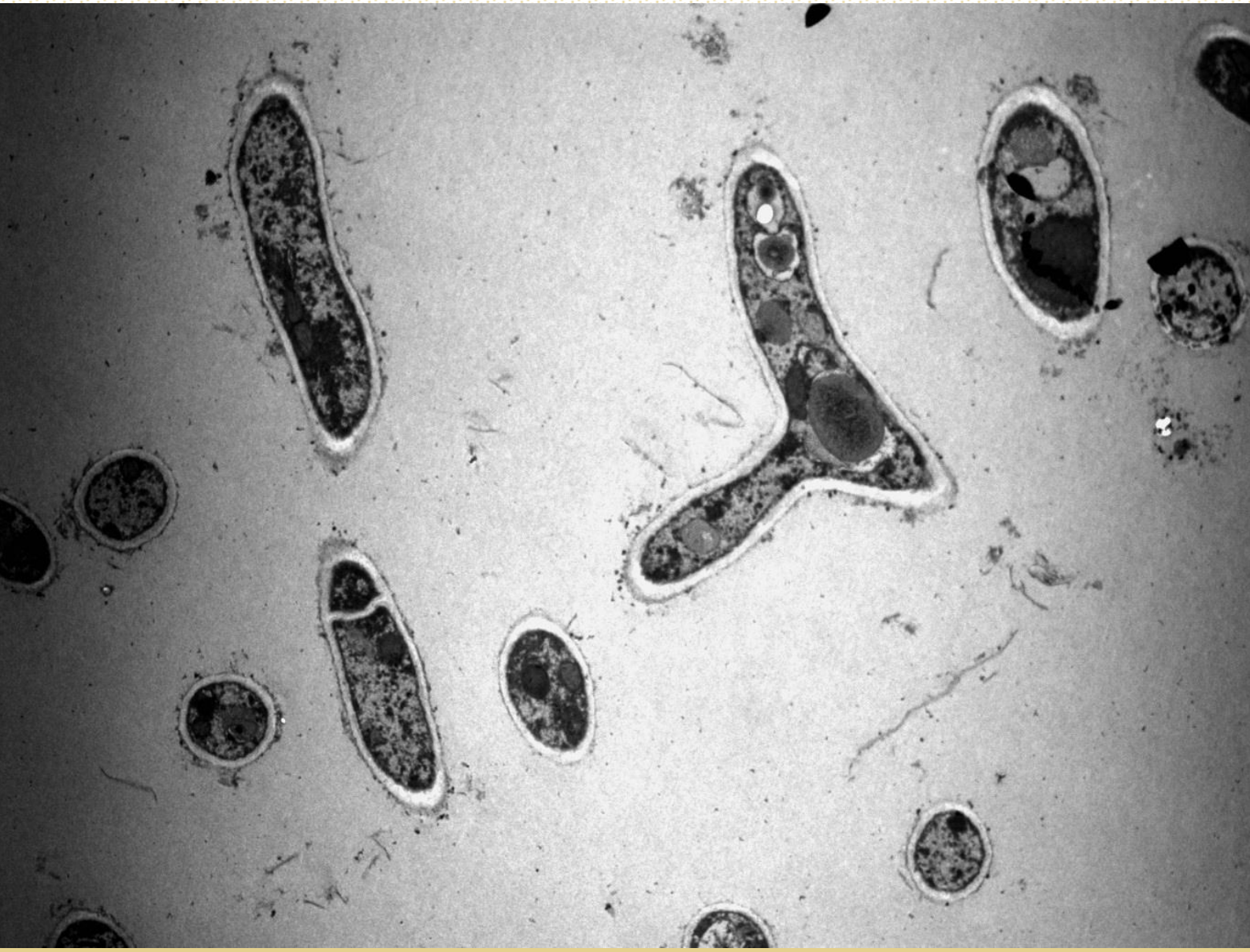

Diogo Matos da Fonseca

THE ANTI-DERMATOMYCOTIC PROPERTIES OF CAFFEINE

Tese de mestrado em Investigação Biomédica, ramo de Infeção e Imunidade,
orientada pela Professora Doutora Teresa Gonçalves e
apresentada à Faculdade de Medicina da Universidade de Coimbra

Junho de 2016

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Universidade de Coimbra

The Anti-Dermatomycotic Properties of Caffeine

Diogo Matos da Fonseca

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica. Trabalho realizado no Centro de Neurociências e Biologia Celular da Universidade de Coimbra, sob a orientação científica da Professora Doutora Teresa Gonçalves

Universidade de Coimbra

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(On the cover: Ultrastructure of *T. mentagrophytes* hyphae grown in the presence of 10 mM caffeine. Image was acquired using a transmission electron microscope)

À minha avó

Agradecimentos

"Success is not final, failure is not fatal:

It is the courage to continue that counts."

(Winston Churchill)

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List of abbreviations

A_1 – adenosine A_1 receptor

A_{2A} – adenosine A_{2A} receptor

ALT – alanine transaminase

AST – aspartate transaminase

ATP – adenosine 5'-triphosphate

cAMP – cyclic adenosine monophosphate

CBC – complete blood count

CTR – control

CWI – Cell Wall Integrity

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DTT – dithiothreitol

eATP – extracellular adenosine 5'-triphosphate

EDTA – ethylenediaminetetraacetic acid

ER – endoplasmic reticulum

FBS – fetal bovine serum

GPI – glycosylphosphatidylinositol

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ITS – internal transcribed spacer

MAP – mitogen activated protein

MIC – minimum inhibitory concentration

MOI – multiplicity of infection

MTT – 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

NA – not available

PAMPs – pathogen-associated molecular patterns

PBS – phosphate-buffered saline

PDA – Potato Dextrose Agar medium

PRRs – pattern recognition receptors

rDNA – ribosomal deoxyribonucleic acid

SDS – sodium dodecyl sulfate

TEM – transmission electron microscopy

UV – ultraviolet

YME – Yeast Malt Extract medium

Abstract

Dermatophytes are a group of filamentous fungi that can cause infections in the skin known as dermatophytoses, a type of dermatomycosis. Although not considered overall alarming, these infections can form skin fissures, therefore creating niches which potentiate the forthcoming of life-threatening opportunistic infections. Antifungal-based therapies for dermatophytoses are expensive and associated with several adverse effects and toxicity. Therefore, new therapeutics research is lagging due to the complexity of designing drugs that effectively target fungi without damaging the host. This work focus on the anti-dermatomycotic potential of caffeine, a compound that is broadly studied in several areas of medicine and widely used in cosmetics, however its antifungal properties remain scarcely explored. The impact of caffeine in dermatophyte growth, morphology and ultrastructure was studied, together with its antifungal properties during an *in vitro* keratinocyte infection by *T. mentagrophytes*, a common dermatophyte as the model pathogen. Results showed that caffeine inhibits *T. mentagrophytes* radial growth and induces an abnormal mycelial and cellular morphology. Two major components of the fungal cell wall, β-(1,3)-glucan and chitin are changed when the fungus is grown in the presence of caffeine. Moreover, the normal ultrastructure of the fungal cells is disturbed, particularly in what concerns mitochondria and endoplasmic reticulum. During dermatophyte-keratinocyte interactions, caffeine rescues keratinocyte cell viability and also delays spore germination, an indication that caffeine can act as a prophylactic agent. As preliminary data, it was verified that caffeine leads to an increase in the extracellular concentration of ATP during keratinocyte interactions with *T. mentagrophytes* which might indicate that this drug also bolsters the immune-inflammatory response upon these type of infections.

Overall the results obtained during this work show that caffeine has a clear potential as therapeutic and prophylactic tool to treat and prevent dermatophytoses.

Resumo

Os dermatófitos constituem um grupo de fungos filamentosos responsáveis por infeções cutâneas conhecidas como dermatofitoses, um dos tipos de dermatomicose. Estas infeções não são consideráveis graves para o indivíduo imunocompetente, mas podem levar ao aparecimento de infeções secundárias potencialmente letais em doentes com o sistema imunitário comprometido. Estas infeções secundárias são causadas por microrganismos oportunistas que invadem o hospedeiro através de lesões da pele com origem na infeção primária por dermatófitos. Para além de geralmente dispendiosas, as terapias antifúngicas prescritas para o tratamento de dermatofitoses estão frequentemente associadas a fenómenos de toxicidade e efeitos secundários nocivos. A investigação nesta área fica comprometida devido à dificuldade de desenhar fármacos que atuem seletivamente no fungo sem causar dano ao hospedeiro. O principal objetivo deste trabalho centrou-se no estudo da cafeína como um agente indutor de alteração da fisiologia das células fúngicas e, deste modo, nas propriedades terapêuticas da cafeína para o tratamento de dermatomicoses, Na verdade, apesar de este composto ser alvo de amplos estudos em várias áreas da medicina, o seu potencial antifúngico ainda foi escassamente explorado. Neste trabalho foi avaliado o impacto da cafeína no crescimento, morfologia e ultraestutura de dermatófitos e estudado o seu poder antifúngico num modelo de infeção *in vitro* de queratinócitos por *T. mentagrophytes* como dermatófito modelo. Os resultados obtidos demonstraram que para além de a cafeína inibir o crescimento de *T. mentagrophytes,* leva também a uma alteração da morfologia do micélio e das hifas. A composição da parede no que diz respeito aos principais constituintes, quitina e β-(1,3)-glucano, é alterada pela presença da cafeína e ocorrem alterações da ultraestrutura celular do fungo, em particular das mitocôndrias e do retículo endoplasmático. Por outro lado, a presença de cafeína preserva a viabilidade de uma cultura de queratinócitos durante a infeção por *T. mentagrophytes, in vitro*, ao mesmo tempo que, nestas condições de interação agente-hospedeiro, retarda a germinação dos esporos, o que pode indicar um potencial profilático para esta droga. Como dado preliminar verificou-se também que, durante estas infeções, a cafeína aumenta a concentração de ATP extracelular, o que pode indicar que a cafeína potencia a progressão da resposta imune-inflamatória.

Os resultados obtidos demonstram que a cafeína apresenta um claro potencial terapêutico para o tratamento ou para a prevenção de doenças causadas por dermatófitos.

Chapter 1

Introduction

1. Introduction

Although not as widely studied as other microbial pathogens, fungi are important microorganisms capable of inducing disease in both immunocompetent and immunocompromised individuals, hence being associated with significant morbidity and mortality worldwide. Only a small fraction of known fungal species are capable of infecting mammals, and even a smaller fraction represents species capable of directly inducing life-threatening diseases.

Dermatophytes are a group of filamentous fungi capable of colonizing keratinized structures and therefore induce superficial infections of the human skin. Although this type of infections are generally not alarming to the immunocompetent individual, they can form fissures in the skin and leave "an open door" for other opportunistic microorganisms to enter the body and cause severe secondary infections. This is particularly harmful in the aged, immunosenescent population. Apart from the biased increase of the target population of these diseases, it is also hypothesized that changes in the planet's climate including the rapid increase in global warming can eventually lead to an increase of fungal diseases prevalence and epidemiology (Garcia-Solache & Casadevall 2010). These facts raised the need and urge to study these microorganisms' pathogenic mechanisms, and to enhance and develop proper treatments not only to clear established infections, but also to serve as prophylactic therapies to prevent their occurrence especially in the prime targets of these diseases.

Current treatments for infections caused by these fungi are generally comprised of topical application of antifungal lotions and/or administration of oral antifungals that have limited effectiveness and can lead to several cutaneous complications. The antifungal properties of caffeine, one of the world's most widely consumed drug, were scarcely explored, and its impact on dermatomycosis and dermatophyte infections has yet to be unravelled.

In the first chapter of this thesis, a general and synthetic literature review regarding this theme will be introduced. It starts by addressing fungi in general, their taxonomy and some of their unique characteristics like their cell wall structure, it provides some epidemiological notes of dermatophyte infections and its associated

risks, and current main therapies to clear these infections. Finally there will be given some insights about caffeine and its beneficial properties, with an emphasis on dermatological conditions and in fungal infections.

1.1. Fungi: taxonomy and cell wall structure

The kingdom Fungi, estimated to comprise more than 1.5 million species (Hawksworth 2001), includes eukaryotic microorganisms, among which yeasts and filamentous fungi (known as moulds). Since unlike plants they cannot perform photosynthesis to fix carbon, fungi depend on other organic sources of carbon to thrive, therefore being considered obligatory heterotrophs (Cain 1972). Most fungal species are saprophytic: they survive by decomposing dead or inert organic matter down to its composites in order to obtain nutrients to survive. Even though Fungi is a highly diverse kingdom that includes a large number of species that range from unicellular (yeasts) to multicellular forms (moulds) and mushrooms (Adams 2004), only approximately 400 species are known to cause disease in animals (Brown et al. 2012) and even fewer (little over 300 species) were identified to be pathogenic to humans (Taylor et al. 2001).

While the majority of yeasts reproduce by budding, the main strategy of reproduction by most filamentous fungi lies in sporulation (or conidiation), by dispersing a high number of metabolically inactive small spores (conidia) coated by a thick wall that protects and makes them prevalent and highly resistant to external threats, until favourable conditions for germination and growth are met (Figure 1). These spores are airborne and depending on their size and aerodynamics, can be dispersed through large areas, one of the reasons why most species are ubiquitous in the environment (Eduard 2009), and why fungi have been estimated to comprise approximately 25% of the Earth's biomass (Miller 1992).

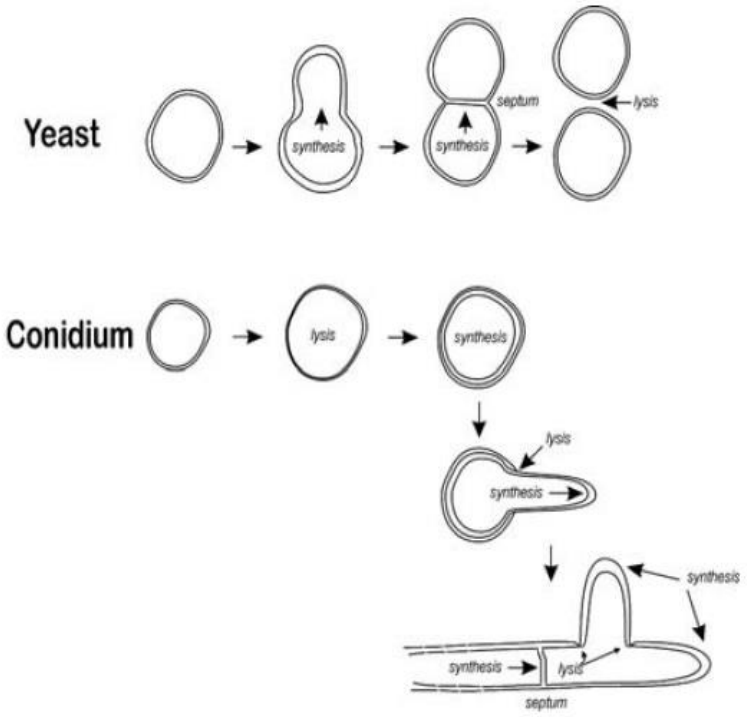


Figure 1: **Morphogenesis of yeasts and fungal conidi**a (*in*: Latgé & Calderone, 2006)

1.1.1. Fungal cell wall

The cell wall is an important dynamic structure of fungal cells. It consists of a natural border between the fungus and its environment, that provides it with the mechanical strength, shape and integrity, and that is sturdy enough to help fungi withstand changes in osmotic pressure while retaining an adequate plasticity to allow cell growth, division, and their subsequent proliferation (Casadevall et al. 2009). All exchanges between the fungal cell and its environment rely upon a functional and permeable cell wall, and since it is the most extrinsic border and the initial defensive barrier of the fungal cell, it is the first cellular component to contact directly with the surrounding hostile environment and the target host, therefore playing an active role during infection.

