorph *Issatchenkia orientalis*).

In modern phylogeny the higher fungi are divided into ascomycetes, basidiomycetes, zygomycetes and chytridiomycetes, the majority of human pathogenic fungi are located in ascomycetes (*Candida*, *Aspergillus*, dermatophytes), some in basidiomycetes (*Cryptococcus*, *Malassezia*) and in zygomycetes (*Mucor*, *Rhizopus*, *Lichtheimia*). In chytridiomycetes no human pathogenic fungi have been found so far, but some species are responsible for the worldwide dying of amphibians (Chytridiomycosis). (Hoog 2000, p. 4-20)

The cell wall distinguishes fungi from other eucaryota. It is the first part of fungal cell to combat with pathogens and to challenge the environment. The synthesis of the fungal cell wall is one of the targets for antifungal drugs, since any disruption will have an impact on growth and morphology of the fungal cell, consequently leading to cell lysis and death.
As a result, cell wall construction is tightly controlled, and the composition, structure and thickness of the wall are modified depending on predominate exterior factors.

The main purpose is maintaining the cell shape, stabilizing the internal osmotic pressure, protecting against physical stress and serving as a framework for proteins.

The cell wall consists of a three-dimensional scaffold of polysaccharides, glycoproteins and proteins. The network contains fibrillar polysaccharides, representing the main inert scaffold, surrounded by a gel-like matrix containing polysaccharides, glycoproteins and proteins, and a variety of molecules such as lipids, pigments, inorganic ions and salts. The latter vary as they are used as nutrient reserves, for transport and translocation, for metabolizing non permeable substances, for communicating and interacting with the environment and protecting against outside attack.

A fundamental of cell wall component are polysaccharides, contributing with 80% to the wall material, and the most important one to maintain structure is chitin, a long polymer of N-acetylglucosamine. Proteins, on the other hand, make up 20%, most of them glycoproteins; they determine surface properties, since situated at the outer surface or close to it. (Moore et al. 2011, p. 157–158)

1.1.2. Medical taxonomy

Up to now scientists have discovered and described more than 100.000 species of fungi and 500 of them have been associated pathogens for human beings and no more than 100 cause disease in individuals with intact immune system.
(Dismukes et al. 2003, p. 3)

In order to keep track of medically relevant fungi a classification for clinical practice is necessary. Therefore fungi of medical significance are subdivided into dermatophytes, molds, dimorphic fungi and yeasts or, alternatively, due to their pathogenic activity as pathogens, opportunists or apathogens.

Dermatophytes are the causative agents of dermatophytosis, living as parasites on keratinized tissues (skin, hair and nails) and including the anamorphic genera *Trichophyton*, *Microsporum* and *Epidermophyton* as well as the teleomorph *Arthroderma*. In general, anthropophilic, zoophilic or geophilic species are known, depending on their natural reservoir. Some human pathogenic dermatophytes causing skin infections are *T. rubrum*, *T. mentagrophytes*, *T. soudanense*, *T. schoenleinii*, *M. canis*, *M. gypseum* and *E. floccosum*.

Molds, a group of heterogenous fungi, present as filamentous, fast growing and colourful colonies forming numerous spores. The majority of molds are opportunistic pathogens and belong to the biological classes of Zygomycota, Ascomycota and Deuteromycota. Some examples are the genera *Mucor, Rhizopus, Absidia, Aspergillus, Botrytis, Monilia, Penicillium, Alternaria* and *Fusarium*.

Yeasts and yeast-like fungi reproduce by budding, considering that some yeasts have the property of dimorphism. Generally, distinction is made between sexual and asexual reproduction. Yeasts undergoing sexual reproduction are Ascomycota (e.g. *Issatchenkia*, the teleomorphic state of *C. krusei* or the baker's yeast *Saccharomyces cerevisiae*) and Basidiomycota (e.g. *Filobasidiella*, the teleomorphic state of *C. neoformans*), those with asexual (anamorphic) life cycle only belong historically to Deuteromycota (*Candida, Cryptococcus, Trichosporon,* *Malassezia, Rhodotorula*). However, currently major changes in the phylogeny and taxonomy of fungi take place.

Dimorphic fungi, as mentioned above, are dimorphic changing from single cell to filament structure in a temperature dependent matter in most cases like *Histoplasma capsulatum*. Other representatives are *Coccidioides immitis*, *Blastomyces dermatitidis* and *Sporothrix schenckii*.

Pathogenic fungi are described as fungi causing disease in healthy hosts, among them are most dermatophytes and dimorphic fungi as *C. neoformans*. Opportunistic fungi, in comparison, are fungi living as saprophytes in the host's environment and revealing their potential as pathogens under certain circumstances causing disease in the colonized host. Some facultative pathogens are *Candida* spp., *Trichosporon* spp. and *Aspergillus* spp.. *Saccharomyces* spp. is regarded apathogenic even in debilitated hosts. (Seebacher 1990, p. 14–21)

1.1.3. Candida species

About 200 species belong to the anamorphic genus *Candida* (Kauffman et al. 2011, p. 167–168) and create a heterogeneous group that altogether grow as yeasts. Most members of the genus are capable of forming a filamentous type of growth and only *C. albicans* and *C. dubliniensis* produce pseudohyphae additionally. This renders both species polymorphic microorganisms. (Calderone 2002, p. 15)

Only a small number of yeasts, approximately 17 different species, have been reported as pathogens. Nevertheless *C. albicans* remains the major fungal pathogen of humans and the most common cause of mucous membrane and fungal bloodstream infection.

The most common and clinically relevant non-*albicans* species are discussed below:

Candida glabrata infections have increased dramatically worldwide and the pathogen can rapidly develop resistance to antifungal drugs.

Candida parapsilosis causes disease especially in patients carrying intravascular catheters, prosthetic devices and in intravenous drug addicts and is associated with candidemia in neonatal intensive care units. As a virulence factor, extracellular polysaccharides (matrix) often referred to as slime have been identified and enable the adherence to skin of hospital personnel and artificial surfaces. Clinical *C. parapsilosis* isolates often show a reduced susceptibility to echinocandin antifungals.

Candida tropicalis is a common agent of fungemia in patients with leukaemia, prolonged neutropenia and prolonged intensive care unit stay.

Candida krusei, a less common bloodstream isolate, has the potential to cause invasive disease in haematological patients complicated by neutropenia. Clinically significant, *C. krusei* is intrinsically resistant to fluconazole and has decreased sensitivity to most other antifungal agents.

Candida kefyr has a predilection to infect immunosuppressed humans in rare occasions.

Candida guilliermondii infection is uncommon, fungemia in neutropenic host and endocarditis in intravenous drug users occur occasionally. In the clinical setting reduced susceptibility to fluconazole and high minimum inhibitory concentrations to echinocandins are important.

Candida lusitaniae is considered a rare contagion among patients with haematological malignancies and in intensive care units, but acquires intrinsic or secondary resistance to amphotericin B.

Candida dubliniensis is a more recently identified *Candida* species from the oral cavity of HIV- infected patients and patients suffering from cystic fibrosis with similar morphologic characteristics seen in *C. albicans*.

Molecular-based methods enable the identification of cryptic species such as *Candida orthopsilosis*, *Candida metapsilosis*, *Candida nivariensis*, *Candida bracerensis* and *Candida fermentati*. (Kauffman et al. 2011, p. 167–168)

1.1.4. Candida albicans

The causative agent of thrush was thought to have been discovered various times in the past, without identifying the appropriate/correct organism. In 1923 the pathogen of thrush lesions was named *Candida* for the first time, *derived from the Latin phrase "toga candida", which was used to describe a special white robe worn by candidates for the Roman Senate.* The term "*albicans*", originating from Latin to whiten, was first mentioned in the year 1847 and in the 1950's the Eighth Botanical Congress determined the binominal expression *Candida albicans* as the *nomen conservandum*. (Calderone 2002, p. 4)

The polymorphic fungus *C. albicans*, as mentioned above, has the ability of morphogenetically switching from unicellular budding yeast form (blastospore) to filamentous form (hyphae and pseudohyphae). (Lim et al. 2012)
The size of the yeast cell is 4-6 x 6-10 µm, and this growth form is favoured at temperatures below 35°C and an acidic pH. While environmental parameters, a temperature above 35°C and an alkaline pH, for example, predispose to form hyphae.

Additionally *C. albicans* can produce large (8-12 µm in diameter) asexual, spherical, refractile, thick-walled cells named chlamydospores, that are found on short lateral and terminal branches. (Calderone 2002, p. 16–17, 391)

The white-opaque switching marks a reversible and spontaneously low frequency transition between the normal yeast phenotype (white) and an elongated cell type (opaque) and is termed according to the morphology of the colonies on solid media:

white phase - a white, hemispherical smooth colony
opaque phase - a grey, flat smooth colony

Undergoing the white -opaque transition modulated by environmental conditions the cellular phenotype is affected and the white budding cell presenting as a round to ellipsoid cell transforms into an elongated or bean-shaped cell phase, that is essential for fungal mating.