For the vast majority of fungal species, the cell wall is comprised of glycoproteins extensively modified with N- and O-linked carbohydrates in some cases containing a glycosylphosphatidylinositol (GPI), and mainly polysaccharides like glucan and chitin

that comprise about 90% of the wall biomass, which are bound together as polymers to form a tight semipermeable fibrillar network (Figure 2) (Latgé & Calderone 2006).

Glucans are the major structural polysaccharides of the fungal cell wall, constituting roughly 50-60% of its dry weight (Kapteyn et al. 1999). These glucans form polymers of repeated glucose residues that end up assembling into long chains. There are several types of glucans found in fungal cell walls, and even though their abundance varies depending on the target species, their contents generally include not only β-(1,3)-glucans, but other glucan types like β-(1,6)-, mixed β-(1,3)- and β-(1,4)-, α-(1,4)- and α-(1,3)-linked glucans that have been found in several fungal walls. From those, β-(1,3)-glucan is the most abundant one, accounting for 65-90% of glucan contents, and probably the most important since it serves as the main structural polysaccharide to which other wall components are covalently attach to, making its synthesis vital for proper cell wall assemblage and consequently for the normal development of fungi (Bowman & Free 2006).

Chitin is the second most abundant natural biopolymer in the world, outweighed only by cellulose in terms of abundance (Rinaudo, 2006). It exists as a long linear polymer of β-(1,4)- linked N-acetylglucosamine that folds back on itself to form immensely strong microfibrils tougher than any other molecule in nature and “stronger, weight-for-weight than bone and steel”, while retaining enormous tensile strength (Lenardon et al. 2010). Although it is considered to be a relatively minor component of the fungal cell wall (it generally accounts for only 1-2% for yeast (Klis 1994) and 10-20% for filamentous fungi (Bartnicki-Garcia 1968) wall by dry weight) it is still a structurally important polysaccharide, since it contributes significantly for the overall integrity, correct formation and osmotic stability of the cell wall (Bowman & Free 2006).

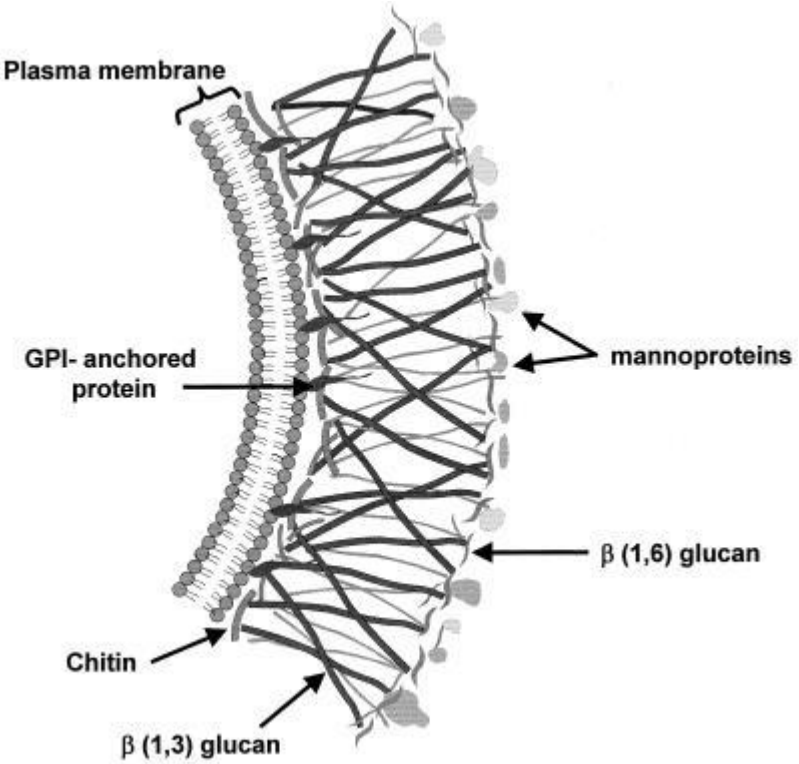


Figure 2: **Schematic of the fungal cell wall** (*In*: Selitrennikoff, 2001)

A well-formed and functional cell wall is required to maintain the osmotic equilibrium between the fungal cell and the surrounding environment, and therefore vital for it to thrive and retain its normal features such as its virulence. Mammalians lack the ability to produce this compounds (Goldman & Vicencio 2012; Eschenauer et al. 2007), thereupon making the cell wall an interesting target for the development of antifungal drugs to clear infections caused by pathogenic species.

1.2. Dermatophytes

Dermatophytes are a group of saprophytic filamentous fungi that are both keratinolytic and keratinophylic since they have the ability to chemically solubilise keratin and other related fibrous proteins such as elastin and collagen through the secretion of proteases, and use these degraded proteins as a source of nutrients to survive (Marchisio 2000; Wagner & Sohnle 1995).

According to their life cycle stage, these microorganisms can be either anamorphic (asexual state, where somatic reproduction occurs) or teleomorphic (sexual reproductive state that differs morphologically from the anamorph). Dermatophytes are most frequently found in their anamorphic state, and as saprophytes, they reproduce asexually through sporulation of arthro-, micro- and macroconidia produced in specialised conidiogenous cells, and exhibit a range of characteristic vegetative structures like hyphae, chlamydospores, spirals antler-shaped hyphae, nodular organs, pectinate organs and racquet hyphae (Emmons 1934).

The dermatophytes are divided into three ecological groups according to their natural reservoirs: geophiles, zoophiles and anthropophiles. Geophilic species like *Microsporum gypseum*, are primarly soil-inhabiting saprophytes that have the ability to invade keratinous substrates successfully. Their distribution is influenced not only by the availability of keratin in the substrates (Marples 1964), but also by the pH of the soil, since these microrganisms tend to prefer near neutral pH (Böhme & Ziegler 1968). Infections by these fungi are generally contracted from soil that contains a high density of conidia and rarely transmitted from host to host (Vroey 1984). Zoophilic species like *Microsporum canis*, are essentially animal pathogens and even though they can be isolated from infections in humans, these dermatophytes rarely grow actively as saprophytes instead they usually survive on a dormant state in contaminated materials of animal origin (English 1972). Anthropophilic species like *Trichophyton rubrum*, occur mainly as human parasites, even though some species can occasionally infect animals. Anthropophiles transmission normally requires human contact, hence the diseases caused by these fungi being strongly associated with community life (Philpot 1977). This type of classification is not only important to determine the major sources of these microorganisms but also to understand the clinical properties of the infections they cause (Woodfolk 2005).

There are only three known anamorphic genera of dermatophytes: *Epidermophyton*, *Microsporum* and *Trichophyton* classified based on spore morphology and accessory structures. Depending on the genus, these fungi can show different pathogenicity and virulent patterns *in vivo* (Weitzman & Summerbell 1995).

1.2.1. *Trichophyton mentagrophytes*

Trichophyton mentagrophytes is a fungal species complex that comprises the second-most common dermatophyte following *T. rubrum* (Nenoff et al. 2007). Apart from its anamorphic state, this complex englobes three sexual species (teleomorphic states): *Arthroderma benhamiae, A. simii* and *A. vanbreuseghemii* (Kano et al. 2011). According to its microscopic morphology, *T. mentagrophytes* shows septate hyphae, very round to tear shaped microconidia of 4-6 µm in diameter (tear shaped ones are rare and easily confused with those produced by *T. rubrum*), thin walled and cigar shaped macroconidia of 4-8 x 20-50 µm that contain 1 to 6 cells (sometimes not present), coiled spiral hyphae and nodular bodies that are often seen (Figure 3) (Larone, 2002)

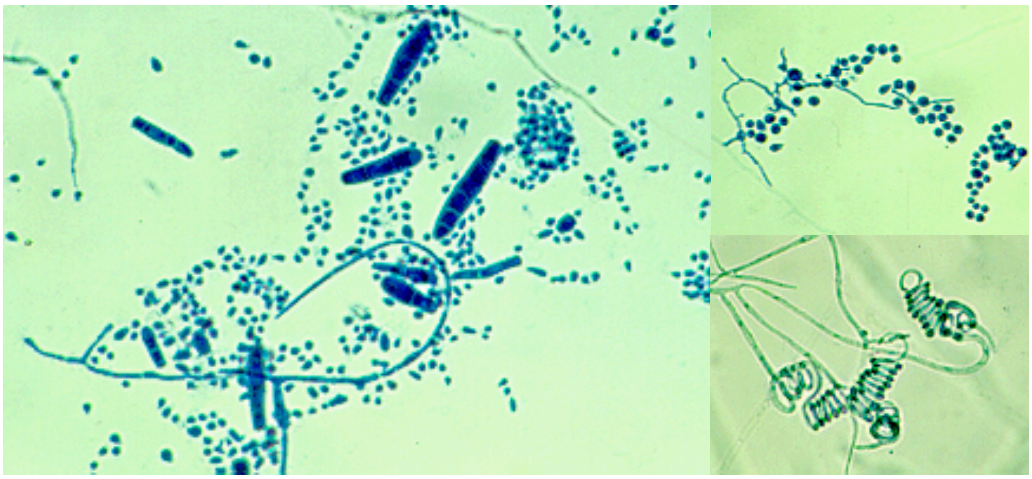


Figure 3: ***Trichophyton mentagrophytes* microscopic morphology**. Left image shows large cigar-shaped macroconidia and small round microconidia, upper right image shows chlamydoconidia and bottom left image shows coiled hyphae. (Adapted from http://www.mycology.adelaide.edu.au/)

This fungal complex has both zoophilic and anthropophilic pathogens and is capable of colonizing all parts of the body surface, including hair and nails, and it is a common cause of athlete's foot (Fréalle et al. 2007).

1.2.2. Dermatophytosis

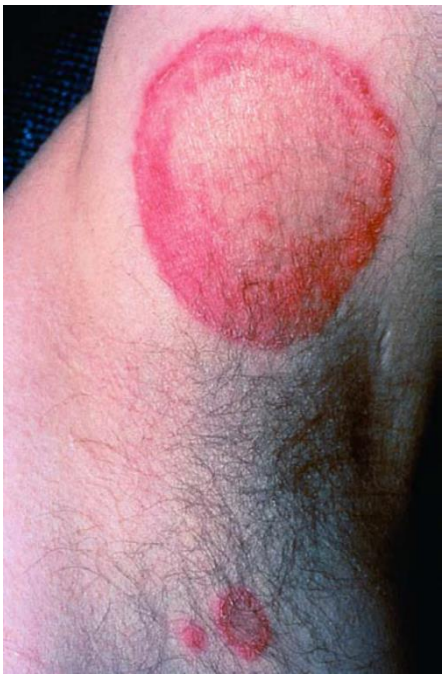


Figure 4: **Tinea corporis of the axilla, a common type of dermatophytosis** (*In:* (Hainer, 2003).

Infections caused by dermatophytes are known as dermatophytoses and are commonly referred to as tinea infections and named according to the infected host body site: tinea corporis (infection of body surfaces other than the feet, groin, face, scalp hair, or beard hair; Figure 4); tinea pedis (infection of the foot - athlete's foot); tinea cruris (infection of the groin); tinea capitis (infection of scalp hair); tinea unguium (infection of the nail - onychomycosis) (Hainer, 2003). These infections have different clinical manifestations; these can depend on the type of pathogen, anthropophilic infections tend to be chronic with almost no inflammatory signs, whereas infections caused by zoophilic and geophylic variants are self-healing and are often associated with acute inflammation (Weitzman & Summerbell 1995).

In general, these infections alone are not considered dangerous to the human host, however these fungi frequently induce damage to the outer layers of the epidermis which can lead to scaling and form lesions of the skin, providing a niche for secondary opportunistic microorganisms like bacteria to proliferate and stablish secondary infections like cellulitis and fasciitis (Björnsdóttir et al., 2005) that can be potentially life-threatening especially to the immunosenescent population like the elderly (Marques et al., 2000; Lübbe, 2003). These type of infections are referred to as “complex dermatophytoses”, and can lower the efficacy of the treatments to clear these potentially severe secondary infections caused by other concomitant microorganisms since dermatophytes secrete anti-bacterial compounds, making them often resistant to penicillin and other antibiotic treatments (Vanhooteghem et al. 2011).

1.2.2.1. Epidemiology

Dermatophytes are the main causative species of superficial mycoses (dermatomycosis) worldwide, estimated to affect 20-25% of the global population which makes them one of the most frequent forms of infections (Havlickova et al. 2008; Brown et al. 2012).

The distribution of these dermatomycoses vary according to geography and a wide range of environmental and cultural factors, however tropical countries have a higher incidence of these kind of infections because most of this countries having low socio-economic status that translates in a suboptimal hygiene, and closer a proximity to carrying agents of these infections like animals. Apart from that, they tend to have the optimal humid and warm climates of 25-28°C required for dermatophytes to thrive (Havlickova et al. 2008).

The sources of dermatophyte infections are usually shower facilities, both private and public, but also changing rooms and mats. Wearing occlusive footwear increases humidity, thus creating a microenvironment appropriate for fungus development (Hayette & Sacheli 2015). In what respects fungal foot infections, in particular onychomycosis, initially the infection does not cause distress in the patient but as the disease progresses, pain, discomfort and paresthesia might arise. Dermatophytosis are not considered life-threatening but can lead to skin fissure formation and ulceration, providing a route for other opportunistic microorganisms to enter the body and cause severe physiopathological conditions (Roujeau et al. 2004), especially in the aged and in the diabetic patient (Vanhooteghem et al. 2011).

Trichophyton rubrum, became the most common dermatophyte detected in patients suffering from dermatophytoses in the last 30 to 40 years in North and Central Europe, followed by *Trichophyton mentagrophytes* being detected twice as often in some countries like Poland and both are mainly isolated from tinea pedis (athlete's foot), the dermatophytosis with a higher incidence rate in those areas. In Mediterranean countries, apart from *T. rubrum,* zooophilic dermatophytes like *Microsporum canis* that are mainly associated with animals like cats and dogs are also common, mainly isolated from tinea capitis (hair ringworm) in children (Seebacher et al. 2008).

In the United States of America, dermatophytosis is the second most frequent reported skin disease, preceded by acne. The most common types of dermatophytosis are tinea pedis, tinea corporis and tinea cruris with *T. rubrum* being the most likely agent in these diseases, followed as well by *T. mentagrophytes*, most frequently found in onychomycosis and tinea pedis (Weinstein & Berman 2002; Seebacher et al. 2008; Kemna & Elewski 1996).

T. rubrum and *T. mentagrophytes* are generally the main cause of dermatophytosis worldwide, making both this fungi a rising epidemiological and economical problem.

1.2.2.2. Therapy

Dermatophytosis, also known as Tinea, is a very common human fungal infection, with high incidence in Europe, and includes athlete's foot and onychomycosis among other diseases (Hayette & Sacheli 2015). The therapeutic approach for these diseases usually consists on an initial long-term topical application of antifungal drugs on the infection site (Rotta et al. 2012), and when proven inefficient, an oral intake of these antifungals is often prescribed. These treatments are usually expensive, have limited effectiveness and can bring further complications like toxicity and undesirable drug interactions (Brown et al. 2012). It was also reported that some antifungals oral administration can generate dangerous adverse effects like inflammatory dermatomycosis flare-ups of infected skin zones, causing severe cutaneous complications (Nikkels et al. 2006).

Depending on the site of infection, the severity of the pathology, and the host, several treatment regimens can be followed, from topical application, especially with ciclopirox, an inhibitor of several enzymes since it chelates trivalent cations, to oral intake of antifungals like azoles, fluconazole and itraconazole, and allylamines, both classes inhibiting the synthesis of ergoesterol,, and griseofulvin, an inhibitor of mitosis through binding of microtubules (Lakshmipathy & Kannabiran 2010). These treatments duration varies from 6 weeks to 26 weeks and are associated with the risk of high

hepatotixicity. In patients under systemic antifungal treatment it is mandatory the monitoring of hepatic enzymes due to the risk of hepatitis (Ely et al. 2014).