Another point to be made, this unique developmental programme leads to host adaptation through expression of different gene loci, indicating the two cell types tend to colonize distinct host niches and developed a path to escape primary host defence mechanisms. (Morschhäuser 2010)

Recent literature indicates that the obligate diploid *C. albicans* has a viable haploid state, which forms true hyphae, pseudohyphae and chlamydospores similar to diploids. Finally haploids obtained through a concerted chromosome loss mate to regenerate diploid cells or undergo auto-diploidisation. Concluding, different ploidity states of *C. albicans* contribute to genetic diversity and promote adaptation to changing host conditions.

Further a diploid tetraploid parasexual cycle was described earlier as involved in the opaque transition, thus opaque diploids conjugate to tetraploids following ploidy reduction into diploid cells. (Hickman et al. 2013)

1.1.5. Medical relevance

Epidemiology

Since the 1940's, when the widespread use of antibiotic drugs was introduced, previously undocumented manifestations of *Candida* infections appeared and the incidence, especially in the last two decades on account of more susceptible hosts, has increased dramatically. (Mandell et al. 2010, p. 3225)

Advances in science have provided improved survival of patients with acquired or hereditary immunodeficiency and those being treated with immunosuppressants or biologicals due to malignancies, autoimmune diseases and organ or bone marrow grafts. (Lilic 2012)

Furthermore the use of broad-spectrum antibiotics, intravascular catheters, prosthetic devices, total parenteral nutrition and surgical procedures, in particular gastrointestinal surgery favour the entry of opportunistic fungal pathogens. (Kindo and Giri 2012)

Candida spp. have been the fourth most common microorganism detected in blood samples of hospitalized patients in the US during recent years. Between 2000 and 2005, the incidence of candidemia-related hospitalizations per 100.000 people has accelerated by 52% (Mandell et al. 2010, p. 3225) and the mortality rate of invasive fungal infections is to be estimated about 30 to 70%. (Lilic 2012)

Despite the use of antifungal agents, candidemia is still the most frequent life-threatening fungal disease and several population-based surveys in the Western World have indicated an increased incidence. The incident rates in most European countries have ranged from 1.9 to 4.8/100.000 population per year. The ratio of *C. albicans* caused candidemia ranges from 37% in Latin-America up to 70% in northern countries and suggests significant geographical differences in *Candida* species distribution. (Asmundsdottir et al. 2012)

Denture stomatitis is recently considered to be the most common form of oral candidiasis and oropharyngeal candidiasis (OPC) is still the most frequent opportunistic infection affecting up to 90% of HIV infected individuals at some point during the course of the disease. (Junqueira 2012a)

Importantly, acute vulvovaginal candidiasis (VVC) affects up to 75% of women at least once during their lifetime, and 40-50% of them will have at least one recurrence. Approximately 5-8% of those will experience recurrent VVC (RVVC), defined as four or more mycologically proven episodes within a year. (Rosa et al. 2012)

Clinical Manifestation

While previously extremely infrequent manifestations of *Candida* infections have become documented frequently, clinical manifestations are subdivided into mucous membrane infections, cutaneous affections, and deep organ involvement. (Mandell et al. 2010, p. 3227)

Oropharyngeal Candidiasis (OPC)

Oral thrush is characterized by creamy white patches on erythematous ground on surfaces of oral mucosa, tongue, throat and gums. When wiped off, they leave a raw, erythematous, bleeding and painful plaque. The symptoms are variable, such as severe asymptomatic lesions, painful mucous membrane and burning tongue and dysphagia.

The most common manifestation of OPC is acute pseudomembranous candidiasis presenting as whitish-yellow thick curd-like exsudate affecting the entire oral cavity although small discrete or confluent plaques are present.

Dental prostheses are a predisposing factor for chronic atrophic stomatitis. The chronic inflammatory reaction develops erythema and oedema under the dental plates and typical signs are chronic soreness and burning of the mouth.

Candida infection is not the only cause of angular cheilitis (perleche), which shows soreness, erythema and fissures at the commissures of the mouth.

Oral transparent to white plaques variant in size localized on the inner surface of cheeks, tongue and lips, so called leukoplakia, are used to describe chronic hyperplastic candidiasis.

Acute atrophic stomatitis or midline glossitis causes a nonspecific atrophy with symmetrical affections on the dorsum of the tongue. (Dismukes et al. 2003, p. 172)

Candida Esophagitis

The classification of *Candida* esophagitis is performed on the basis of endoscopic appearance. Type I describes a few white or beige plaques, Type II numerous plaques with more than 2 mm in diameter, Type III confluent, linear and nodular elevated plaques surrounded by hyperaemia and beginning ulcers and Type IV like Type III with increased mucosal involvement and possible luminal constriction. Dysphagia, odynophagia and retrosternal pain may occur but entirely asymptomatic courses are described. (Dismukes et al. 2003, p.174)

Despite there have been a small number of reported cases of *Candida* esophagitis occurring in patients with unknown underlying disease, it is more commonly associated with treatment of malignant hematopoietic or lymphatic disorders and in AIDS patients. (Mandell et al. 2010, p. 3227)

Candida Vaginitis

Vulvovaginal candidiasis (VVC) presents as itching and irritating sensation at the vulva and vaginal introitus. Patients report white, watery and thin or sometimes cottage cheese-like and thick discharge. On gynaecological examination the vulva appears normal to red and edematous. Excoriations and fissures may accompany thin and waxy skin surface. In severe cases the itching sensation forces the patients to scratch and ulcers develop. On speculum inspection white plaques of discharge adhering to the epithelium cover the vaginal mucosa. When removed, bleeding may be visible together with thin and watery secret. (Kruger and Botha 2007, p. 139–140)

Acute VVC can be idiopathic with unknown predisposing factors or caused by several distinct mechanisms: familial susceptibility, pregnancy, systemic antibiotic or oral contraceptive usage, diabetes mellitus, sexual behaviour and immunosuppressive therapy. (Rosa et al. 2012)

Candida Balanitis

Sexual intercourse with a partner suffering from vaginal candidiasis leads to the formation of vesicles on the penis, that develop into patches. This process may be accompanied by severe itching and burning sensations and may extend to the thighs, gluteal folds, buttocks and scrotum. (Mandell et al. 2010, p. 3229)

Intertrigo

This common skin condition occurs in occluded body regions where skin surface is in close proximity and remains moist and warm e.g. groins, armpits, breast folds and interdigital space. It starts with vesiculopustular eruptions, which enlarge and rupture, leading to maceration and fissures of the skin. The area of infection is surrounded by a white border composed of necrotic epidermal cells. Sometimes satellite lesions consolidate causing an extension of the infected area. (Mandell et al. 2010, p. 3230)

Onychomycosis and Paronychia

The inflammation of the nailfold appears as rather well-localized, warm, glistening and tense spreading extremely under the nail and secondary involving the nails, resulting in thickening, ridging and discoloration and - in severe infections - actually nail loss.

Onychomycosis and paronychia are associated with frequent immersion of hands in water and contracts dishwashers, laundry workers and parents of young children, and there is a higher incidence among diabetics. In addition, *Candida* is a cause of onychomycosis itself. (Mandell et al. 2010, p. 3230)

Diaper rash

The reaction begins in the perianal region and extends to the perineum where the skin is exposed to the diaper. Maceration caused by wet diapers promotes the inflammatory process. Perianal skin gets erythematous and macerated resulting in intense pruritus. As a consequence the anal canal is involved and the skin condition spreads over the perineum affecting the whole area covered by diaper. (Mandell et al. 2010, p. 3230)

Chronic Mucocutaneous Candidiasis (CMC)

Chronic mucocutaneous candidiasis denotes a heterogeneous group of infections of the skin, mucosa, hair and nails, which have in common a protracted and persistent course despite what is usually adequate therapeutic intervention. Most forms of CMC manifest in infants or young adults with oral thrush followed by onychomycosis and skin affections. These infections have been associated with immunologic defects such as T-cell function disorders and selective immunoglobulin deficiency, and with several endocrine disorders (autoimmune polyendocrine syndrome type-1) that follow CMC, among the most common are hypoparathyroidism and Addison's disease. (Mandell et al. 2010, p. 3230–3231)

Syndrome of disseminated Candidiasis and Candidemia

Patients most commonly predisposed to the disseminated form of infection are those with neoplastic disease (especially acute leukaemia), burn and organ transplant patients, patients who have complicated postoperative course (following heart and gastrointestinal surgery) and low-birth-weight neonates. (Mandell et al. 2010, p. 3234)

Following *Candida* dissemination, either spread from localized primary disease of deep organs or as haematogenously disseminated infection (Calderone 2002, p. 327), multiple organs are affected, with the kidney, brain, myocardium and eye as the most common. In the neoplastic group, patients undergoing extensive immunosuppressive therapy, liver and spleen involvement is accelerated. Other organs less frequently involved encompass the lungs, the GI tract, skin and endocrine glands.

Pathological findings of invaded organs include diffuse microabscesses with a combined acute suppurative and granulomatous reaction and small macroabscesses and remain in most cases *postmortem* diagnosis. The *premortem* diagnosis is impeded by negative blood cultures in many patients with disseminated candidiasis and the recovery of an increased number of the organisms from sputum, urine, feces and skin for example, where commensalism is frequent without any pathological significance.

Certainly, the list of clinical manifestations of *Candida* infections described above is incomplete and comprehends merely the most widespread presentations. (Mandell et al. 2010, p. 3234–3235)

Pathogenic Characteristics

During both superficial and systemic infection, *C. albicans* depends on a wide range of virulence factors and fitness attributes. Virulence factors include morphological transition between yeast and hyphal forms, expression of adhesins and invasins on the cell surface, thigmotropism, biofilm formation, phenotypic switching and secretion of hydrolytic enzymes. Among the fitness attributes are rapid adaptation to pH change, metabolic adaptability, nutrient acquisition systems and stress response systems.

Polymorphism such as transition between budding yeast cell and pseudohyphal or true hyphal form (dimorphism), and white to opaque switch, as well, are considered important virulence factors. Especially yeast and true hyphae play a role in host invasion, albeit the hyphal form has been considered to be more invasive.

The presence of N-acetylglucosamine, physiological temperature, presence of CO_2 and starvation induce hyphal growth. Another regulation mechanism is quorum sensing, a microbial molecular communication system, that promotes yeast growth within high cell densities ($>10^7$ cells ml^{-1}) and hyphal formation in low cell densities ($<10^7$ cells ml^{-1}).

On the other hand contact sensing, upon contact with abiotic and biotic surfaces, induces the switch to hyphal growth and to biofilm formation. On surfaces with specific topologies directional hyphal growth, known as thigmotropism or (Mayer et al. 2013) contact guidance, takes place. (Junqueira 2012a)

The cornerstone to enable invasion is the ‘commensal‘ or ‘pathogenic’ adhesion to epithelial cells mediated by surface adhesins (Naglik et al. 2011), specialized proteins, which attach to other *C. albicans* cells, other microbes, abiotic surfaces and host cells. Within this group is the agglutinin-like sequence (ALS), a gene encoding glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins and, ALS3 being a highly expressed hypha-associated adhesin whilst infection. (Mayer et al. 2013)

While adhesion is the first mean to interact with host cells, invasion is the first step in infection specific activity causing damage of epithelial cells. *C. albicans* is able to utilize two different pathways for host cell entry, whether induced endocytosis or active penetration. (Naglik et al. 2011)

In order to achieve induced endocytosis the pathogen expresses particular proteins on the cell surface, referred to as invasins, which interact with host ligands, such as E-cadherin on epithelial tissue, and lead consequently to pseudopod formation triggering engulfment into the host cell. Indeed, this process is considered a passive mechanism, as killed hyphae are endocytosed (Mayer et al. 2013), as well as viable ones and happens within four hours after fungus-cell interaction. Observations suggest that vital engulfed *C. albicans* hyphae prevent endolysosomal maturation, thus enabling prolonged survival and growth within epithelial cells causing disruption of cell membrane and invasion of neighbouring cells. (Naglik et al. 2011)

In contrast, active penetration, either directly into host cell or between host cells, (Gow and Hube 2012) occurs at later time points and requires viable hyphae. Up to now it is still unclear which fungal factors contribute to this fungal-driven process.

As soon as *C. albicans* hyphae have successfully invaded they cause tissue damage either inducing necrosis directly by hyphal factors or apoptosis. (Naglik et al. 2011)

Biofilm formation on catheters, dentures and mucosal cells, the most commonly, start with the adherence of yeast cells to the substrate following proliferation and formation of hyphae in the upper layer of the biofilm, accumulation of extracellular matrix and dispersion of yeast cells. The capacity to form biofilms renders the fungus more resistant to antimicrobial agents and host immune factors due to complex architecture, increased amount of drug efflux pumps, metabolic plasticity and the biofilm matrix itself. Recent literature states that *C. albicans* biofilms develop resistance against neutrophilic killing and do not serve as a trigger for reactive oxygen species (ROS) production. (Mayer et al. 2013)

Further, *C. albicans* inhibits various host niches including changing pH from very acidic (pH 2) in the stomach to more alkaline (pH 8) in the lower digestive tract. Neutral and alkaline pH induce stress response in the yeast constricting nutrient acquisition. *C. albicans* can sense pH, adapt to surrounding pH and even modulate extracellular pH. (Mayer et al. 2013)

Within 12 hours *C. albicans* is able to accelerate the environmental pH from 4 to 7, (Mayer et al. 2012) while *C. albicans* actively alkalinizes the environmental milieu by ammonia excretion, undergoing nutrient starvation and autoinducing hyphal morphogenesis. Hyphae are able to secrete hydrolases that play a role in active penetration and extracellular nutrient acquisition.
Nutrition determines survival and growth of living organisms whereby metabolic flexibility, such as glycolysis, glyconeogenesis and starvation responses (initiation of glyoxylate cycle), serves a prerequisite in changing surroundings.

Being a natural commensal in the gastrointestinal tract the fungal growth is controlled through the competitions with other residential microbes. Once *C. albicans* has gained access to the bloodstream, where glucose concentrations are relatively high, it can be engulfed by phagocytic cells. Inside a macrophage nutrient availability is restricted and AMP's, ROS and RNS are produced, forcing the yeast to switch into hyphal morphogenesis in order to escape and pierce through the host immune cell. In extracellular milieu with poor glucose concentrations *C. albicans* switches to alternative metabolic pathways to hydrolyse host proteins, amino acids, lipids and phospholipids.

Regulators of stress response and detoxifying enzymes are essential to combat host derived stresses including oxidative, nitrosative, thermal and osmotic stresses and to develop full virulence. Such a stress-responsive regulatory pathway is mediated by heat shock proteins (HSP), specialized molecules of living organisms that act as chaperons and impede protein unfolding and aggregation, and are expressed as a response to stressful conditions like high temperature, starvation and oxidative stress via the induction of the transcription factor heat shock factor 1 (Hsf1). Additionally, small Hsps defined as low-molecular-mass chaperons to prevent protein aggregation, were found in *C. albicans*.

Finally metal acquisition, a fundamental for the growth and survival of all living organisms as being responsible for the adequate function of many proteins and enzymes, is being restricted by the host. Nonetheless pathogens have evolved elaborate mechanisms to acquire trace metals such as iron by a reductive system, a siderophore uptake system and a heme-iron uptake system. (Mayer et al. 2013)

This flexibility enables *C. albicans* to gain access to nearly all natural iron sources of both the host and the commensal microbes and escape nutritional immunity. (Brunke and Hube 2013)

Commensal or Pathogen

Keeping in mind the above described pathogenic features of *C. albicans* it is somewhat controversial that the common fungal pathogen is tolerated as a residential commensal of the physiological microbial flora.
Indeed, 30-70% of healthy persons are being colonized on the skin and mucosal surface of genital and intestinal tract without showing any signs of disease, but slight alterations in the physiological state give rise to life threatening systemic infection. Thus the interaction between the human host and the normal human microbiome and the ability to discriminate invasion from colonization is crucial for the immunological tolerance and the prevention of tissue invasion. (Gow et al. 2011)

Small amounts of yeast cells do not cause epithelial cell damage and consequently do not induce a cytokine response of epithelial cells. At first, epithelial cells respond to *C. albicans* by the enhancement of a MAPK pathway, which does not make a difference between commensal and invasive yeast forms. In a second phase the presence of hyphae activate a transcription factor responsible for the cytokine production of oral epithelial tissue, which cannot be induced from the budding morphology. (Gow et al. 2011)

The interplay between immune system and changing morphogenesis is still a target of scientific research. During infection pathogen-associated molecular patterns (PAMPs), e.g. cell wall proteins, are recognized by pattern recognition receptors (PRRs) on the surface of innate immune cells triggering immune response. Toll-like receptors (TLR) are able to recognize *C. albicans* and induce the production of pro-inflammatory cytokines.
Nevertheless there are some differences in recognition of blastospores and hyphae: the hyphal form induces IL-4 production on dendritic cells, but yeasts the upregulation of IL-12. (Gow et al. 2011)

Other immune cells, controlling *C. albicans* at the mucosa, are TH17 cells; this type of cells is not correctly working in a subtype of CMC (and leads to insufficient control of the yeast at the mucosal surface of the oral cavity and the skin). In order to activate the TH17 cell subset cytokine production a milieu of specific cytokines including IL-β1, which is solely produced in the presence of hyphae by macrophages, is required. (Gow et al. 2011)

On the other hand, disease does not only occur due to direct fungal pathogenic factors, but may be also enhanced by an overwhelming immune system response causing massive recruitment of neutrophils or, in extreme situations, leading to inappropriate systemic response with consecutive life threatening septicemia. (Gow and Hube 2012)

Diagnostic and Therapeutic Options

Systemic candidiasis with high attributable mortality needs rapid species identification and differentiation techniques, in order to increase the chance of survival. (Calderone 2002, p. 427–428)

Conventional strategies include microscopy, cultivation, histopathological examination and imaging techniques and are regarded gold standard although having limited sensitivity and specificity. Thus, non-invasive and culture independent diagnostic approaches such as fungal antigen detection are emerging and widely used diagnostic kits for cell wall components, e.g. galactomannan and 1,3-β-D-glucan, still yield controversial findings. (Bašková and Buchta 2012)