Table 1: Treatment of Tinea Capitis and Onychomycosis (Adapted from (Ely et al. 2014)

Common treatment options	Route	Laboratory monitoring	Duration of treatment	Estimated drug cost
Tinea capitis				
Griseofulvin	Oral	No baseline testing in absence of liver disease If required for longer than eight weeks, ALT, AST, bilirubin, and creatinine measurements and CBC every eight weeks	Six to 12 weeks (continue for two weeks after symptoms and signs have resolved)	Microsize: $44 ($165) for 300 mL of 125-mg-per-5-mL solution Ultramicrosize: $263 ($430) for 60 250-mg tablets
Terbinafine (Lamisil)	Oral	Baseline ALT and AST measurement CBC at six weeks for courses lasting longer than six weeks	Six weeks; longer for Microsporum infections Assume *Trichophyton* unless culture reveals *Microsporum*	Tablets: $4 ($660) for 30 250-mg tablets Granules more expensive
Fluconazole (Diflucan)	Oral	Baseline ALT, AST, and creatinine measurement and CBC	Three to six weeks for daily dosing Eight to 12 weeks for weekly dosing	Tablets: $100 for 30 150-mg tablets ($1,185 for 90 50-mg tablets) Suspension: $33 ($290) for 35 mL of 40-mg-per-mL suspension
Itraconazole (Sporanox)	Oral	Baseline ALT and AST measurement	Four to six weeks	Solution: NA ($265) for 150 mL of 10-mg-per-mL solution Capsules: $102 ($590) for 30 100-mg capsules
Onychomycosis				
Ciclopirox (Penlac)	Topical	None	48 weeks	$16 ($540) for one bottle
Terbinafine	Oral	Baseline ALT and AST measurement, CBC ALT and AST measurement, CBC at six weeks	Six weeks for fingernails; 12 weeks for toenails	Tablets: $4 ($660) for 30 250-mg tablets Granules more expensive
Fluconazole	Oral	Baseline ALT, AST, alkaline phosphatase, and creatinine measurements, CBC No repeat needed for once weekly therapy	12 to 16 weeks for fingernails; 18 to 26 weeks for toenails	Tablets: $100 for 30 150-mg tablets ($1,185 for 90 50-mg tablets) Suspension: $33 ($290) for 35 mL of 40-mg-per-mL suspension

(ALT = alanine transaminase; AST = aspartate transaminase; CBC = complete blood count; NA = not available)

1.3. Skin and keratinocytes

Skin, the largest organ in the human body that covers an extensive area of 1.5-2.0 m^2 and constitutes roughly 15% of the body weight, is a natural barrier between the organism and the environment. This organ plays important functions such as: providing resistance against trauma; thermoregulation, since it helps the body regulate its temperature through sweating, preventing an unregulated loss of water and solutes

(and consequently desiccation); acting as a natural body guard since it protects living cells from being exposed to harmful environmental aggressions such as UV radiation, fending off external chemical and physical assaults; and especially preventing the invasion of pathogenic microbes, hence playing an important role in the immune response (Bruls & Leun 1984; DeBenedictis et al. 2001; Koster & Roop 2004).

This organ is divided into three main layers, each one showing distinct properties: the epidermis, dermis and hypodermis. The epidermis is the outermost layer of skin and is constituted by cells from the three different germ layers like keratinocytes, melanocytes, Langerhans cells and Mercke cells (Koster & Roop 2004). The epidermis itself is divided in five different layers, and each one performs different functions. The outermost layer of the skin, the stratum corneum is comprised essentially of dead cells and keratin, which along with the cell – cell junctions from the nucleated epidermis and associated cytoskeletal proteins, makes it a perfect physical barrier against external threats. Apart from that, this layer also provides the host with other biochemical and cellular defences since it secretes acids, hydrolytic enzymes, antimicrobial peptides and has circulating macrophages that help clear invasive pathogens (Freinkel & Woodly 2001; Proksch et al. 2008).

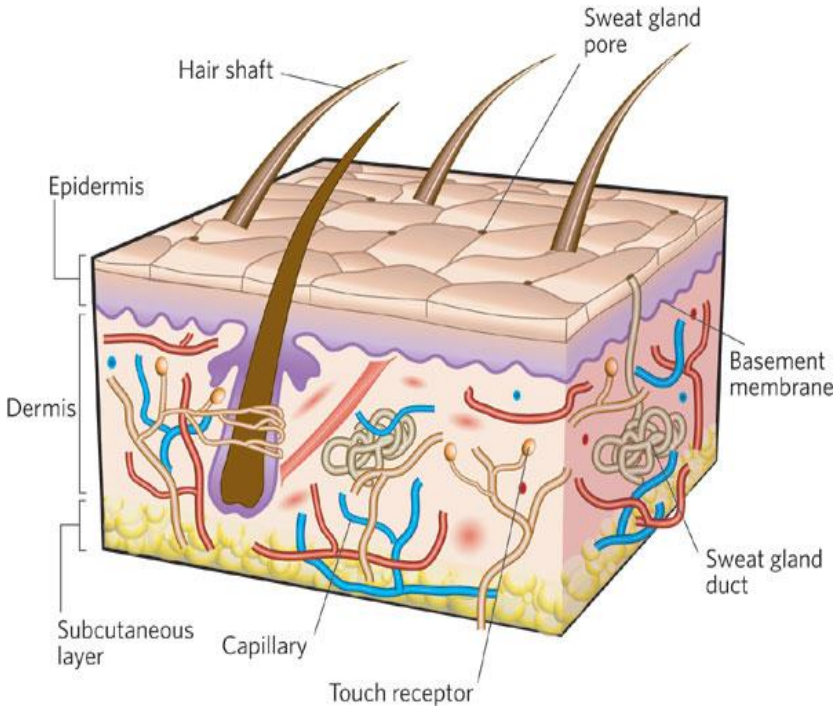


Figure 5: The structure of human skin (*In*: MacNeil, 2007).

Even though the stratum corneum is the “dead” prime protective layer of the epidermis, the deeper layers comprised of living cells that are normally free of infections need to have the ability to fight against invasive pathogens.

Keratinocytes are the main cells that constitute the epidermis, accounting for 95% of all epidermal cells and its morphology varies according to the layer they are located in (Pivarcsi et al. 2005). Apart from playing an important role in maintaining the integrity and mechanical strength of the epidermal layer via the production of several cytokeratins, they are the first cellular line of defence against cutaneous infections (Tizard, 2000).

They also play an important role in the skin immune response, since they are capable of expressing and respond to a wide variety of cytokines and chemokines (Chan, 2004). Another reported trait characteristic of these cells is that they also release ATP, a purine signalling compound, upon an infection insult to initiate inflammation and amplify cell-mediated immunity (Takahashi et al. 2013; Bours et al. 2006).

1.4. Caffeine

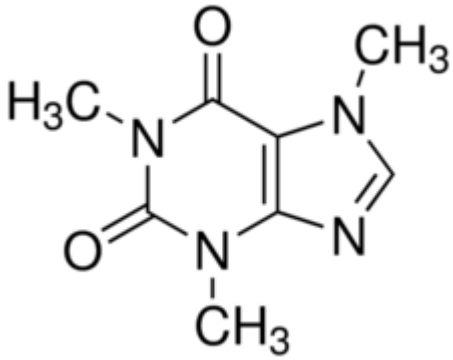


Figure 6: **Caffeine chemical structure**
(*In*: www.sigmaaldrich.com)

Caffeine (1, 3, 7-trimethylxanthine) is an alkaloid stimulant from the methylxanthine family and since it is present in coffee, tea, sodas, chocolate derivatives and various medicinal products, it is probably the most widely consumed psychoactive drug known to man. This compound is endowed with medicinal properties since it is implicated to affect a variety of cellular processes in different organisms including mammals, plants and fungi (Kuranda et al. 2006).

Apart from being a mild stimulant of the nervous system that causes mood enhancement, improves concentration and eliminates physical fatigue, its wide spectrum of medicinal applications include its use in appetite stimulants, analgesics,

some antiviral drugs and it also has anti-tumorigenic properties since it has chemopreventive effects against skin cancers (Bahi et al. 2001; Han et al. 2011). Since this compound is a non-selective antagonist of A_1 and A_{2A} adenosine receptors, it is also broadly used in neonatology at concentrations of 5 to 15 µg/mL to block these receptors, therefore stimulating ventilation and curing apnea of premature new-born babies. (Fredholm et al. 1999; Herlenius & Lagercrantz 1999).

Caffeine and its derivatives are also widely used in cosmetics. This compound is endowed with anti-cellulitic properties since it has an anti-inflammatory effect and prevents excessive accumulation of fat in adipocytes cells by stimulating its degradation during lipolysis, hence its frequent use in both skin lotions and textile fibres. It is also used in topical anti-dark circles lotions since it promotes microcirculation of blood in the skin, effectively reduce the swelling of skin tissue around the eyes, it is used as a stimulator for hair growth because of its combined ability to improve microcirculation and to reduce smooth muscle tension near hair follicles causing nutrients to be easier delivered through the blood vessels of the papillae of hair, and is frequently used in beauty lotions since its potent antioxidant properties help protect cells against UV radiation and therefore slowing down the process of photo aging of the skin (Rubio et al. 2010; Herman & Herman 2012).

Caffeine as also been described as interfering in fungal cells growth and is widely used in *in vitro* assays aiming to study the mechanisms of cell wall synthesis regulation (Klis, 1994; Kuranda et al., 2006; Munro et al., 2009; Levin, 2011).

1.5. Aims

The aim of this work was to explore some of the anti-dermatomycotic properties of caffeine during infections by dermatophytes. To achieve this goal, the structure of this thesis was divided in three major parts: part A, B and C.

Part A focus mainly on the fungus itself, and its main objective is to study the impact of the drug in dermatophyte growth and morphology. To achieve this goal, firstly, the effect of caffeine in dermatophyte growth was assessed and how this drug

modulates two important cell wall polysaccharides that are responsible for the maintenance of fungal normal morphology and its virulence. To get a visual idea on how this morphology is impacted, we resorted to microscopy techniques to evaluate both how this drug affects fungal colonies morphology, and how it alters its cellular morphology.

Next two parts B and C focus mainly on caffeine's impact on target host cells – fungal pathogen interactions.

In part B, the antifungal properties of the compound during the course of an infection insult were explored first by assessing how it affects keratinocytes' viability and secondly how this drug affects fungal spores germination upon infection.

Part C focus mainly on how the drug modulates extracellular ATP released during the course of an infection, as an indicator of inflammatory response.

Chapter 2

Materials & Methods

2. Materials & Methods

2.1. Fungal strains

Trichophyton mentagrophytes was the selected fungal pathogen used in all the experimental assays performed throughout this work. This fungus, kindly provided by Professor Carmen Lisboa of the Laboratory of Microbiology, Faculty of Medicine, University of Porto, was isolated from an infected patient and later identified by sequencing the ITS-5.8 S fragment of the 18S rDNA (Paulo et al. 2009).

2.1.1. Fungi maintenance and culture conditions

T. mentagrophytes were cultured and maintained in an incubator at 30°C in Potato Dextrose Agar medium (PDA) (0.4% potato starch (w/v), 2% dextrose (w/v), 1.5% agar (w/v)) (BD Biosciences®, San Jose, California, USA) sterilized by autoclaving at 121°C for 20 min under a pressure of 1.2 atm. To obtain an optimal spore yield, fungi were cultured in small petri dishes (with approximately 10 mL of culture medium per dish) for a period of 21 days minimum and 42 days maximum prior to microconidia harvest for further use in *in vitro* assays.

For fungal polysaccharides quantification assays, for transmission electron microscopy (TEM) and optical microscopy analysis of acquired images, *T. mentagrophytes* was previously grown in liquid Yeast Malt Extract medium (YME) (0.4% yeast extract (w/v), 1% glucose (w/v), 1% malt extract (w/v)) supplemented with caffeine (Sigma-Aldrich®, St. Louis, Missouri, USA) at concentrations of 1 mM, 5 mM and 10 mM by inoculating 2 x 10^5 fungal microconidia in Erlenmeyer flasks filled with 100 mL of medium sterilized by autoclaving. Caffeine was added to the media posterior to autoclaving, by aseptically adding 10 mL of each caffeine stock solutions (w/v) sterile filtered through 0.22 µm filtration membranes beforehand. Cultures were incubated at 30°C with constant orbital shaking at 120 rpm from 3 to 25 days upon inoculation (control cultures (no caffeine) and cultures supplemented with 1 mM caffeine were grown for 3 days, 5 mM caffeine cultures for 9 days and 10 mM cultures for 25 days).

The maximal concentration used was 12 mM caffeine based on the one reported as the minimum inhibitory concentration (MIC) value for *Candida albicans*: 12.5 mM (Raut et al. 2013).

2.1.2. Microconidia harvest

To harvest *T. mentagrophytes* spores and obtain purified microconidia suspensions for further use in all experimental assays, two to four petri dishes of PDA inoculated with the dermatophyte were removed from the incubator and fungal aerial mycelia from all plates were rinsed twice with the same volume of 2 mL 0.1% Tween 80 solution (v/v) per dish (Sigma-Aldrich®, St. Louis, Missouri, USA) to help detach conidia from hyphae. After proper washing, all the volume of 0.1% Tween 80 solution containing the harvested conidia were collected from the rinsed dishes and filtered through sterile handmade sacred linen filtration systems to remove media remains and hyphae fragments. The filtered suspension was then centrifuged twice at 16.060 g for 10 min at 4°C, supernatants were discarded, pellets were ressuspended first in 1 mL cold Phosphate Buffered Saline (PBS) (10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl (w/v), pH 7.3) and after the final centrifugation, pellet resuspension was done either in 1 mL cold PBS for posterior use in radial growth inhibition assays, with YME for microconidia incubation with caffeine in liquid cultures, or DMEM D5648 (Sigma-Aldrich®, St. Louis, Missouri, USA) supplemented with 10% non-inactivated Fetal Bovine Serum, 10 mM HEPES, 12 mM $NaHCO_3$ and 2 mM L-Glutamine for later use in infection assays.

Spore concentrations of the purified suspensions were estimated in microconidia/mL respectively using a haemocytometer.

2.2. Radial growth inhibition assays

Radial growth inhibition assays were performed by inoculating 1.25 x 10^4 microconidia in regular Petri dishes with 20 mL of PDA supplemented with different caffeine concentrations (50 µM, 1 mM, 5 mM and 12 mM). Spores were aseptically inoculated in the centre of the dishes, and fungal radial growth was monitored from the third until the seventh day of incubation at 30°C by measuring the aerial mycelium length in four different axis centred on the inoculation site in each dish. Mycelia daily measurements were performed roughly at the same time in each day of the assay, using the same regular office ruler.

2.3. Fungal cell wall polyssacharide quantifications

To prepare *T. mentagrophytes* mycelia for β-(1,3)-glucan and chitin quantification, fungal colonies were grown in YME in the same conditions as described before in section 2.1.1. After each set incubation period, colonies were collected from the media, washed twice with sterile distilled water to remove media remains, stored in previously weighted micro tubes and frozen at -80°C overnight. Samples were then lyophilized for 6 h and tubes containing dried mycelia were stored at room temperature until further processing. (Mycelia dry weight was calculated by subtracting each empty micro tubes weight to its respective weight after lyophilisation).

2.3.1. β-(1,3)-glucan quantification

β-(1,3)-glucan contents were determined by the Aniline Blue Assay (Fernandes et al. 2014).

Lyophilized fungal colonies were sonicated with 1 mL NaOH 1 M until mycelia were properly homogenized. After sonication, samples were incubated for 52 °C for 30 min and samples were transferred to a microtiter plate wells in order to perform the Aniline Blue Assay. At the end of the assay, samples in the microtiter plate were incubated again at 52 °C for 30 min and kept at room temperature for another 30 min

before their emitted fluorescence was read using a SpectraMax® GEMINI EM fluorimeter. For this assays, standard calibration curves were plotted using several dilutions from a stock solution of 50 g/L curdlan (Sigma-Aldrich®, St. Louis, Missouri, USA) in NaOH 1 M.

2.3.2. Chitin quantification

Chitin contents were determined as described in Fernandes et al. (2014). This method is based on the quantification of the glucosamine released from fungal cells walls upon acid hydrolysis.

Lyophilized fungal colonies were sonicated in 1 mL sterile distilled water until proper mycelia homogenization was achieved. Samples were then spun at 16.060 g for 20 min at 4°C, and after centrifugation water was discarded and pellets were extracted in SDS-Mer-OH buffer (50 mM Tris (w/v); 17.34 mM SDS (w/v); 0.3 M β-mercaptoethanol (v/v); 1 mM EDTA (w/v); pH 8.0) at 100 °C for 10 min. Mycelia samples were centrifuged again at 16.060 g for 20 min at 4°C, supernatants were discarded, 0.5 mL of HCl 6 M were added to each sample and micro tubes were incubated for 18 h until all HCl 6 M has evaporated. Sterile distilled water was then added to the dried samples and micro tube were sonicated until the pellets were completely hydrated and properly homogenized. 100 µL of Solution A (1.5 N Na_2CO_3 in 4 % acetlyacetone (w/v)) was added and samples boiled for 20 min. Finished the boiling period, 700 µL of 96 % ethanol and 100 µL of Solution B (β-dimethylaminobenzaldehyde in 50% concentrated HCl and 50% absolute ethanol solution (w/v)) was added, and samples were incubated for 1 h at room temperature. Following that, 220 µL of each sample was transferred to 96 well microtiter plates and their absorbance at 520 nm were measured using a SpectraMax® PLUS 384 spectrophotometer. For this assays, standard calibration curves were plotted using several dilutions from a stock solution of 5 g/L glucosamine (Sigma-Aldrich®, St. Louis, Missouri, USA).

2.4. Optical microscopy analysis

Liquid *T. mentagrophytes* cultures were started with and without 10 mM caffeine supplementation and grown in the same conditions and for the same time as described before in section 2.1.1. After each growth period, media was removed, colonies were collected from the cultures flasks and assembled between regular glass microscope slides and coverslips to be posteriorly visualized at 120 h under a Nikon® Eclipse E400 microscope equipped with a digital camera (Nikon® Digital Sight DS-L1).

2.5. Transmission electron microscopy (TEM) analysis

Liquid *T. mentagrophytes* cultures were started with 10 mM caffeine supplementation and with no drug supplementation (control cultures) and grown in the same conditions and for the same time as described before in 2.1.1. At each time point, samples were washed and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. Postfixation was performed using 1% osmium tetroxide for 1 h. After rinsing with the buffer, the samples were dehydrated in a graded ethanol series (30 to 100%), impregnated, and embedded in Epoxy resin (Fluka Analytical®). Ultrathin sections (80 nm) were mounted on copper grids (300 mesh) and stained with uranyl acetate 2% (15 min) and 0.2% lead citrate (10 min). Observations were carried out on an FEI-Tecnai® G2 Spirit Bio Twin™ transmission electron microscope at 100 kV.

2.6. Infection assays

2.6.1. Cell line maintenance and culture conditions

The HaCaT cell line of immortalized human keratinocytes was selected for this study. Cells were obtained from the German Cancer Research Centre (Heidelberg, Germany), seeded in 75 cm^2 cell culture flasks and maintained in Dulbecco's Modified Eagle Medium D5645 (Sigma-Aldrich®, St. Louis, Missouri, USA) supplemented with 10% non-inactivated Fetal Bovine Serum (FBS), 10 mM HEPES, 12 mM sodium bicarbonate

and 2 mM L-Glutamine at 37°C (DMEM) in a humidified atmosphere with 5% CO_2. Cell cultures were maintained from 3 to 4 days until keratinocytes reach a state of approximately 70% confluence. Upon reaching said confluence state, media contained in the culture flasks was rejected, cells were washed twice with PBS at 37°C to remove media residues and then detached from the bottom of the dish by incubating with 2 mL of trypsin-EDTA solution (Sigma-Aldrich®, St. Louis, Missouri, USA) for 10 min at 37°C with 5% CO_2. After incubation, flasks were lightly tapped to help cell dislodging and trypsin activity was neutralized by adding 8 mL of fresh DMEM (37°C) to each flask. Cell suspensions were properly homogenized, and to start cultures anew suspended keratinocytes were split 1:5 (2 mL of suspension) and seeded in new flasks with fresh media.

All infection assays described in this work were performed using HaCaT cells from passages #35 to #55.

2.6.2. *In vitro* infection assays using HaCaT cells and *T. mentagrophytes* microconidia

For *in vitro* infection assays, HaCaT cells were grown and detached as previously described in section 2.6.1. After cell detaching and new cultures establishment, the remaining volume of cells suspended in medium-neutralized trypsin was collected to a single sterile conical centrifuge tube and posteriorly centrifuged for 4 min, 4°C at 500 g. Supernatant was discarded after centrifugation, the pellet was ressuspended and properly homogenized in DMEM and an aliquot (10 µL) of the suspension was collected to count cells and estimate keratinocyte concentration using a haemocytometer. Following cell counting, 2 x 10^5 keratinocytes were seeded in 12-well assay plates (for MTT assays, 10^5 cells were plated in each well instead) and incubated overnight in a cell incubator at 37°C in a humidified atmosphere with 5% CO_2 for 18 h to allow keratinocytes to settle, adhere to the bottom of the plate, and to duplicate. After the incubation period, it was expected that keratinocyte doubling was achieved, hence upon addition of conidia, it was assumed to have a density of 4 x 10^5 keratinocytes per seeded well (2 x 10^5 for MTT assays).

Before addition to HaCaT cells, *T. mentagrophytes* microconidia were harvested as described before in section 2.1.2., and added to wells containing adhered keratinocytes in a multiplicity of infection (MOI) of one microconidia per HaCaT cell (1:1).

To assess the impact of caffeine in keratinocyte-microconidia interaction, caffeine solutions were also added to some wells at different concentrations. To minimize media deprivation, the caffeine volume added to each well was always 10% (100 µL) of its final volume (1 mL). For eATP quantifications, cells were incubated with fungal conidia in the presence or absence of caffeine in DMEM without phenol-red.

2.7. MTT assays

To perform MTT assays, 10^5 HaCaT cells were plated in 12-well assay plates as described before in 2.6.2. 2 x 10^5 *T. mentagrophytes* microconidia and caffeine solutions at different concentrations were added 18 h posterior to cell seeding and after culture media renewal. Plates were then incubated at 37°C in a cell incubator with 5% CO_2 for 6, 9 and 12 h and after each incubation period has ended, media from each well was rejected and 900 µL of new media was added per well along with 100 µL of a 5 mg/mL MTT solution (Sigma-Aldrich®, St. Louis, Missouri, USA) in PBS (w/v) in order to achieve a final MTT concentration of 500 µg/mL per well. Plates were incubated again for 2 h to allow keratinocytes to metabolize the compound. Prior to this incubation period, media was discarded, 500 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich®, St. Louis, Missouri, USA) was added to each well, plates were wrapped in tin foil to avoid photo degradation and were spun at 120 rpm for 5 min in an orbital shaker to dissolve formazan crystals. Contents in each well were transferred to 96-well microtiter plates in 100 µL triplicates and absorbance at 570 nm was measured in a SpectraMax® PLUS 384 spectrophotometer.

2.8. Ungerminated microconidia assays

Infection environments were established using HaCaT cells and *T. mentagrophytes* microconidia in 12-well assay plates as described before in section 2.6.2. Cells were incubated at 37°C in a cell incubator with a humidified atmosphere of 5% CO_2 for 6 h and 9 h. After each incubation period, assay plates were removed from the incubator, kept in ice, and the bottom of each seeded well was scratched using sterile 1000 µL filtered tips. After proper scraping, well contents were homogenized, transferred to 1.5 mL sterile micro tubes and then centrifuged at 16.060 g for 10 min at 4°C. After centrifugation, supernatants were rejected, pellets were ressuspended in 100 µL cold PBS and posteriorly to homogenization, 10 µL aliquots of each tube were collected to count ungerminated microconidia using a haemocytometer. Only ungerminated microconidia contained in each aliquot were counted in the central square of the haemocytometer and their concentration was estimated in ungerminated microconidia per mL.

2.9. Extracellular ATP quantification

To quantify extracellular ATP (eATP) during the course of a *T. mentagrophytes* infection, *in vitro* infection assays were performed as described in section 2.6.2., and two infection time points (6 h and 12 h) were selected. After each time point, 12-well assay plates were removed from the incubator and placed on ice. Supernatants from each well were collected to sterile 1.5 mL micro tubes and kept ice-cold until processing. Micro tubes were then centrifuged at 14.825 g at 4°C for 5 min, and at the end of the centrifugation, 500 µL of supernatants from each micro tube were transferred to new sterile 0.5 mL micro tubes and stored at -80°C until quantification. ATP quantifications were performed as previously described (George et al. 2015) using the Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma-Aldrich®, St. Louis, Missouri, USA), to record luminescence with a VICTOR3 Multilabel Plate Reader (PerkinElmer®).

2.10. Statistical analysis

Statistical analysis of the obtained results were performed in order to assess about the statistically significant differences between different data sets obtained in this work. Data were analysed using GraphPad® Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Data are presented as means ± standard errors (SEM), and significance values are presented as $p<0.05$, $p<0.01$ or $p<0.001$ (*, ** and *** respectively).

Chapter 3

Results

3. Results

3.1. A – Impact of caffeine in dermatophyte growth and morphology

Altering fungal morphology and consequently delaying or inhibiting fungal growth is vital for most antifungals to be successful in clearing infections caused by these pathogens. To assess if caffeine has a similar effect on both dermatophyte growth and morphology, radial growth inhibition assays were performed in an effort to understand if *T. mentagrophytes* growth is impaired upon incubation with the drug and its impact on fungal morphology was assessed by resorting to microscopy techniques.

3.1.1. Radial growth inhibition assays

In order to effectively colonize the target host, dermatophytes need to proliferate and spread their hyphae, therefore growing through the substrate they feed on. Radial growth inhibition assays were performed to understand how caffeine affects dermatophyte growth. For that, *T. mentagrophytes* microconidia suspensions were inoculated in PDA plates supplemented with several caffeine concentrations. Figure 7 shows the obtained results for these assays.

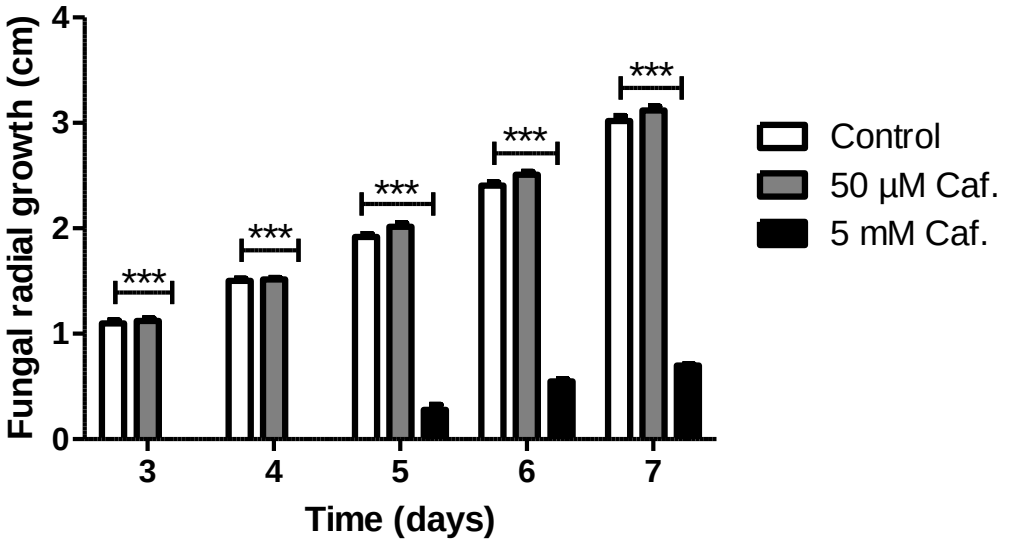


Figure 7: Impact of caffeine on *T. mentagrophytes* radial growth. Microconidia were inoculated in PDA plates in the presence or absence of caffeine, and mycelia growth was monitored for 5 days. “Control" stands for the negative control, were the fungus was grown in plates containing no caffeine, “50 µM caf.” represents plates inoculated with fungal microconidia in the presence of 50 µM caffeine and “5 mM caf.” with 5 mM caffeine supplementation. Results were plotted as mean ± SEM from quadriplicates (n=3), repeated measures ANOVA, ***p<0.001 (Control vs 5 mM caf)

By analysing the results it is possible to assess that mycelial growth was significantly inhibited in the presence of 5 mM caffeine comparing with control conditions where no caffeine was added. Otherwise, 50 µM caffeine does not seem to have a direct impact in *T. mentagrophytes* radial growth since a similar growth pattern was observed, with no statistically significant differences when compared with control growth conditions. Fungal growth in the presence of 5 mM caffeine was only registered after the fifth day of growth because only from that day it was possible to observe and measure mycelial growth with the same measurement methodology used to register dermatophyte growth in control and 50 µM caffeine conditions.

Whilst this pattern was observed for 5 mM caffeine concentration, an even more pronounced radial growth inhibition was reported for 12 mM caffeine. This latest concentration of the drug, was also tested, however, throughout the seven days of the assay, fungal growth was strongly inhibited disabling growth registration using the adopted measurement methodology. However, a vestigial and minimal mycelial growth was only visible to the naked eye at the eleventh day after inoculation (data not shown).

3.1.2. Cell wall components quantification

With the purpose of understanding caffeine's mode of action and its impact in fungal growth, assays were performed to understand if this drug modifies the fungal cell wall structure of *T. mentagrophytes*. The two cell wall components studied, β-(1,3)-glucan and chitin, were selected because apart from being two structurally important polysaccharides present in this cellular structure, that strongly account for its integrity, they are also important pathogen-associated molecular patterns (PAMPs) to consider when studying infection and inflammation phenomena triggered by these fungi. Therefore, modulation of the normal levels of these molecules can not only be important to somehow change fungal cell wall conformation and structure, which might impact fungal growth rate and consequently affect dermatophyte proliferation, but also alter these fungi normal virulence patterns and subsequently trigger different responses by host cells.

3.1.2.1. β-(1,3)-glucan quantification

Apart from being the major structural polysaccharides present in the fungal cell wall, glucans are the main "building blocks" of the cell wall of fungi. There are several types of glucans that can be found in this structure, however, β-(1,3)-glucan is one of the most important, because apart from being more abundant and serving as an important constituent to which other cell wall components are bound to, therefore accounting for its stability, it also plays an important role in pathogen recognition by host cells upon infection.