Confirmed diagnosis of the contagion in early stages of infection and promptly optimum systemic antifungal therapy are the main factors to combat this life-threatening disease. (Calderone 2002, p. 427–428)
Antifungal agents should be administered within 24 to 48 hours from the time the first positive blood cultures was drawn as mortality rates were below 15% in patients receiving antifungals within 12 hours and mortality rate exceeded 40% in patients treated with antifungal therapy 3 days or later after admission to hospital. (Karthaus and Cornely 2007)

Unfortunately, many diagnoses of systemic infection cannot save the patients as diagnosed *post-mortem*. What is more, without identification of the causative agent, antifungals are administered empirically and facilitate the emergence of resistant *C. albicans* and non susceptible *Candida* species. (Calderone 2002, p. 427–428)

Since the late 1950's the polyene amphotericin B, which acts via the penetration of fungal membrane and binds to ergosterol causing membrane disruption, is available (Belenky et al. 2013) and is today still effective. Nevertheless AMB is no longer treatment of choice due to its high toxicity levels compared with azoles and echinocandins (Karthaus and Cornely 2007) and reserved to treatment of disseminated and deep organ infections rapidly progressing or refractory to azoles or echinocandins administration. (Mandell et al. 2010, p. 3235)

It is available only in parenteral formulations, amphotericin B deoxycholate and in three lipid formulations. Pharmacokinetics and toxicity profiles depend on the formulation, (Lass-Flörl et al. 2011) while lipid formulations show lower renal toxicity. (Mandell et al. 2010, p. 3235)

The most commonly used antifungals are azoles (including fluconazole, itraconazole, voriconazole and posaconazole), which are able to inhibit the biosynthesis of ergosterol, encoded by ERG11, resulting in the accumulation of a toxic sterol molecule, that disrupts the cell membrane. Due to the frequent administration and prophylactic intervention connected with fungistatic effect allowed the development of drug resistance, overexpression of drug efflux pumps and alteration of the drug target. (Shapiro et al. 2012)

Even the class of echinocandins, inhibitors of β-1,3-glucan synthesis, that cause osmotic lysis of the fungal cell wall of all *Candida* sp. (Cappelletty und Eiselstein-McKitrick 2007), have reported cases of drug resistance, but exhibit potent concentration-dependent fungicidal activity against azole-resistant *Candida* sp.. (Chen et al. 2011)

This group encompasses caspofungin, micafungin and anidulafungin in parenteral application with favourable toxicity profiles, so that solely dose reduction of caspofungin is recommended in patients with impaired liver function (Mandell et al. 2010, p. 3236), as the three agents do not interfere with the cytochrome P450 or P-glycoprotein systems. Of the three, anidulafungin has a unique elimination mechanism via the chemical degradation in bile, resulting in longer half-life than the other two. (Chen et al. 2011)

Interestingly, increased growth of *Candida* sp. in vitro was observed at MIC drug concentrations above the organism's MIC, a trait termed ‘Eagle effect’. (Pound et al. 2010)

1.2. Galleria mellonella

The pathogenicity of *C. albicans* can be assessed using the greater wax moth *Galleria mellonella* as a model host. In studies the infection model *G. mellonella* indicates comparable results to mammalian models such as mice, and commonality between virulence factors causing disease in mice models and for killing of the arthropode. (Junqueira et al. 2011)

1.2.1. What is G. mellonella*?*

G. mellonella, an insect (arthropode), also known as the greater wax moth, belongs to the order of *Lepidoptera* and the family *Pyralidae*. It is a natural combat in apiculture. To be exact, larvae, hatching from eggs laid into bee hives, burrow a gallery in the combs whilst feeding on honey, pollen and wax.

The life cycle of *G. mellonella* ranges from four weeks to six months, spanning the metamorphosis from egg via caterpillar and spinning a cocoon after 18 days, to finally develop into the adult moth.
Young caterpillars are coloured white and turn grey, when they grow older. (Lewbart 2012, p. 313–314)

The size of full grown caterpillars extends from 23 to 28 mm. Their head is coloured dark brown with four pairs of ocelli. (Robinson 2005, p. 339)

1.2.2. Is there any homology to vertebrates?

The insect's immune system provides some structural and functional similarities to the innate immune system of vertebrates.
From the point of structure, the insect's cuticle is comparable to the mammalian skin serving as barrier to infective agents. The haemolymph, similar to human blood, transports waste products, nutrients and signal molecules, as well as immune cells. (Kelly and Kavanagh 2011)

The immune cells present in haemolymph consist of six different types, namely prohemocytes, coagulocytes, spherulocytes, oenocytoids, plasmatocytes and granulocytes. (Junqueira 2012a)

Those immune cells are summarized haemocytes, and represent vertebrate monocytes and granulocytes from the point of function. They phagocytise and encapsulate invading organisms, and form nodules around larger foreign structures.

Nevertheless there is also a humoral immune response mediated by the formation of antimicrobial peptides and melanin, haemolymph clotting (Mowlds et al. 2008) and wound healing.

AMPs are released from haemocytes, other cells and organs into the haemolymph, where they accumulate on the site of infection and attack components of the bacterial and fungal cell membrane and wall. (Kelly and Kavanagh 2011)

In the absence of an adaptive immune system the literature states that exposure of insects to low amounts of pathogen or pathogenic material including glucan or LPS induces priming of an immune response, and results in fortified protection against lethal infection. Conducted studies show that low doses of yeast or fungal cell wall components exposed to *G. mellonella* induce the expression of antimicrobial peptides. (Mowlds et al. 2008)

1.2.3. What features render **G. mellonella** *a valuable model host?*

“Over the past decade invertebrate mini-host models with well-characterized genetics and simple immunity have been effectively used to explore several aspects of both fungal pathogenicity and host immune response. Several factors sparked the development of these models. Innate immune mechanisms are evolutionarily conserved between invertebrates and mammals and several common virulence factors are involved in fungal pathogenesis in phylogenetically diverse hosts, such as fruit flies, nematodes, and mammals.” (Junqueira 2012a)

First the breeding of large numbers is involved to low overall costs and provides cost-effective infection systems. Next those insects are commercially available worldwide and easily achievable as sold in pet shops as bites and feeding. The insect does not need any special environment and can be kept in smaller laboratories in contrast to mammalian animal models.
The rather large size of larvae allows the precise injection of a certain amount of pathogens or antibiotics and thus enables standardized conditions for pathogenicity and drug effect studies.
Most essentially *G. mellonella* can be adapted to human physiological temperature (37°C), what makes it the ideal mini host model for *C. albicans*. (Vogel et al. 2011)

1.2.4. Animal testing versus invertebrate model organism

Scientific research on live humans involves the ethical dilemmas and individual differences in terms of nutrition, social habits and individual immune status. Those aside from racial, ethnic and cross-cultural variations afflict the etiology and pathogenesis of infective diseases like candidiasis.
In contrast the use of animal models such as murine models bears ethical dilemmas as well, whereas the interindividual diversity can be controlled and manipulated to maintain comparable data regarding etiology, pathology, diagnosis and disease treatment. (Junqueira 2012a)

Insect hosts such as *G. mellonella* emerge model systems for innate immunity studies and human pathogens because there is a lack of ethical restrictions and they are not included in animal model legislation. They are ethically better acceptable than vertebrate hosts, for example, mice, rats and rabbits (Vogel et al. 2011), and large-scale studies can be conducted without any logistical obstacles. (Junqueira 2012a)

Based on the fact that invertebrate model organisms facilitate experiments to explore evolutionary preserved elements of fungal virulence and host immunity, research with insect hosts reaches its peak in popularity. (Junqueira et al. 2011)

1.3. Aim of the study

This study examined the pathogenicity of *Candida albicans* isolates, obtained from diverse host environments, namely blood or oral infection sites, in an insect model organism, the greater wax moth *Galleria. mellonella*. The objective of this work was to determine whether there is a difference in survival of *G. mellonella* infected with either invasive or superficial host isolates of *C. albicans*.

The work presented here was thought to establish whether the insect *G. mellonella* is an appropriate model organism to monitor *Candida* pathogenicity.

2. Material & Methods

2.1. Material

2.1.1. Larvae, fungal strains

- *Galleria mellonella* larvae obtained from a supplier (pet shop) in Graz on the day of delivery and used at least within the following 48 hours
- Fungal strains: *Candida albicans* isolated from blood cultures or oral candidiasis of patients courteously provided by Prof. Krause, University Hospital Graz, Internal Medicine.