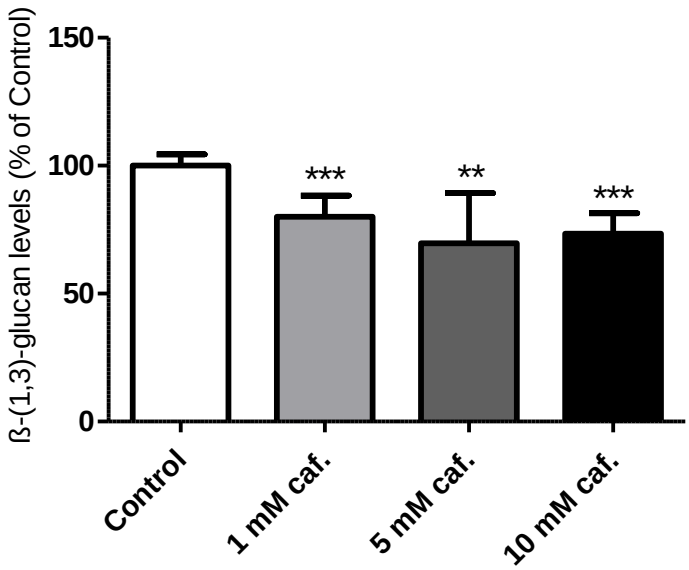


Figure 8: **Effect of caffeine on β-(1,3)-glucan contents of *T. mentagrophytes* cell wall.** Microconidia were inoculated in YME supplemented with different caffeine concentrations prior to fungal colonies processing and further polysaccharide quantification. "Control" stands for the negative control, were the fungus was grown in YME containing no caffeine, "1 mM caf." represents media flasks inoculated with fungal microconidia in the presence of 1 mM caffeine, "5 mM caf." with 5 mM and "10 mM caf." with 10 mM caffeine supplementation. Results are presented as mean ± SEM (n=3), unpaired t-test, **p<0.01, ***p<0.001 (vs control).

The results obtained for β-(1,3)-glucan quantifications show that all the caffeine concentrations tested a significant decrease in cell wall β-(1,3)-glucan levels in *T. mentagrophytes* (Figure 8). The decrease in β-(1,3)-glucan is similar, regardless of the caffeine concentrations used, meaning that low caffeine concentrations such as 1 mM, have a similar dampening effect on β-(1,3)-glucan present at the dermatophyte cell wall as the other studied concentrations of this drug, five and ten times higher.

3.1.2.2. Chitin quantification

Chitin is the second most abundant natural biopolymer in the world, outweighed only by cellulose in terms of abundance. Even though it is generally considered to be a minor component of the fungal cell wall (roughly 2% for yeast and up to 20% for filamentous fungi), chitin is an important structural component of the cell wall since it greatly contributes to its overall integrity and to maintain the osmotic equilibrium

between the fungal cell and the surrounding environment. Like β-(1,3)-glucan, chitin is also an immunoreactive compound capable of triggering an immune response by host cells upon recognition and consequently eliciting the production of cytokines by activation of a range of pattern recognition receptors (PRRs).

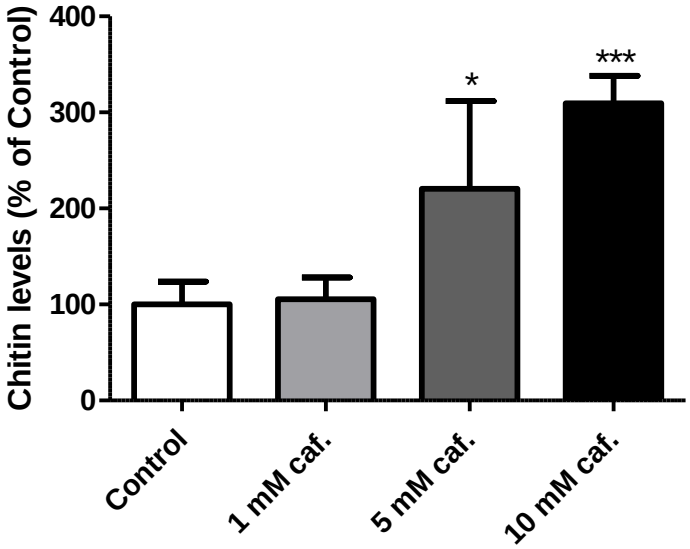


Figure 9: **Effect of caffeine on chitin contents of *T. mentagr*ophytes cell wall.** Microconidia were inoculated in YME supplemented with different caffeine concentrations prior to fungal colonies processing and further polysaccharide quantification. "Control" stands for the negative control, were the fungus was grown in YME containing no caffeine, "1 mM caf." represents media flasks inoculated with fungal microconidia in the presence of 1 mM caffeine, "5 mM caf." with 5 mM and "10 mM caf." with 10 mM caffeine supplementation Results are presented as mean ± SEM (n=3), unpaired t-test, *p<0.05, ***p<0.001 (vs control).

Chitin quantification in fungi grown in media supplemented with several concentrations of caffeine showed that different concentrations of this drug affect differently the chitin cell wall content. While 1 mM caffeine does not show a clear change of the chitin cell wall level, 5 mM and 10 mM concentrations lead to significant increase of this cell wall component of *T. mentagrophytes* (Figure 9). At 10 mM caffeine, the highest tested concentration of the drug, a roughly fourfold production of chitin occurred, when compared with control conditions, indicating that high concentrations of this drug lead to an upregulation of chitin synthesis by this dermatophyte.

3.1.3. Optical microscopy analysis

To understand how caffeine affects *T. mentagrophytes* colonial morphology, the fungus was cultured in liquid YME medium supplemented with 1 mM, 5 mM and 10 mM caffeine and after each set growth period, colonies were collected and photographed using a digital camera for macroscopical analysis. After that, microscope slides were prepared to assess how caffeine impacts in morphology at a microscopic level. Figure 10 shows plates with the macroscopic morphology of *T. mentagrophytes* colonies (each looking yellowish cotton balls) obtained in liquid media containing different concentrations of caffeine.

Control (CTR) colonies have heterogeneous shapes and sizes; the density of each colony is lower than that of those obtained in the presence of caffeine. Considering that the cultures started with the same initial inoculum (same microconidia density), more fungal colonies are visible in the control flasks than in the flasks containing caffeine-supplemented medium (Figure 10).

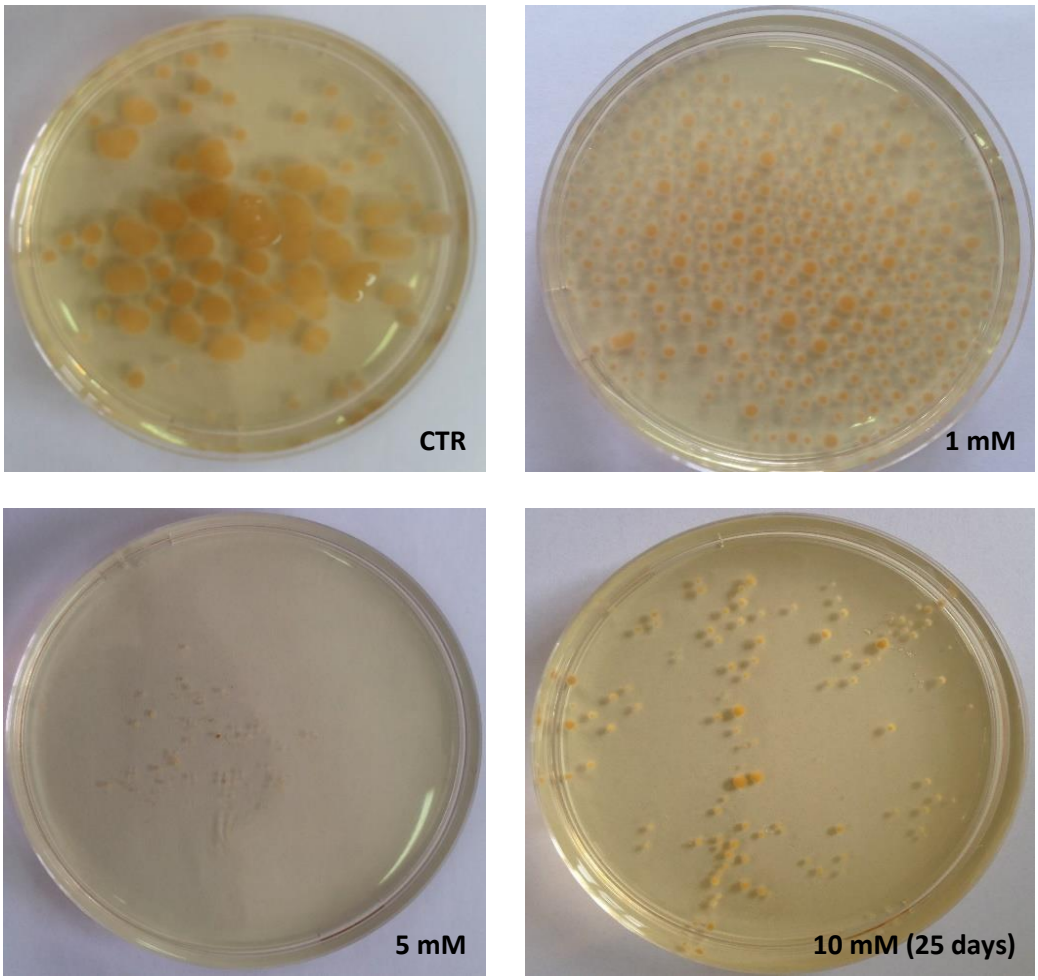


Figure 10: **Impact of caffeine on *T. mentagrophytes* colonial morphology and growth in liquid cultures.** Microconidia were inoculated in YME supplemented with different caffeine concentrations. "CTR" stands for control cultures were the fungi were grown in the absence of caffeine and the remaining markings define the caffeine concentrations that were added to the culture media prior to microconidia inoculation. Control, 1 mM and 5 mM caffeine-supplemented cultures were incubated for 4 days, whilst 10 mM caffeine-supplemented cultures were maintained for 25 days. Images were acquired using a regular digital camera.

Colony morphology is altered when the fungus is incubated with the drug. Colonies grown in the presence of 1 mM caffeine appear in slightly lower number and are much smaller in comparison with those grown in the absence of caffeine. Apart from that, colonies incubated with 1 mM caffeine are much smaller in size, but more condensed than those collected from control cultures. The mycelial aspect of 5 mM caffeine-supplemented cultures clearly shows a decrease in the number of fungal balls (colonies) with a marked smaller size than those obtained in the previous conditions (Figure 10, "CTR" and "1 mM" images).

These observations led us to believe that caffeine had a modulatory impact on *T. mentagrophytes* hyphal morphology, since its growth rate was impaired when the fungus was incubated with the compound, even in small concentrations. To tackle this hypothesis, a higher concentration of caffeine, 10 mM, was tested. It was observed that during the growth period assay (4 days), there were no visible fungal colonies in the flasks, so the cultures were kept until colonies were visible in the liquid medium. This was achieved by the twenty-fifth day of growth, looking smaller, rounder and denser than those obtained in flasks containing 1 mM caffeine (Figure 10, "10 mM (25 days)" image).

With the microscopic observation of the fungal balls corresponding to individual colonies (Figure 11) it was possible to get a better glimpse on how dermatophyte morphology is altered by caffeine. By comparison with colonies incubated with caffeine, it is possible to confirm what was observed before in Figure 10 since control colonies are apparently less dense than those incubated in culture media with caffeine. Apart from that, these colonies display longer hyphae than those grown in the presence of the drug. Both these effects seem to be more marked especially in 5 mM and 10 mM caffeine-supplemented cultures.

To better understand how the two highest concentrations of the drug selected for these assays influenced colonial morphology, single colonies grown in YME with 5 mM and 10 mM caffeine were harvested from the media and photographed in the same microscope with a lower magnification. By comparing both colonies it is possible to infer that the one grown with 10 mM caffeine shows higher density and smaller hyphal size than the one grown with 5 mM, even though the colony appears larger, (probably due to the longer incubation period of twenty five days in comparison with the four days of growth of 5 mM caffeine-cultures.