2.1.2. Reagents

- Viabank TM Medical Wire Equipment MWVIM Tubes (mwe, United Kingdom)
- SAB (Sabouraud Glucose Agar plates with Antibiotics) containing Sabouraud Dextrose Agar, Refobacin and Chloramphenicol (prepared at our Department of Microbiology)
- IPS (Insect Physiological Saline) consisting of 8.76 g NaCl, 0.36 g KCl, 15.76 g Tris HCl, 3.72 g EDTA, 4.72 g Sodium citrate adjusted with destillated H_2O to pH 6.9

- petri dishes containing kimwipes
- 95% ethanol (Fuchs et al. 2010)

2.1.3. Equipment

- Incubator (Binder, Germany)
- Maldi TOF (bioMérieux, France)
- Vitek DensiCHECK (optical density of 550 nm) (bioMérieux, France)
- Microscope (Zeiss, Austria)
- Vortex mixer (VWR, USA)
- Brucker Türck haemocytometer
- Insulin syringe (30’s gauge; 0,30 mm x 8 mm) (Fuchs et al. 2010)

- MALDI-TOF (Matrix Assisted Laser Desorption Ionisation-time of flight) (bioMérieux, France)

2.2. Methods

2.2.1. **Candida albicans** ***strains, conservation and culture conditions***

We used 20 different clinical *C. albicans* strains isolated from either superficial (n= 10), in our case oral infection sites, or from systemic candidiasis (n= 10), namely blood culture, of various patients in this study. (Junqueira et al. 2011)

In order to identify the *Candida* species, the fungal strains were grown on SAB plates and subsequently analysed by MALDI -TOF (Matrix Assisted Laser Desorption Ionisation-time of flight) analysis. (Junqueira et al. 2011)

For analysing the fungus, a small quantity of intact yeast cells was transferred to the MALDI target plate and covered with a small amount of matrix solution, which is necessary for cell lysis.
Drying embeds the cell structures into a matrix, which serves both for energy transfer and for chemical ionisation. Laser beam desorbs the ions from the target plate, which are accelerated (depending on size and electric charge) in an electric field and detected in a detector. Each fungus has a characteristic mass spectrum undergoing this procedure and the obtained results are compared with reference spectra used for precise identification of the species. (Bader 2013)

Afterwards the identified fungi were suspended in VIABANK TM tubes and subsequently maintained at -80°C in the freezer, in order to obtain fresh strains at each repetition of the experiment. (Junqueira et al. 2011)

2.2.2. **Galleria mellonella** *larvae and storage*

For the experiment larvae of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) in the sixth instar level were used. After delivery they were placed in plastic boxes with ventilation louvres and filled with wood shavings and stored in a refrigerator in the dark at 5°C to a maximum of seven days. For each experimental group medium sized larvae without any grey markings were randomly chosen. (Mowlds and Kavanagh 2008)

2.2.3. Pre-incubation

However, 24 hours prior to infection with *C. albicans* larvae were incubated at room temperature (25°C) in the dark.

On three independent occasions the experiment was performed, at a time ten larvae were infected with one *C. albicans* strain.

Furthermore two control groups were introduced consisting of a native group that received no treatment, and a group that received an injection of 20 µl IPS. (Mowlds and Kavanagh 2008)

2.2.4. Preparation of inoculum

Regarding the preparation of the inoculum only fresh fungal strains were used. Therefore strains were grown on SAB plates overnight and transferred to new ones the following day. Freshly grown (not older than 48 h) *C. albicans* colonies were suspended in buffer (IPS) into SARSTED 15-ml tubes and vortexed shortly (at 2500 rpm). The cell density was checked with densicheck and adjusted to a value above 4.5 McFarland.

Consequently the yeast cells were counted with a haemocytometer and by diluting with IPS or by suspending more cells into the tube the appropriate concentration of 2,5x10^9 cfu/ml was achieved. (Fuchs et al. 2010)

2.2.5. *Injection*

*G. mellonella wer*e injected with 20 µl of inoculum through the last, left proleg directly into the haemocoel. During that procedure larvae were held stationary using the thumb, pointer and middle finger. (Fuchs et al. 2010)

After the injection the insects were transferred to a petri dish lined with a kimwipe in order to recover. If the insertion of suspension was carried out correctly, the leakage of haemolymph of the injection site would terminate within a minute. Consequently the infected larvae were kept in a petri dish covered with a kimwipe and incubated at 25°C. (Fuchs et al. 2010)

2.2.6. *Survival assay*

The larvae were controlled daily and the number of dead larvae was scored. Larvae were considered dead when they displayed no movement in response to touch and were removed from the petri dish. Killing curves were plotted and statistical analysis was performed. (Junqueira et al. 2011)

The remaining (surviving) larvae were returned to the incubator. The endpoint of the experiment was the death of all larvae in the experimental groups, or the transition of larvae into pupa. Survival assays were carried out until seven days *post infectionem*. (Fuchs et al. 2010)

2.2.7. *Statistical analysis*

All experiments were performed on three independent occasions. The results presented are expressed as the mean ± SE. Survival curves were analysed with student t-test and differences were considered significant at $p \leq 0.05$.

3. Results

In the work presented here the virulence of *C. albicans* isolates in the insect model *G. mellonella* was studied. The pathogenicity between superficial and invasive clinical *Candida* isolates was assessed by a survival assay.
The results indicate that there is no statistically significant difference between superficial and invasive isolates of *C. albicans* (p=0.6) in killing the model organism.

The killing curves of larvae infected with superficial and invasive isolates show similar linear curve progression. Although between the first 24 and 48 hours slight differences in curve progression are discernible. Their statistical relevance is after 24 hours (p=0.40) and after 48 hours (p=0.90); i.e. no statistically significant difference between the first 24 and 48 hours of oral and systemic isolates.

In more detail, after the injection of the fungal inoculums the lively larvae were less motile, in comparison with the IPS control group, those larvae showed the same motility as before the injection.
We introduced a native control group to monitor natural ageing and to recognize old larvae, only once we had to abort a series of experiments (as mentioned below). In general, the native control group showed similar surviving curves as the IPS group.

What is more, we observed less viability of infected larvae 24 hours post-injection. This was determined by the protocol for survival assay, as larvae infected with *C. albicans* displayed poor response to physical stimuli. Additionally we noticed discolouring of the insect's cuticle (the cuticle showed grey colouration), this discolouration process happened within the first hour after inoculums delivery. Within 24 (48) hours 72% (48%) of the superficial and 64% (47%) of the invasive experimental group were dead.

Dead larvae were coloured dark brown to intense black, had reduced turgor and seemed somehow edematous. Sometimes the dead larvae seemed to be dissolved.

Once we tried a series with humid kimwipe, because the larvae seemed to shrink while in the incubator. The results were growth of *Aspergillus fumigatus* on the dead bodies of larvae. Because of possible contamination with *Aspergillus fumigatus* the series of experiments was aborted. (Picture 3)

In order to proof successful infection of larvae with *C. albicans* we prepared histological sections for histological examination. The larvae were prepared following histological protocols and the histological sections were stained with fluorescing colour. On examination under a fluorescing microscope, hyphal and single cells were present within the larvae’s haemocoel.

The use of *G. mellonella* as an alternative model for the study of virulence mechanisms of *C. albicans* was studied on animal hosts. We discovered that it is possible for the fungus to proliferate inside the haemocoel of the insect's larvae and to colonize and kill the caterpillar.
Our results suggest potential charge of *G. mellonella* larvae as an invertebrate host for studying infection mechanisms of *C. albicans*.

Table 1 Clinical isolates of *C. albicans* and their effect on *G. mellonella*

Strain of *C. albicans*	Clinical isolate	Number of killing/total	Mean survival (d)
68	Oral	9/10	3,67
69	Oral	9/10	3,13
81	Oral	9/10	3,50
83	Oral	8/10	3,19
87	Oral	8/10	4,04
97	Oral	7/10	5,83
102	Oral	8/10	4,71
111	Oral	9/10	4,42
115	Oral	5/10	6,71
120	Oral	8/10	4,50
132	Blood culture	7/10	5,08
133	Blood culture	8/10	4,33
134	Blood culture	8/10	4,54
142	Blood culture	7/10	5,09
143	Blood culture	7/10	4,58
144	Blood culture	10/10	2,96
145	Blood culture	8/10	4,71
146	Blood culture	8/10	3,75
149	Blood culture	9/10	3,85
152	Blood culture	9/10	2,71
Oral		8/10	4,30
Blood culture		8/10	4,17

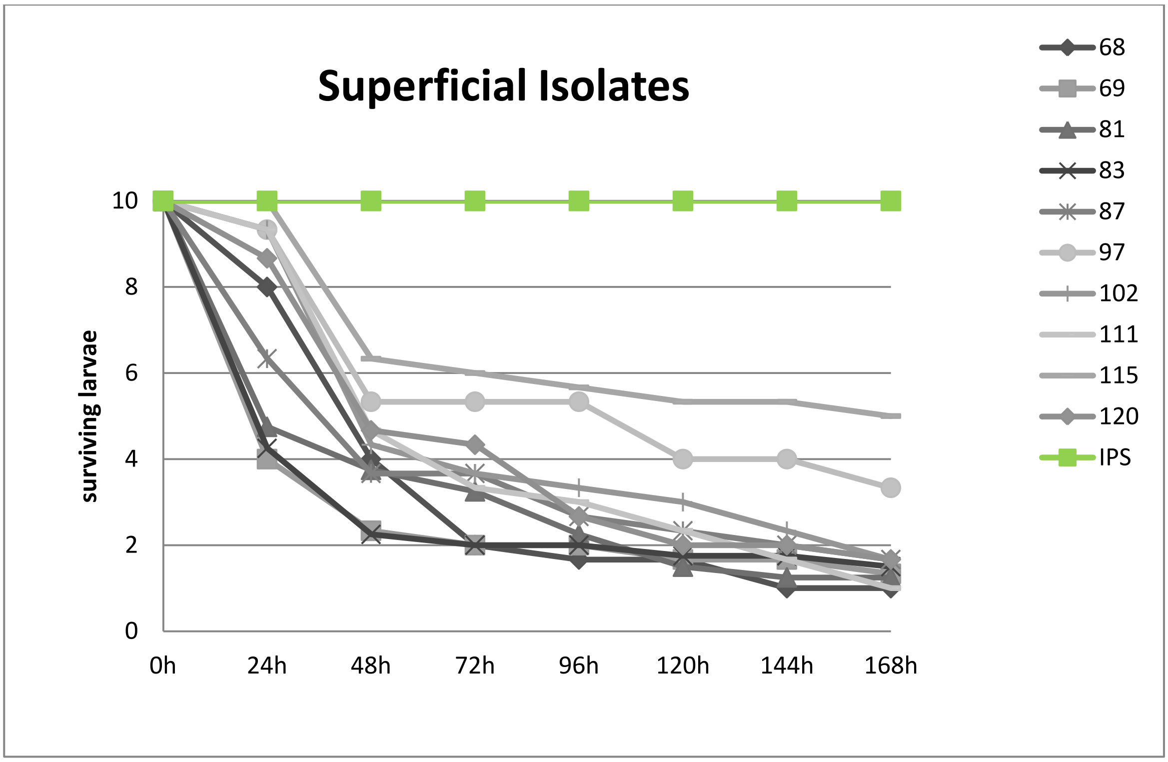


Figure 1 Killing of *G. mellonella* by superficial isolates

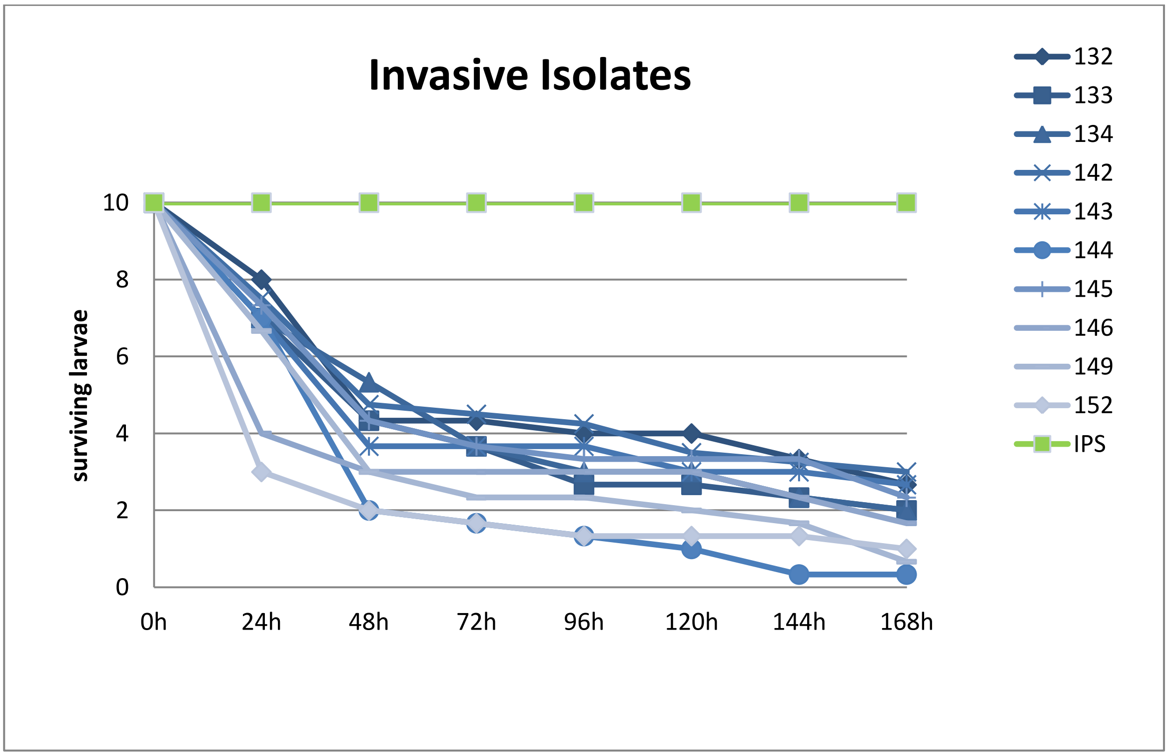


Figure 2 Killing of *G. mellonella* by invasive isolates

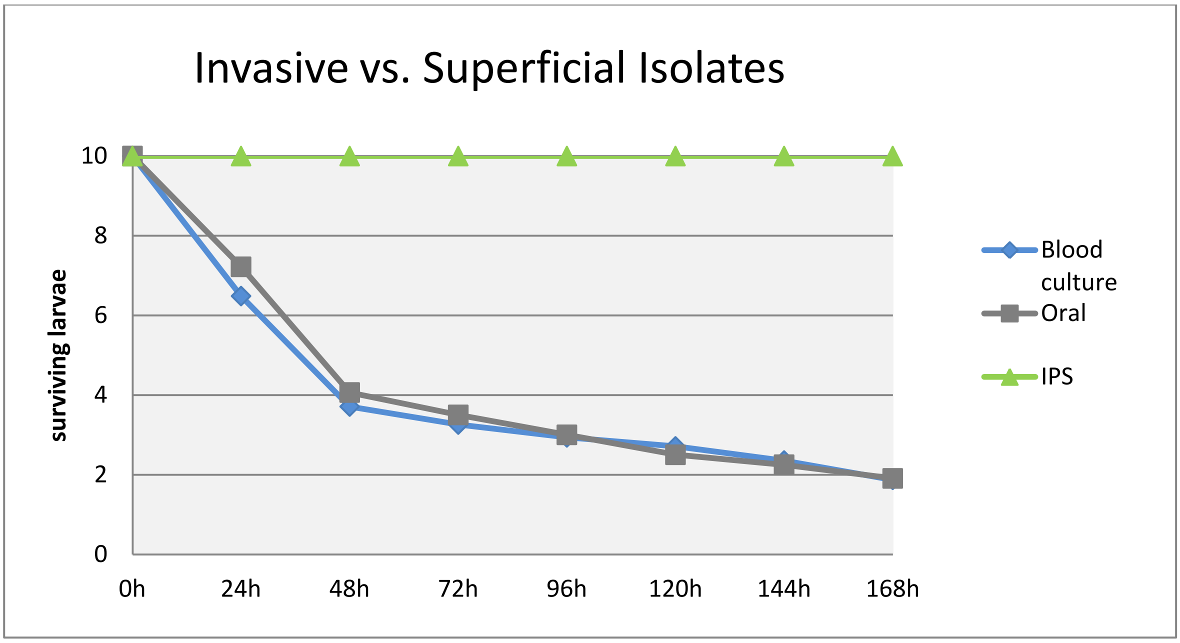
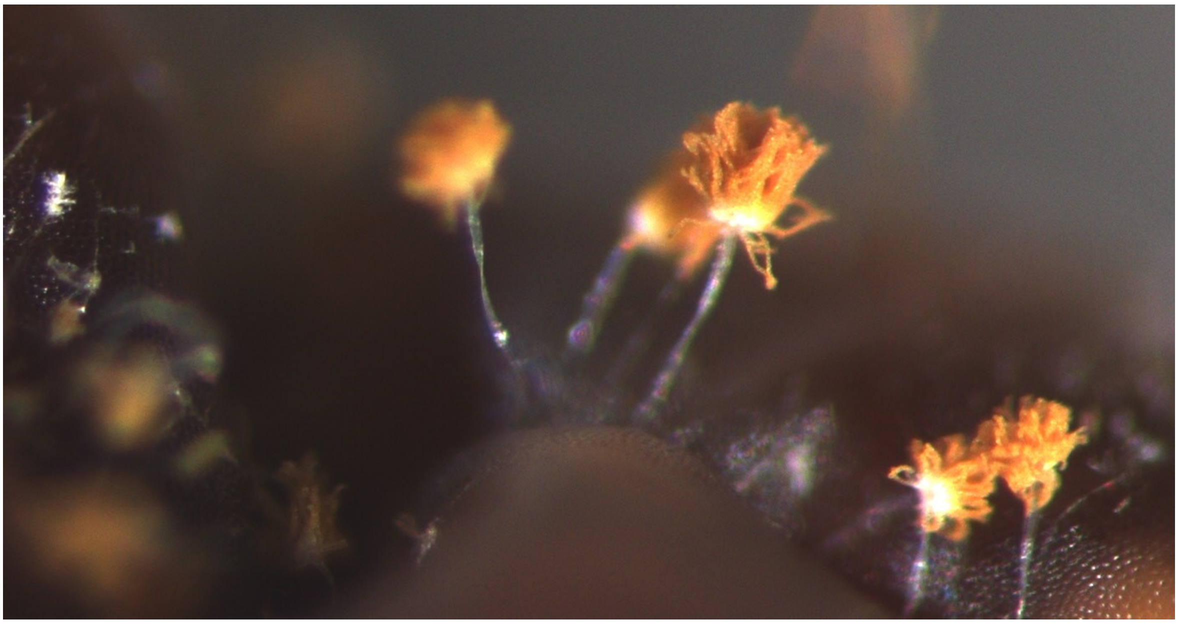