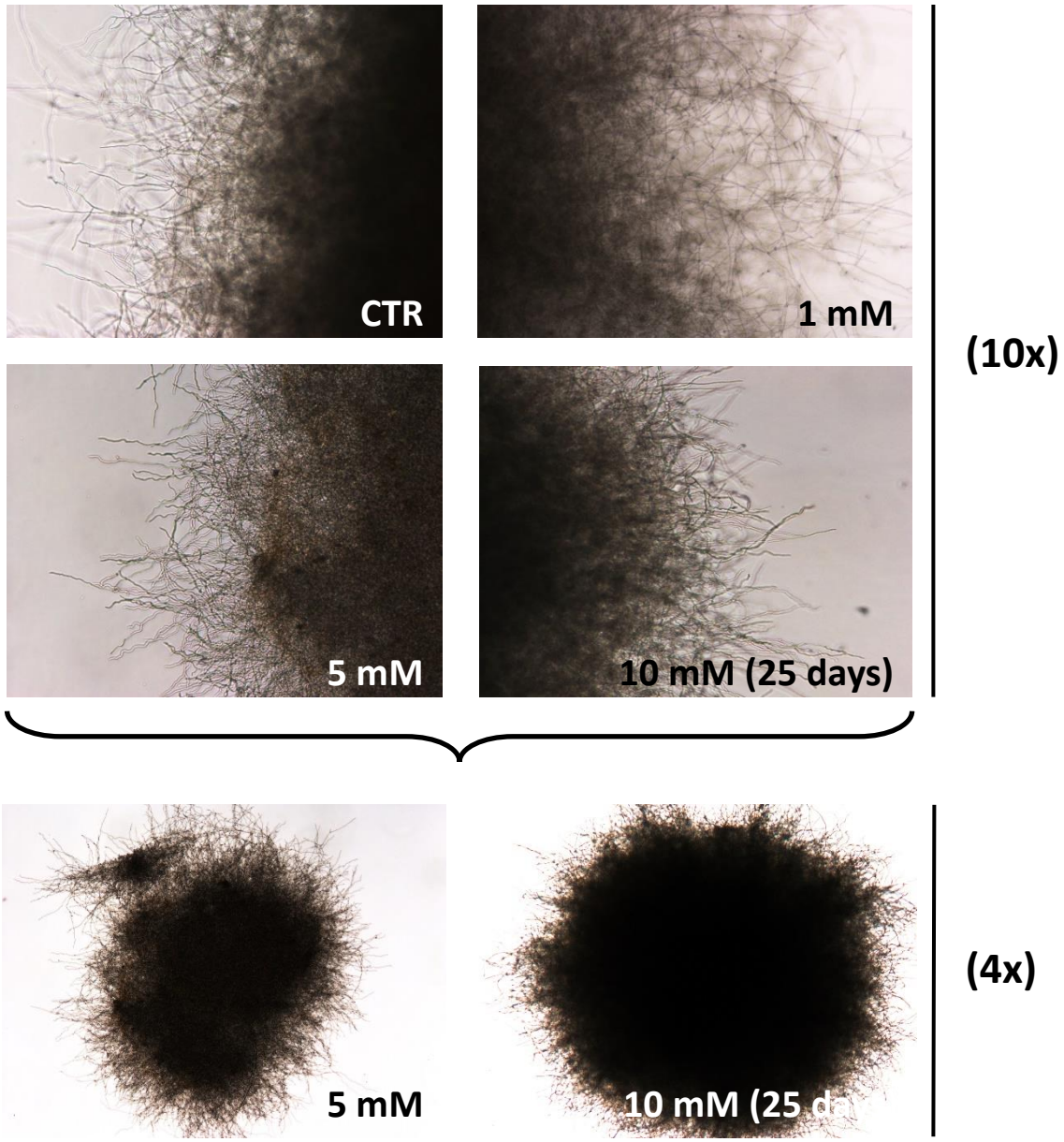
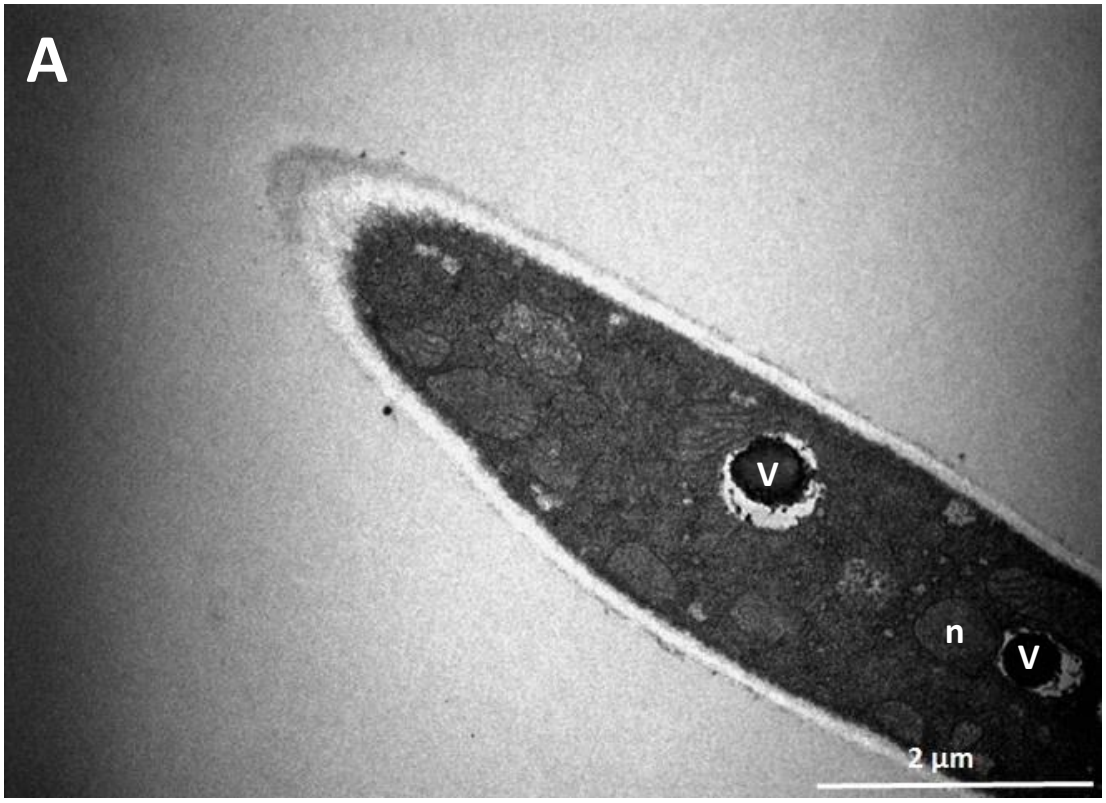
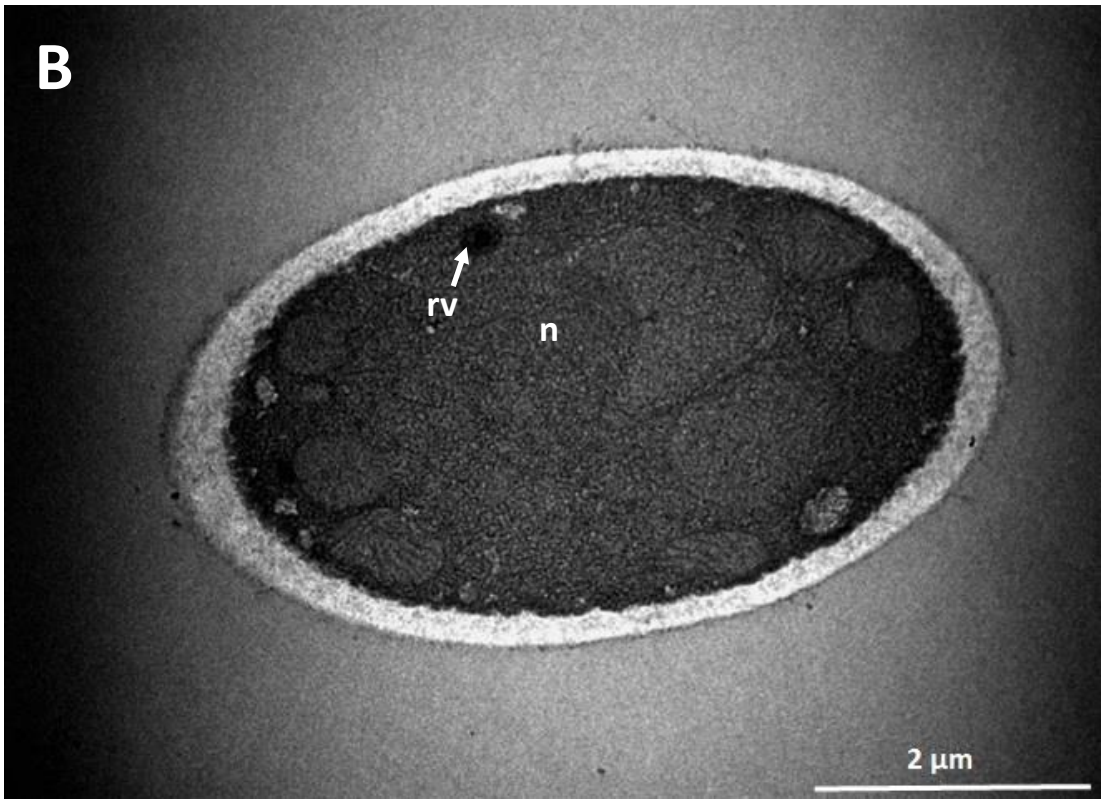


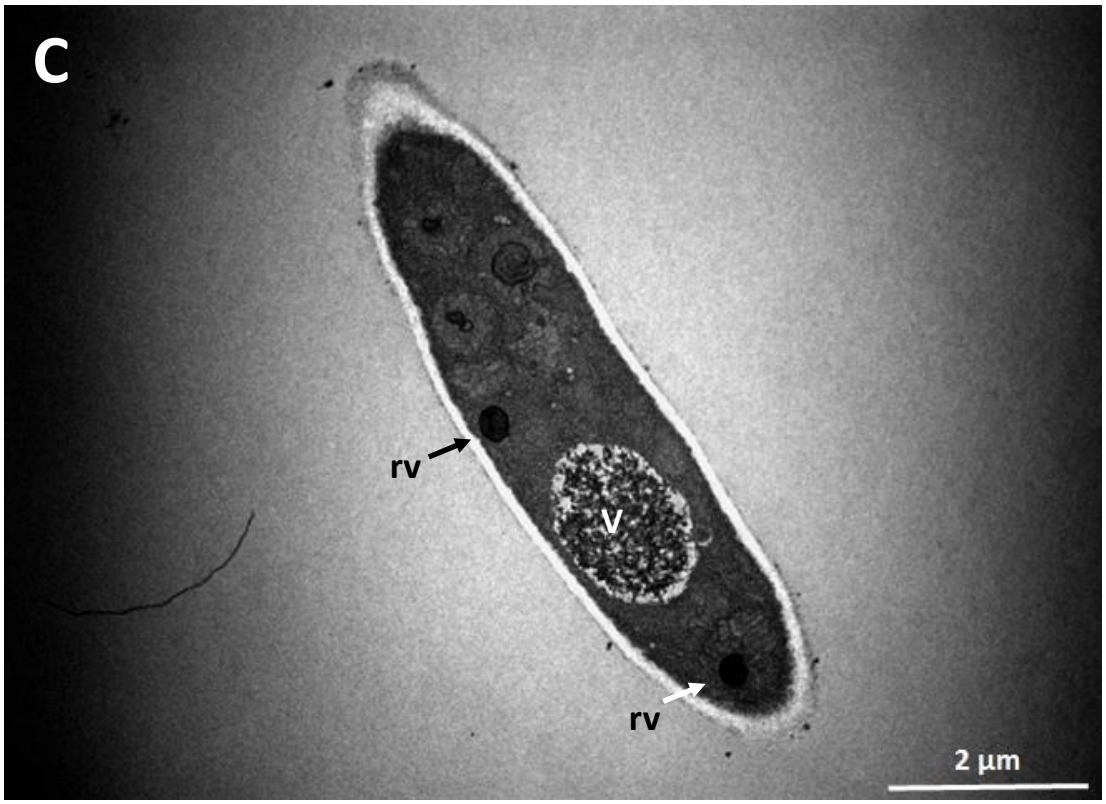
Figure 11: **Impact of caffeine on *T. mentagrophytes* microscopical colonial morphology in liquid cultures.** Microconidia were inoculated in YME supplemented with different caffeine concentrations. "CTR" stands for control cultures were the fungi was grown in the absence of caffeine and the remaining markings define the caffeine added at different concentrations. The images were obtained with a Nikon® Eclipse E400 microscope equipped with a Nikon® Digital Sight DS-L1 digital camera.

3.1.4. Transmission electron microscopy (TEM) analysis

After performing an optical microscopy analysis of *T. mentagrophytes* colonies to assess how caffeine impacts in the morphology of colonies, we decided to perform a TEM analysis of the cultured colonies to understand how the ultrastructure of the fungus is affected by this drug. Figure 12 shows the compiled images acquired using this microscopy technique.




B
rv
n
2 µm


C
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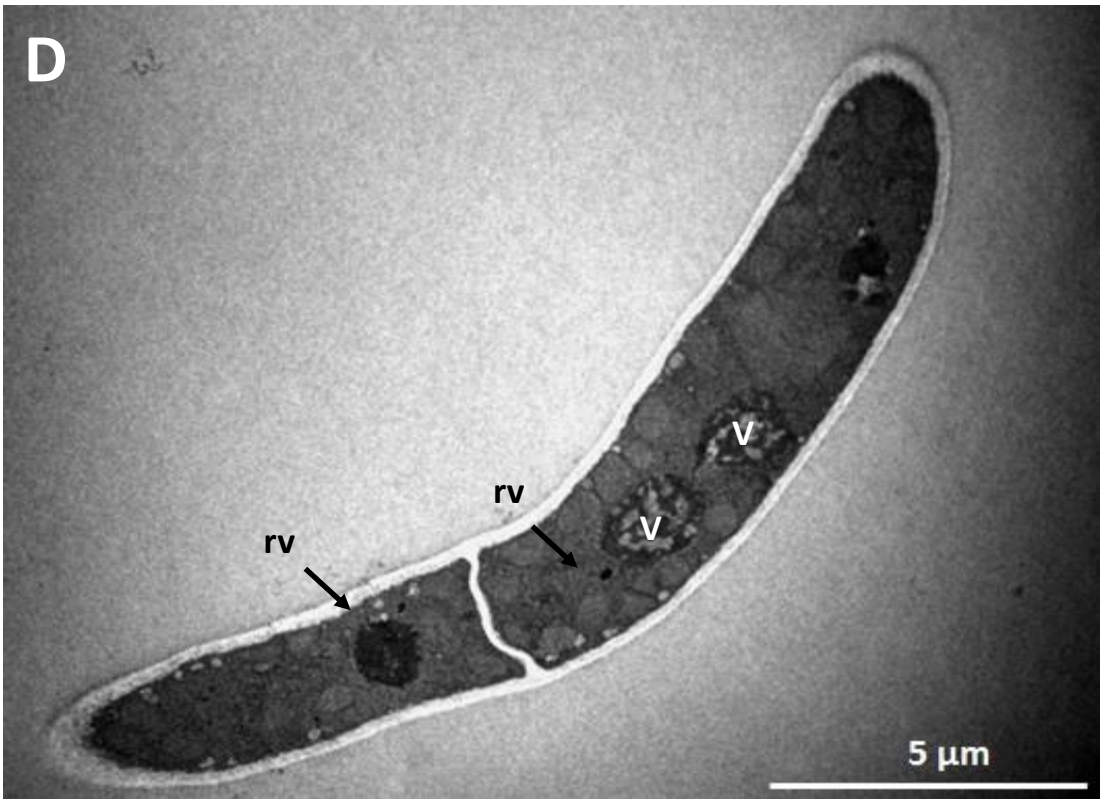
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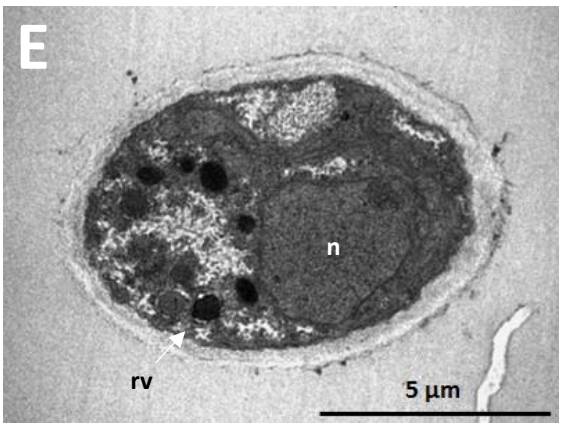
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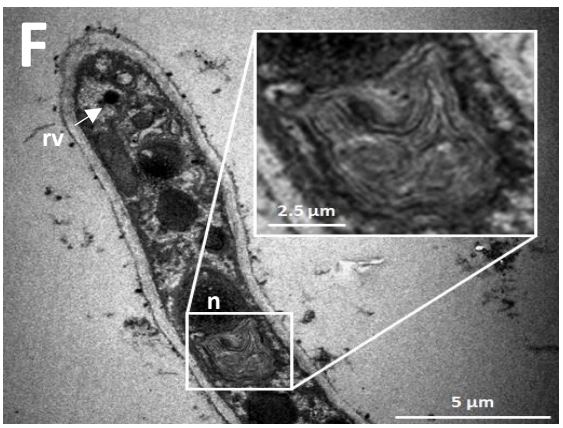
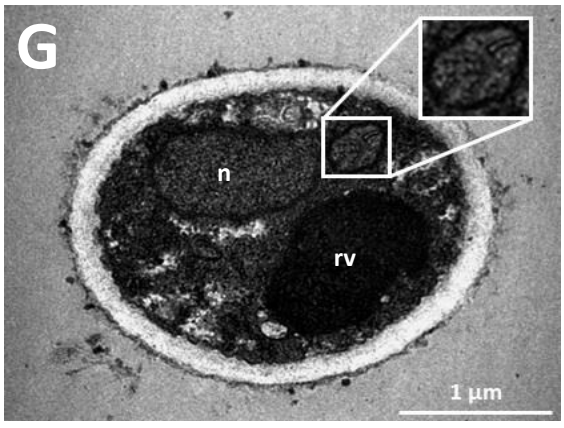
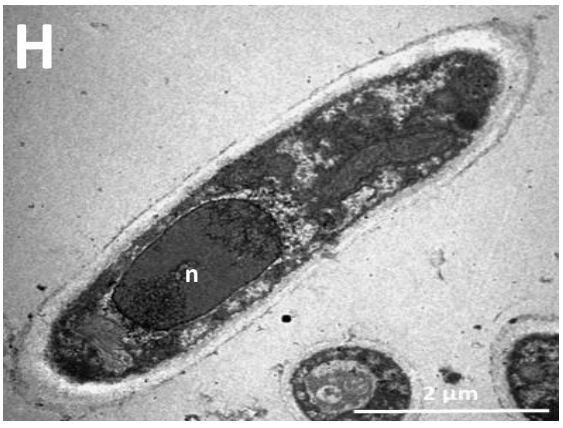
5 µm