Figure 3 Invasive (blood culture) and superficial (oral) isolates summarized results

Picture1 Larvae of *G. mellonella* IPS group

Picture 2 Larvae showing discoloration



Picture 3 Dead larvae infected with *Aspergillus fumigatus*

Picture 4 Larva of *Galleria mellonella* with four pairs of prolegs

Picture 5 Magnified image of two pairs of larval prolegs

Picture 6 Insertion of the needle in the right last proleg for inoculum delivery (citied from Fuchs et al. 2010, p. 477, Figure 1 (D))

Picture 7 Moths (adults) of *Galleria mellonella*

4. Discussion

‚Conventional assays to examine the virulence of various microbial pathogens have traditionally relied upon the use of vertebrate species. In recent years it has been demonstrated that insects may be employed to evaluate the virulence of fungi.‘ (Kelly and Kavanagh 2011)

The use of invertebrate mini host models with well-characterised genetics and simple immunity rose to explore several aspects of fungal pathogenesis and host immune response. (Chamilos et al. 2007)

Consequently non-vertebrate pathosystems ranging from single cell protozoa to insects (*Drosophila melanogaster*, *Caenorhabditis elegans*, *Acanthamoeba castellanii*, *Dictyostelium discoideum*, *Bombyx mori*, *Culex quinqefasciatus*, *Blattella germanica* and *Arabidopsis thaliana*) with conserved innate immunity have been introduced that enable high-throughput screening of fungal pathogens (Lionakis 2011) and can provide valuable data for both the infectious process in vivo and the host response. (Navarro-Velasco et al. 2011)

‘A critical step in addressing a question or hypothesis regarding host-pathogen interaction is to determine which infection model fits best into the experimental design.’ (Desalermos et al. 2012)

Choosing an adequate heterologous host for the investigations of *Candida* pathogenicity comparative characteristics among different host systems (such as Lionakis published) provided valuable information about the adequate model. The decisive factors that consider *G. mellonella* within the various invertebrates suitable for our studies were:

- need for simple laboratory resources
- low overall cost
- correlation of virulence factors with mammalian models
- survival at mammalian physiological temperature

- potential for large-scale screening studies of fungi
- precision in fungal inoculum delivery with injection (Lionakis 2011)

Further beneficial characteristics include sufficiently protracted tempo of infection as in human beings, inoculum standardisation and ethical acceptance.
Important aspects of innate immunity are evolutionarily conserved between invertebrates and mammals, and similar fungal virulence factors are present in distinct hosts. Reasonably, comparative genomic studies reveal homology of proteins responsible for pathogen recognition, signal transduction and innate immune response between non-vertebrates and vertebrates. (Chamilos et al. 2007)

For example, the *Galleria* model comprehends six classes of haemocytes, which display homologous mechanisms of oxidative killing such as reactive oxygen species in mammals. (Lionakis 2011)
Another process conserved between mammals and invertebrates is phagocytosis. It is mediated by the adherence of opsonic ligands to the surface of the particle and results in recognition of specific receptors. Those pattern recognition receptors (PRRs) are activators of the complement system in vertebrates and inducers of the Prophenoloxidase activating system (ProPO-AS) in insects, explained in details below. (Kavanagh and Reeves 2004)

Concluding, there is little doubt that invertebrate studies are fundamental for immunology studies of vertebrates.

Conducting experiments with *G. mellonella* some unexpected problems may occur such as the death of the control group caused by contaminated IPS or defective needles that lead to intense trauma during the injection. Metamorphosis to pupa stage may start if the storage temperature is too high or old larvae are selected. (Fuchs et al. 2010)

Relevant to this discussion, larval age and shipping conditions that may influence the vitality of insects, are difficult to evaluate. Preferably, the trials are conducted whilst cooler environmental conditions in order to prevent premature ageing caused by elevated temperature levels in estate.

Based on the recent increase of studies using *G. mellonella* as a model host, standardized conditions for experimental design need to be introduced. A study, for example, investigated the effect of starvation on larval survival, concluding that nutritional deprivation for seven days reduces haemocyte density, but does not afflict their functionality, decreases AMPs as well as immune proteins and leads to increased susceptibility to infection. (Junqueira 2012b)

Mowlds and colleagues demonstrated the effect of pre-incubation temperature on the synthesis of AMPs (taking place in the insect’s fat body, haemocytes, digestive tract, salivary glands and reproductive tract). According to their results pre-incubation at 37°C and 4°C showed increased survival rates and higher haemocyte density per larvae compared to pre-incubation at 30°C for 24 hours, indicating that incubation temperature has a strong effect on the insect's immune response. (Mowlds and Kavanagh 2008)

Data obtained from a study, where larvae were physically stressed 24 hours prior to inoculation with *C. albicans*, showed a reduced sensitivity of larvae to microbial infection. This increased ability of larval survival is reflected in elevated haemocyte density, possibly due to release through physical disturbance of haemocytes attached to internal organs. Further analysis of peptides found in haemolymph demonstrated homology to proteins important for the insect's immune response. (Mowlds et al. 2008)

Based on these investigations, in this study larvae were not used immediately upon arrival in the laboratory and were supplementary pre-incubated at the temperature level, identical to the incubation temperature, for 24 hours in the dark. To avoid effects of nutritional deprivation on larval survival petri dishes, as well as shipping devices were covered with wood shavings to provide a food source during experiments.

Indeed, non-lethal inocula of *C. albicans* induce the production of protective AMPs combating subsequent lethal fungal re-challenge. Despite conserved antifungal innate immune mechanisms between mammals and insects, the latter deprive orthologs for adaptive immune signalling pathways and specialized immune cells such as natural killer cells, dendritic cells and T-lymphocytes. (Lionakis 2011)

Though a single pathosystem cannot serve all scientific interests and the so-called multi host approach, the use of an array of complementary model hosts may help to understand the various virulence factors enabling growth within another organism. (Desalermos et al. 2012)

The model organisms provide a primary analysis of relative pathogenicity of various pathogenic yeasts and can be established for the screening of yeast mutants' pathogenicity and for a large amount of yeast isolates before the validation in animals takes place. (Cotter et al. 2000)

Due to the fact that insect model organisms represent solely non-adaptive immune response and more detailed investigations on adaptive immunity mechanisms may be carried out, experiments could be expanded on conventional animal models of infection and thus minimise the number of animal testing and animal suffering.

In our study we discussed the infection model *G. mellonella* as a non-vertebrate pathosystem for the pathogenicity of *C. albicans* and adapted available experimental protocols for wild-type *G. mellonella* larvae to our research project. We compared superficial and invasive *C. albicans* clinical isolates.
We confirmed that the pathogenicity of superficial *Candida* isolates is similar to invasive *Candida* isolates, indicating that the pathogenicity of the yeast *C. albicans* is not correlated with the infected site. A study conducted by Junqueira (2011) showed similar results.
After having successfully obtained access to bloodstream, *C. albicans* is able to cause systemic infection with lethal outcome. In this context there seems to be no

difference whether the *Candida* isolate was discovered on mucosal surface or not. Therefore, even superficial infection with *C. albicans* should be treated seriously to prevent possible systemic infection and spread of the pathogen resulting in inner organ involvement.

Junqueira et al. (2011) verified mortality rates of 100% within larvae injected with 10^5 cfu per larvae in a volume of 10 µl. The incubation temperature level was 37°C in their investigations.
Of interest, Mowlds (2008) demonstrated that temperature variation has a strong effect on insects' immune response and on gene expression and may explain higher mortality within incubation temperature at 37°C and higher replication rates of *C. albicans*.
What is more, Junqueira et al. (2011) included 5 systemic isolates and 11 oral isolates from different geographical sites in their investigations. The systemic ones were obtained in the US (Boston) and the oral isolates were recovered in Brazil. Considering that there are geographical strain distribution differences within *Candida albicans* from north to south, as mentioned above, there may be also geographical strain differences regarding virulence attributes.

Consistent with that Cotter et al. (2000) reported greater mortality rates within a group inoculated with clinical isolates compared to laboratory isolates (cultured in the laboratory for at least 3 years). This indicates higher expression of virulence factors in clinical isolates of *C. albicans*, than in laboratory isolates.

Cotter et al. (2000) obtained mortality rates of 90% for *C. albicans* after the injection of $2x10^6$ cfu per larva and 20% for larva injected with $2x10^5$ cfu per larva after 72 hours. The incubation temperature in this experimental setting was determined 30°C. These results reinforce the influence of temperature-dependent pathogenicity.
Similar arguments can be made for the concentration of suspended cells. Higher densities of cfu per larvae show higher mortality rates.
Notably, the data from a study of Fuchs and colleagues, (2010) demonstrated that killing curves for 10^2 and 10^4 *C. albicans* yeast cells per larvae are comparable to

killing curves of non-pathogenic yeasts. These results show that *G. mellonella* is, in fact, capable of combating fungal infection successfully.

More in general, it shows that *C. albicans* adapted perfectly to human physiological temperature levels that renders it the most prevalent fungal pathogen.

Notably to this issue Cotter et al. (2000) demonstrated that heat killed *C. albicans* strains showed mortality values of 20% (10^6 cfu/larva), and suggested that death of *G. mellonella* due to allergic alterations (an immune response initiated by APC, as part of inborn immunity and carried out by adaptive immune cells) caused by a huge number of yeast cells injected in haemolymph did not lead to larval death.

Another point to be made, Cotter et al. (2000), observed discolouration of the insects' cuticle following injection.
This melanisation process is part of the immune response to (restrict invading) fungi and bacteria, (Cotter et al. 2000) together with phagocytosis and nodule formation. Those nodules are within short time melanized by pro-phenoloxidase pathway. (Jackson et al. 2009)

Pro-phenoloxidase (ProPO), a zymogen situated in haemocytes, is released by rupture and is transported to the cuticle, and accumulates around wounds or encapsulated parasites. (Kavanagh and Reeves 2004)

Thus, it may appear that melanisation is induced by disruption of physical barriers such as inoculum injection via the last pro-leg followed by extensive wounds, but the injection side re-seals after removal of the syringe needle without leaving a scar. (Kavanagh and Reeves 2004)

This observation of the discolouration process indicates that this unique cellular response can be used for the determination of successful *C. albicans* inoculum delivery within one hour post-infection.

A study carried out by Fuchs et al. (2010), states that larvae become less motile 24 hours after infection and respond poorly to physical stimuli (such as poking), when still alive. In regard to the infection-induced hypomotility larvae need to be carefully observed during survival assays to recognize living larvae.

However, recent investigations showed that RVVC can be regarded an exceptional infection site.

Patients suffering from CMC, a deficiency in Th17 immune response, do not develop VVC. As follows, adaptive immune response does not protect against VVC and symptomatic infection arises both from neutrophilic infiltration of the vaginal lumen and from augmented fungal burden. What is more, *C. albicans* interaction with vaginal epithelial cells triggers the production of neutrophil-chemotactic factors, which enhance a strong neutrophil migration in VVC and an overreactive immune system is assumed to cause disease manifestation, rather than immune deficiency (Gow et al. 2011) as RVVC is not correlated with HIV positive individuals. (Cassone and Cauda 2012)

This example constitutes reinforcing host factors, that can exacerbate a harmless infection and, finally in the worst case lead to high mortality rates.
However, therapeutic intervention with antifungals bears the risk of developing resistance mechanisms, some of them have been described above,
and the emergence of drug-resistant strains among previously drug-susceptible populations through a process of adaptive evolution. (Huang and Kao 2012)

C. albicans resides as an ubiquitous commensal already during delivery or soon after birth within the human microbial community in the gastrointestinal tract and is frequently recovered at any given time on the skin, oral and vagina cavity of otherwise healthy individuals, as long as their microbial pathogens are kept at bay. (Cassone and Cauda 2012)

The finding that superficial and invasive *C. albicans* isolates have an equivalent pathogenic potential in an invertebrate model of infection in conjugation with clinical impressions has led ourselves and others to postulate that putative fungal infection necessitates prompt therapeutical intervention in order to prevent invasion and dissemination to other organ systems especially in the case of systemic fungal infection.
To address this, OPC, a common chronic clinical manifestation should be treated seriously with antifungal drugs, keeping in mind that oral isolates once gained access to the bloodstream lead to candidemia.

Therefore effective therapy of mucosal candidiasis with oral triazole drugs is necessary in the case of failing an approach with a combination of old (nystatin, AMB) and new (echinocandin derivates) drugs is introduced. (Cassone and Cauda 2012)

Nevertheless azole resistant *Candida* species may be selected without prolonged triazole administration. In non-immunocompromised individuals mild clinical manifestations can be treated with topical polyenes. (Ullmann et al. 2012)

Of interest, a study noted that septic patients in the intensive care unit have an increased risk to develop *Candida* infection, since endotoxemia impairs Th 17 cell response and may render individuals with endotoxin-related systemic infection more susceptible to *Candida* infection. (van de Veerdonk et al. 2012)

As follows, when bloods are drawn and in face of antibiotic intervention no clinical improvement is present, bloods should be searched for fungal infection.

Most essentially, identification of the causative species of candidiasis is to be established and susceptibility to the chosen and alternative antifungals is to be ensured. (Ullmann et al. 2012)

Despite potent antifungal therapy the diagnostic methods remain the time limiting factor.

Lack of significant signs and symptoms combined with low specificity of available non-invasive diagnostic procedures contribute to difficult diagnostic approach of fungal infections. Consequently, treatment with antifungal drugs is applied empirically upon clinical suspicion and may involve the administration of potentially toxic and expensive pharmaceuticals. (Fontana 2012)

Once candidemia is detected, blood cultures should be repeated daily until at least two negative consecutive samples, and antifungal therapy should be continued for 14 days after the last positive blood culture. (Ullmann et al. 2012)

Additionally to prompt antifungal treatment intravenous catheters and indwelling devices should be changed.

Prophylactic treatment of *Candida* infection is a highly controversial topic, but prospective controlled trials observed positive results in allogenic bone marrow transplanted patients. In general, for prophylactic use fluconazole, posaconazole and micafungin are recommended. Current recommendations focus on solid organ transplant recipients with high-risk liver, pancreas and small bowel grafts. Postoperative prophylaxis with fluconazole is recommended at surgical intensive care units.
Empiric administration of antifungals is recommended for suspected systemic candidiasis with no neutropenia. Primary fluconazole is recommended with consideration for the predominant *Candida* spectrum. (Mandell et al. 2010, p. 3235)

Doubt remains as the toxicity profiles of the vast majority of antifungal agents especially AMB are numerous e.g.:
Acute toxicity of the natural antibiotic polyene AMB is infusion-related and presumably a result of proinflammatory cytokine production by innate immune systems. It comprehends nausea, vomiting, rigors, fever, hypertension/hypotension and hypoxia. Delirium, depression, psychotic behaviour,

tremors, akinetic mutism and diffuse cerebral leukoencephalopathy are a few examples for the neurotoxic adverse effects of AMB.
Even fatal cardiac arrhythmias due to hyperkalemia are well-documented in the toxicity profile, if intravenous infusion occurs rapidly.
Nevertheless chronic nephrotoxicity clinically manifested by renal insufficiency, urinary potassium wasting, hypomagnesemia, metabolic acidemia and polyuria remains the dose-limiting factor in many patients. (Laniado-Laborín and Cabrales-Vargas 2009)

‚An invertebrate model can be an asset in screening fungal strains in order to identify mutant strains that can then be followed for further study to assess the genetic contributions to virulence. Strain pathogenicity is tested using the survival assay comparing the length of survival.‘ Furthermore, comparing the length of survival rate hypervirulent clinical isolates may be identified. It is quite convenient to ascertain the virulence of *C. albicans* in infected larvae as they begin to die within 24 to 96 hours after injection. (Fuchs et al. 2010)

Additionally, in order to obtain more data internal structures of the larvae can be extracted and there may be a relation between the yeasts' morphology and killing curves. (Fuchs et al. 2010)

Extracted haemolymph allows the harvest of haemocytes and can be followed by Fluorescence-activated cell sorting (FACS), in order to evaluate haemocyte density and determine their pathogenic capacity. (Lionakis 2011)

Even proteins extracted of larval haemolymph can be evaluated regarding their purpose, changes in the protein profile following injection and homology to human proteins.
Another point to be made, insect models of microbial infection can be used to demonstrate the antimicrobial activity of various antifungal drugs (Kelly and Kavanagh 2011) and susceptibility of different yeast strains to antifungal agents.

Fundamental for the development of new antifungal agents and diagnostic strategies is to gain insight into the virulence attributes and mechanisms of *C. albicans* responsible for its pathobiology.
Among the promising targets there are the major and small heat shock proteins that are unique to fungi and the elaborate fungal nutrient acquisition mechanisms focussing on iron, zinc and copper. (Mayer et al. 2013)

In particular, the transition from commensal to pathogenic state is a key factor for infection and needs to be investigated in more details.

Summarizing, invertebrate studies offer an approach of ethically accepted, first line methods for the evaluation of pathogenicity of fungal strains and can be regarded as an initial screen for further studies conducted on animal models that entail ethical and legal concerns.
The insect model organism *G. mellonella* represents, in consideration of our investigations, a suitable alternative model for the evaluation of *Candida* pathogenesis, although aforementioned shortcomings and potential limitations have to be taken in account.
C. albicans, a common fungal pathogen with complex structure and ability to adapt to changing host environment, is tolerated as a harmless commensal of the human microbiome and under adequate circumstances emerges as a life-threatening pathogenic organism. Without doubt this property renders it a potent fungal pathogen and together with the results of this study - that the superficial isolates have the potential to cause elevated morbidity (just as bloodstream isolates), if locally invasion or systemically dissemination takes place in the cardiovascular system.

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