F
rv
2.5 µm
n
5 µm


G
n
rv
1 µm


H
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2 µm

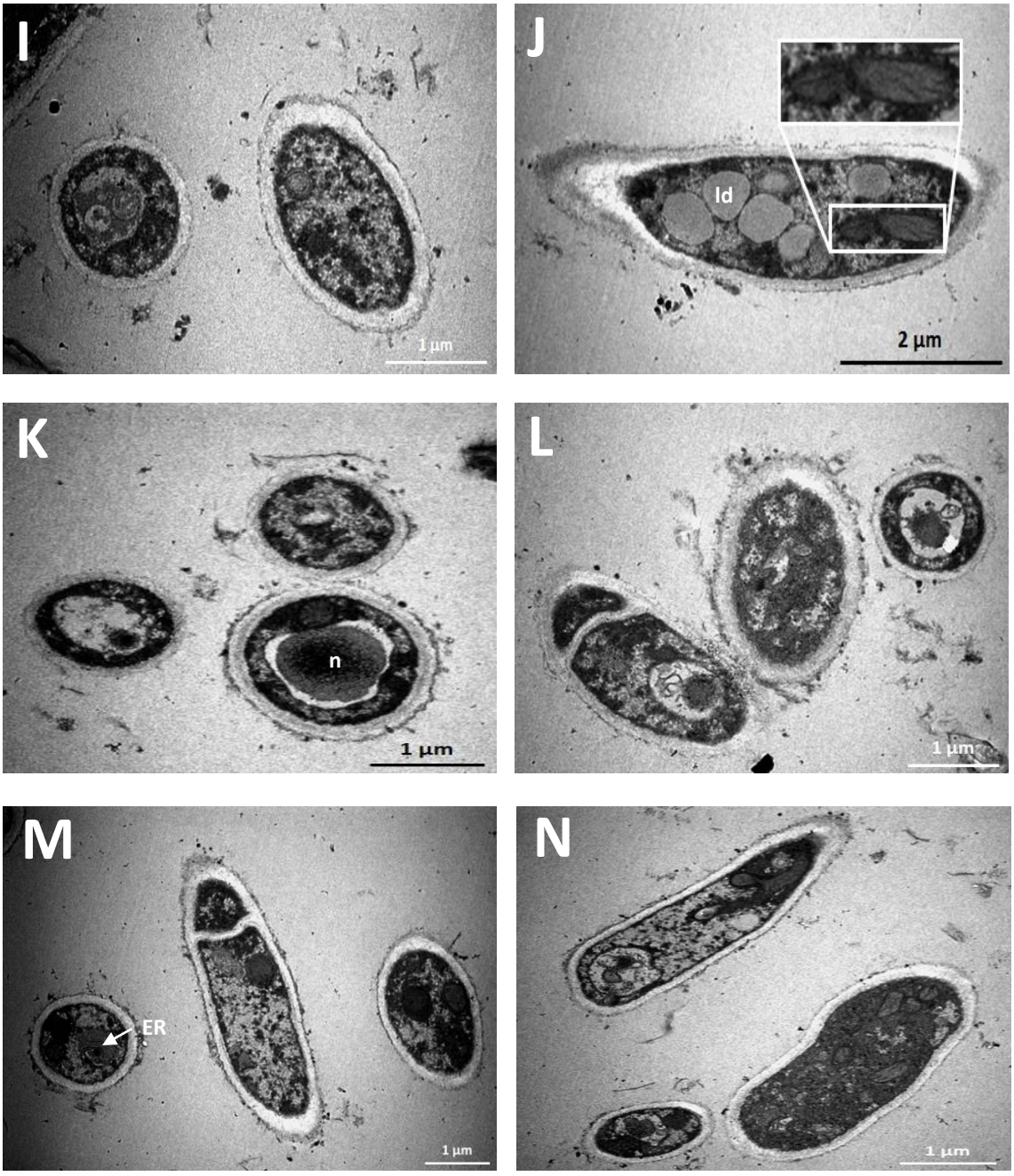

Figure 12: **Impact of caffeine on *T. mentagrophytes* cellular morphology**. Fungal microconidia were inoculated in YME in the presence or absence of 10 mM caffeine. After each set growth period, colonies were collected and processed to be later observed in a transmission electron microscope. Acquired images tagged with letters from A to D represent fungi grown in the absence of the drug (controls), and letters from E to N represent fungi grown in media supplemented with 10 mM caffeine. "V" identifies intracellular vacuoles, "rv" reserve vesicles, "n" nuclei, "ER" endoplasmic reticulum and "ld" lipid droplets.

The morphology of *T. mentagrophytes* grown in control conditions, i.e. in the absence of caffeine, as observed by TEM (Figure 12-A to -D), shows fungal hyphae with

a regular clear cytoplasm and a homogeneous cell wall with a “smooth” surface. One of the most marked features of the ultrastructure of fungal cells is a high number of mitochondria, with normal morphology characterized by well-defined cristae and regular nuclei. In images 12-A, -C and -D, large vacuoles with denser electronegative compounds inside them are visible, probably representing reserve materials required for hyphal growth.

The TEM study showed that *T. mentagrophytes* grown in the presence of 10 mM caffeine (Figure 12-E to -N) exhibits abnormal cellular morphologies and ultrastructure modification when compared with control (Figure 12-A to -D).

Nuclei, unlike those of the control cells (Figure 12-A to -C), show irregular shape and larger size. Apart from that, in Figure 12-F, -G and -K, the intranuclear content is more electronegative, most probably corresponding to condensed chromatin. Nevertheless, in Figure 12-H, the chromatin seems to be migrating to opposite poles of the nucleus, which suggests that the fungal cell is undergoing mitosis and consequently dividing.

The fungal cell wall ultrastructure of fungi grown in 10 mM caffeine also presents changes when compared with control cells, not exposed to caffeine. The cell wall surface of fungi grown in caffeine-supplemented media is “rougher”. To this irregular surface adds a not so homogeneous structure as observed in control cells. This indicates that the drug is affecting the arrangement of the cell wall components. Moreover, the stability of this structure seems compromised since there are materials detaching from it and being leaked to the media (as seen in the central cell in Figure 12-L). Apart from that it looks like septa are also affected by this phenomenon since in Figure 12-L and -M they seem to be have a thicker inner layer, and are not as uniform as they are in control cells.

Another cellular components that seem modified upon incubation with 10 mM caffeine are mitochondria. When compared with cells grown in the absence of caffeine, the fungal cells/hyphae have less mitochondria. Strikingly, the ultrastructure of these organelles show aberrant morphology with abnormal cristae (see zoomed crops in Figure 12-G and -J).

The endoplasmic reticulum (ER) also exhibits a remarkable modification of the normal morphology, as seen in the zoomed crop of Figure 12-F and -M where the ER is expanded and coiled in whorls.

Reserve vesicles seen in fungal cells incubated with caffeine also apparently exist in larger number and size (especially seen in Figure 12-E and G) than in the control condition where the drug was not added. In Figure 12-J there is also seen a huge number of accumulated lipid droplets that are not seen in control. The cytoplasm is also affected by this compound. Unlike control cells, caffeine-treated fungi have a heterogeneous cytoplasm.

Another aspect worth to note is that the extracellular media of fungi incubated with caffeine is unclear, with residual materials not observed in the extracellular milieu of fungi grown in control conditions.

3.2. B – Antifungal properties of caffeine during keratinocyte infections by *T. mentagrophytes* spores

In order to study the antifungal properties of caffeine during a dermatophyte-host interaction, the HaCaT cell line of immortalized keratinocytes was selected. A well-known model of *in vitro* infection was established using cellular monolayers of these cells incubated with *T. mentagrophytes* microconidia. In this part of the work, it was studied the impact of caffeine on keratinocyte viability upon infection with the dermatophyte, assessed using MTT viability assays and its effect on spore germination during this host-pathogen interactions by counting ungerminated conidia.

3.2.1. Viability of keratinocytes upon infection with *T. mentagrophytes* spores

The quantification of keratinocyte viability was obtained using the MTT viability assay, a colorimetric assay that allows the quantification of cells viability according to their metabolic activity. This method is based on cells capacity to metabolize MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), a water soluble tetrazolium salt, and metabolically convert it to formazan salts, that are later dissolved in a set volume of DMSO. Viable cells actively convert MTT into formazan, therefore forming light pink to dark purple solutions after proper dissolution in the organic solvent. Absorbance at 570 nm of the formed solutions is measured, and colour intensity shift is assumed as a surrogate measurement of cellular viability (the more purple the formed solution is, the higher the cellular viability).

For these experiments, three incubation time points were selected: 6 h, 9 h and 12 h. Before testing the impact of different concentrations of caffeine in keratinocytes viability during an infection insult with *T. mentagrophytes* microconidia, control assays were performed first by incubating cells with the selected drug concentrations without the addition of fungal microconidia in an effort to understand how stimulation with caffeine alone impacts HaCaT cells viability throughout the assay. Figure 13 shows a graph with the obtained results for these assay.

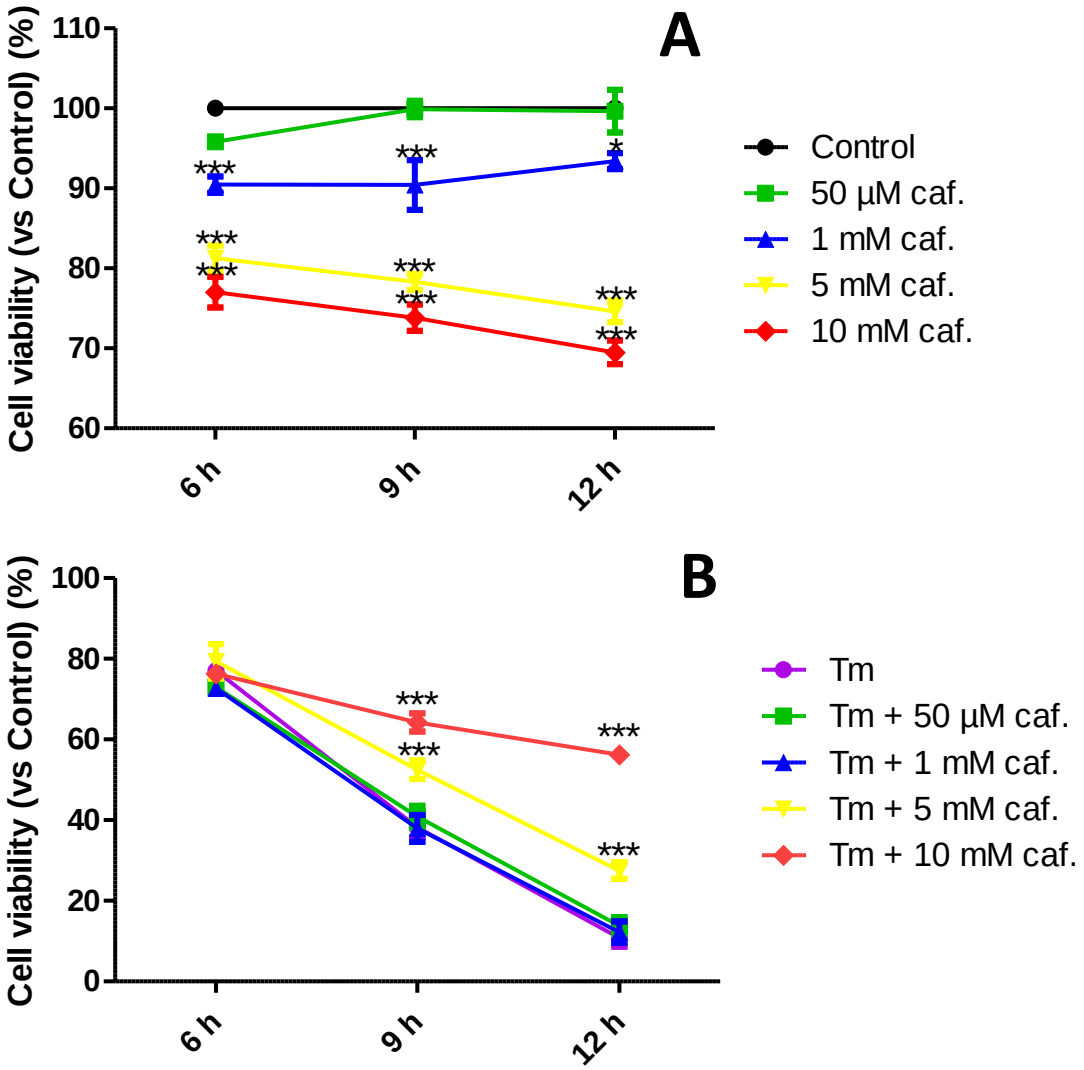

Figure 13: **Modulatory effect of caffeine on HaCaT cells viability and its impact during an *in vitro* infection insult with *T. mentagrophytes* microconidia.** In the upper graph (A), keratinocytes were stimulated with caffeine at different concentrations for 6, 9 and 12 hours, and posteriorly incubated with an MTT solution for 2 h in order for cells to metabolize the compound. "Control" stands for the negative control, were no caffeine was added to cells, "50 µM caf." defines the condition where cells were incubated in the presence of 50 µM caffeine, "1 mM caf." in the presence of 1 mM caffeine, "5 mM caf." with 5 mM caffeine and "10 mM caf." with 10 mM caffeine. In these assays, no fungal microconidia were added to the cells. Results are presented as mean ± SEM (n=3), two-way ANOVA, *p<0.05, ***p<0.001 (vs control). In the bottom graph (B), fungal microconidia were added to keratinocytes in the presence or absence of caffeine at different concentrations for 6, 9 and 12 hours, and posteriorly incubated with an MTT solution for 2 h in order for cells to metabolize the compound. "Tm" defines the condition where cells were incubated with *T. mentagrophytes* without the presence of caffeine, "Tm + 50 µM caf." where cells were incubated with both fungal microconidia and 50 µM caffeine, "Tm + 1 mM caf." With both conidia and 1 mM caffeine, "Tm + 5 mM caf." with conidia and 5 mM caffeine and "Tm + 10 mM caf." with conidia and 10 mM caffeine. Results are presented as mean ± SEM (n=4), two-way ANOVA, ***p<0.001 (vs "Tm").

During the interaction assay period (12 h), there are no differences in keratinocyte viability when cells are incubated in the presence of 50 µM caffeine, however there is a viability decay observed in the conditions were cells were stimulated with 1 mM, 5 mM and 10 mM caffeine (Figure 13-A). Nonetheless, this results show that that caffeine has a negative effect on HaCaT cells viability with a marked viability decrease with increasing concentrations of caffeine (Figure 13-A).

Surprisingly, this effect reverses when caffeine is added during the course of a keratinocyte infection by *T. mentagrophytes* (Figure 13-B). In fact, the cell viability of HaCaT dramatically decreases to 11 % when the cell cultures are infected by fungi during 12 h. However, this decline in cell viability is attenuated by the presence of 5 mM and 10 mM caffeine concentrations to 27% and 56%, respectively, at the end of the 12 h hour infection assay (Figure 13-B).

The comparison of both infection and control assays results show evidence that caffeine has a rescuing effect on cellular viability of keratinocytes during the course of a *T. mentagrophytes* infection.

3.2.2. Ungerminated microconidia assays

For dermatophytes, hyphae formation and polar growth is a determinant requirement for infection and efficient substrate colonization. Apart from that, the virulent characteristics of most pathogenic moulds manifests only after spore germination and subsequent hyphal development. Therefore, these assays were performed in an effort to understand how caffeine impacts in dermatophyte microconidia germination during the course of an *in vitro* keratinocyte infection. The results obtained show that 10 mM caffeine has a delaying effect on *T. mentagrophytes* spore germination (Figure 14).

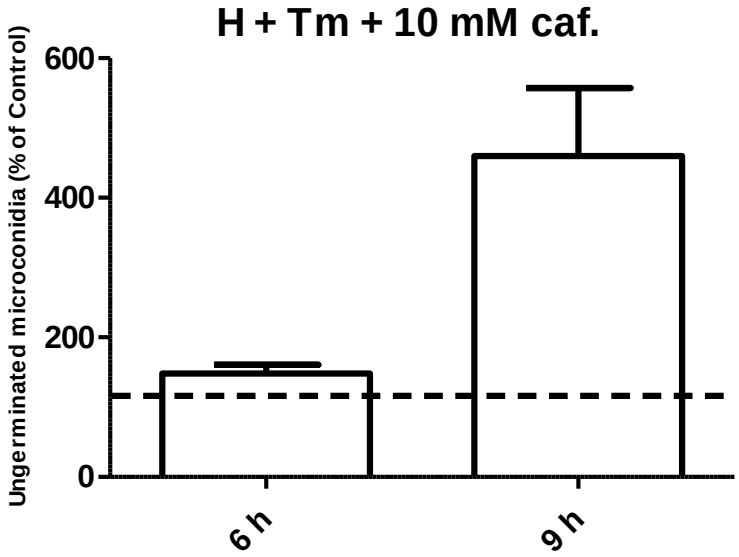

Figure 14: Effect of caffeine on *T. mentagrophytes* microconidia germination during the course of an *in vitro* infection. Keratinocytes were incubated with fungal microconidia in the presence or absence of 10 mM caffeine. After each set incubation period, well contents containing both cells and spores were collected and only ungerminated microconidia were counted. "H + Tm + 10 mM caf." refers to HaCaT cells infected with *T. mentagrophytes* microconidia in the presence of 10 mM caffeine and the defined control condition was the one where cells were infected in the absence of the drug. The percentage of ungerminated microconidia were calculated as a percentage of the ungerminated microconidia counted in the control conditions (no caffeine) normalized to 100%. Shown results are presented as percentage of control as mean ± SEM (n=3).

The presence of caffeine leads to an increased number of ungerminated conidia when compared with the number of ungerminated conidia in control conditions, i. e., when the infection progresses without caffeine. This effect, noted after 6 h of interaction between the two groups of cells, the infection agent and the host cells, is much more marked at 9 h of infection, with around 360 % more ungerminated microconidia than the ones counted in the control conditions at this time period (Figure 14).

Initially, a 12 h infection time point was also set for these assays, however, for technical reasons at this time point it was not possible to accurately count the number of ungerminated spores, so it was decided not to present these results.

3.3. C – Modulation of inflammation during keratinocyte-dermatophyte interactions by caffeine

In order to effectively clear invasive fungal pathogens, the host immune system needs to respond by rapidly activating inflammation. This is a complex process and its fine-tuning is essential not only for the host to eliminate the pathogenic threat but also to minimize the collateral damage it can suffer from this process. Inflammation can be triggering by a myriad of molecular signals that are released and/or synthetized by host cells upon efficient recognition of the pathogen like the production of cytokines and the release of ATP.

3.3.1. Extracellular ATP quantification assays

Cells tend to release considerable amounts of ATP when subjected to some infection stresses caused by pathogenic microorganisms. This released extracellular ATP (eATP) has recently being broadly studied, since it is considered an endogenous danger signal molecule that stimulates inflammation and therefore contributes to the clearance of infectious agents. A preliminary eATP quantification assay was performed to assess how caffeine modulates inflammation during an infection by *T. mentagrophytes* through this eATP release.

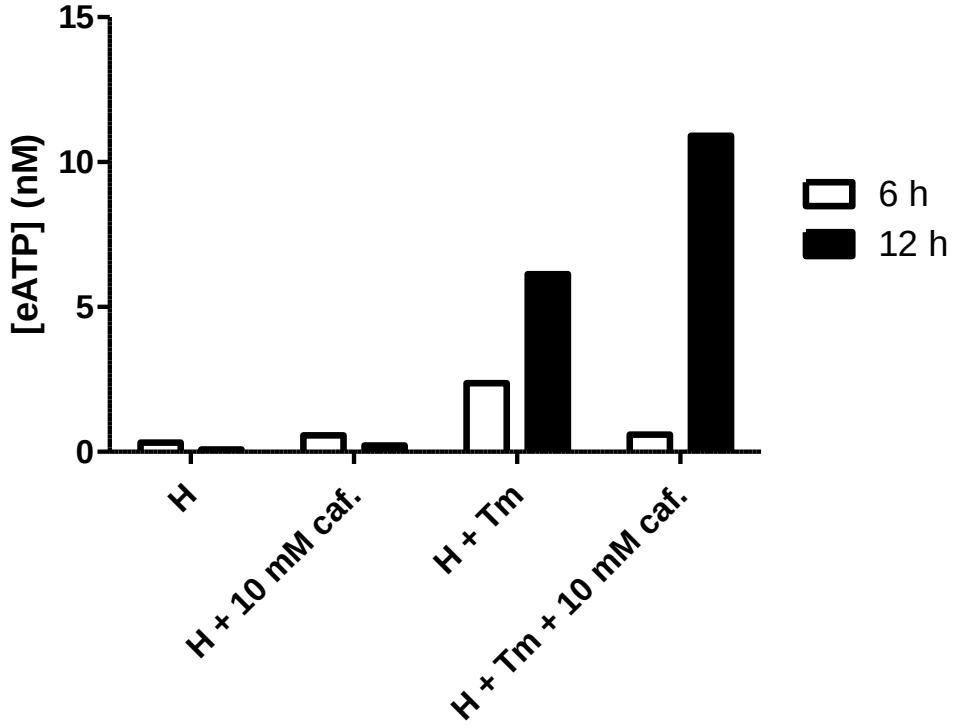

Figure 15: **Effect of caffeine on extracellular ATP levels secreted during an infection by *T. mentagrophytes***. Fungal microconidia were added to keratinocytes in the presence or not of 10 mM caffeine for 6 and 12 hours, and supernatants containing ATP released to the culture media were collected for further quantification as described before. "H" defines the control condition where keratinocytes were not incubated with either *T. mentagrophytes* or caffeine, "H + 10 mM caf." represents the condition where cells were stimulated with 10 mM caffeine alone, "H + Tm" the infection condition where microconidia were added to cells in the absence of caffeine and "H +Tm + 10 mM caf." the condition where keratinocytes where stimulated with both fungal conidia and 10 mM caffeine. (n=1)

Caffeine at a concentration of 10 mM, did not affected ATP release, regardless of exposure period (Figure 15). However, when cells are subjected to an infection insult with dermatophyte microconidia the release of ATP changes. During infection, more ATP is increasingly accumulated in the extracellular milieu than in control or when cells are subjected to the presence of 10 mM caffeine. Nonetheless, when 10 mM caffeine is added during the infection the eATP concentration increases, in comparison with the infection condition where no drug was added.

Chapter 4

Discussion

4. Discussion

Dermatophyte infections (dermatophytoses) are one of the most common infections in humans worldwide. Although these infections are generally not life-threatening to the infected host (Vermout et al. 2008), they can be associated with other potentially severe complications caused by other microorganisms, especially bacteria that take advantage of the fissures formed in the epidermis caused by those fungi and posteriorly constituting a risk factor for the incidence of grave physiopathological conditions that appear in consequence of this secondary subcutaneous infections like cellulitis and fasciitis, hence posing a danger especially to immunocompromised and immunosenescent hosts that are impaired to fight these infections, like diabetic patients and the aged population. (Al Hasan et al. 2004; Roujeau et al. 2004; Bristow & Spruce 2009).

There is broad spectrum of antifungal drugs designed to clear fungal infections. The most common antimycotic drugs prescribed in medical practice belong to the classes of echinocandins, polyenes, alllylamines and azoles (Lewis 2011). Of these, echinocandins are not used in the clearance of dermatophyte infections (Arendrup & Perlin 2014). Several antifungals are used for topical administration in dermatophyte infections including azoles, allylamines, other antifungals with a mechanism of action similar to allylamines (butenafine and tolnaftate), amorolfine also inhibiting ergosterol synthesis, and ciclopirox that binds fungal microtubules thus inhibiting mitosis (Rotta et al. 2012; Hainer 2003). Azoles and allylamines show their antimycotic properties by inhibiting ergosterol synthesis. The most common azoles, itraconazole, ketoconazole and fluconazole, act by inhibiting the synthesis of the enzyme lanosterol demethylase that is crucial in the ergosterol biosynthetic pathway (Ha & White 1999), while allylamines like terbinafine also affect ergosterol biosynthetic pathway by targeting squalene instead, an early precursor of lanosterol that which in turn is involved in the ergosterol synthesis. The lack of ergosterol as stated above makes fungal cellular membranes lose their fluidity, stability and leads to the leakage of intracellular components and ions that compromise the osmotic equilibrium of the fungal cell therefore leading to their death (Lakshmipathy & Kannabiran 2010).

Most of these antimycotic drugs are frequently prescribed in topical lotions to treat body regions infected by dermatophytes depending on the severity of the infection, however this topical therapies have limited effectiveness and sometimes oral administration of this antifungals is required. The major problem regarding oral antifungals is that their intake is frequently associated with toxicity phenomena and can cause other severe complications like inflammatory exacerbations of the infected areas in the body (Al Hasan et al. 2004; Nikkels et al. 2006; Hryncewicz-Gwóźdź et al. 2015).

Caffeine (1,3,7-trimethylxanthine) is a member of the methylxanthine family of drugs and due to its presence in coffee, tea and medicinal products it is probably the most widely consumed psychoactive substance (Fredholm et al. 1999). Historically, caffeine has been the subject of extensive research, and studies have been conducted in various species to determine the impact of caffeine on various biochemical and physiological processes. Regarding the implications of these studies, there is often intense debate and controversy over the general impact of caffeine on human well-being. This controversy has probably been greatest in the field of cardiology (James 2004; Ziegelstein 2004). In addition, evidence is now accumulating to indicate that caffeine and its methylxanthine metabolites can alter the functioning of the immune system, but hitherto caffeine has escaped the scrutiny of a consolidated review dealing with its ability to impact the immune system. Although caffeine as never been described as an efficient antifungal, there are multiple descriptions of caffeine as an inducer of damage to fungal cells. The mechanism of caffeine toxicity in yeast is not well established, but it is clear that the presence of caffeine weakens the cell wall of yeasts, resulting in cell death. Early studies described caffeine as a protein kinase antagonist (Wang et al. 1999), affecting the phosphorylation state of different proteins, some of which involved in the cell cycle progression (Xiang et al. 1994). Caffeine also inhibits cAMP phosphodiesterase, DNA repair, and interferes with MAP kinase pathways (Benko et al. 1997). Several studies showed that the protein kinase-C-dependent signal transduction pathway plays an important role in the resistance to caffeine (Schmitz et al. 2002; Pinto-De-Oliveira et al. 2005). The usage of caffeine has allowed the elucidation of mechanisms of cell wall regulation since its presence activates the Cell Wall Integrity (CWI) pathway (Fuchs & Mylonakis 2009).

This work was designed to explore the antifungal potential of caffeine towards dermatophytes, in particular *T. mentagrophytes*. The results showed that caffeine at concentrations of 5 mM and 10 mM leads to a significant decrease of the fungal radial growth. This same growth inhibition was observed in another important agent of tinea, *Trichophyton rubrum* (Annex 1), leading to the conclusion that this growth inhibition extends to other dermatophytes. The radial growth inhibition results (obtained in solid media) were also confirmed in liquid media with a decreased size and number of the fungal balls mycelia. Moreover, while at concentrations of 1 or 5 mM of caffeine the fungal growth was macroscopically visible after 4 days of growth, only after 25 days of growth in the presence of caffeine 10 mM it was possible to observe fungal growth. Microscopic analysis also showed that caffeine decreases the hypha length and leads to a highly condensed mycelia. These morphological changes were concomitant with modifications in the cell wall composition, with a decrease in β-(1,3)-glucan and an increase in chitin at 5 and 10 mM caffeine. The fluctuation of these main components of the cell wall has been observed in other fungal species when exposed to echinocandins. In fact some fungi also increase chitin levels in response to echinocandins as a salvage mechanism to compensate for decreased Fks activity, responsible for the synthesis of β-glucan (Lee et al. 2012; Walker et al. 2008; Walker et al. 2013). Although most of these observations were done in *Candida albicans*, this was also reported in filamentous fungi such as *Alternaria infectoria* (Fernandes et al. 2014).

The TEM study of the cellular ultrastructure of *T. mentagrophytes* grown in the presence of caffeine 10 mM also showed profound changes. The cell wall surface changes to a rougher aspect, loosening materials to the extracellular millieu. This ultrastructure modification might be due to changes in the cell wall components as observed with other fungi treated with glucan synthesis inhibitors, chitin synthase inhibitors and melanin synthesis inhibitors (Fernandes et al. 2016). Caffeine-grown fungi have an aberrant and clearly disorganized cytoplasm. This characteristic disorganization was already reported for *C. albicans* upon exposure to capric acid and it was suspected to be due to changes in hydrostatic turgor pressure within the cell (Bergsson et al. 2001), since the same effect was also verified for caffeine, it might suggest that the disorganization observed is also due to the ability of the compound to somehow

modulate this parameter. Some nuclei of caffeine-treated fungi also have irregular shape and size with most of these displaying more electronegative intranuclear content. Similar nuclear morphology was also verified in another dermatophyte, *Microsporum gypseum* upon exposure to α-bisabolol, a compound found in the essential oils of several plants, and the authors speculated that this effect might be due to the compound affecting microtubules, which are responsible for the maintenance of the nuclear shape. Authors also verified anomalous septa in *M. gypseum* ultrastructure, which might also happen due to microtubules disruption since they are also associated with an irregular build-up of wall materials to apical regions of the cell wall (Romagnoli et al. 2015). In this work similar morphologies were observed with caffeine for both nuclei and septa, it is hypothesised that the drug can alter the normal microtubules morphology. It was also found that mitochondria are scarce in caffeine-grown fungi when compared with the control, with irregular morphology and abnormal cristae. Little is known about caffeine's effect in mitochondria, however it was already shown that this compound has the ability to promote mitochondria biogenesis in human cancer cells (Vaughan et al. 2012) which apparently is not verified for *T. mentagrophytes* since there are much less mitochondria observed in caffeine-grown cultures. Mitochondria are linked to antifungal tolerance in multiple ways. Its dysfunction is controversially associated to both susceptibility and resistance to plasma-membrane targeting drugs like azoles and polyenes, however reports show that mitochondria dysfunction leads to susceptibility to echinocandins like caspofungin in the yeast model like *S. cerevisiae* (Shingu-Vazquez & Traven 2011).

The results showed that fungi grown in the presence of 10 mM caffeine have irregular endoplasmic reticula (ER). These abnormal structures appear rolled in whorls, which are coiled ER sheets that usually form ring-shaped structures. These abnormal structures were reported before as being indicators of ER stress, caused by the accumulation of misfolded proteins in the ER lumen, as described when *S. cerevisiae* is incubated with DTT, a compound known to induce protein misfolding, massively expanding ER and that lead to their degradation by autophagy processes (Schuck et al. 2014). Vacuoles play an important role in homeostasis such as the maintenance of a balanced chemical composition of the cytoplasm in the face of fluctuating external

conditions, being one of these mechanisms the drawing of water into the cell to compensate for an unbalanced turgor pressure (Weber 2002). Since these structures appear in higher number and some of them are abnormally large, it might be a mechanism deployed by *T. mentagrophytes* to compensate for the changes in the osmotic equilibrium due to a more fragile cell wall, deprived of β-(1,3)-glucan. Lipid droplets were also verified inside caffeine-grown *T. mentagrophytes* hyphae. These structures act mainly as intracellular storage and even though they were early assumed to be inert fat particles used to store carbon and energy, recently it was also discovered that they play a role in multiple cellular functions. One of these functions recently reported was regarding toxin trapping and consequent cytoplasm detoxification in the yeast models *C. albicans* and *S. cerevisiae*. Authors also reported that by mutating these two species therefore making them lose their ability to form this droplets made them more susceptible to antifungal drugs like amphotericin B and miconazole (Chang et al. 2015).

Another aspect explored in this work was the impact of caffeine during the interaction of *T. mentagrophytes* spores and keratinocytes. Keratinocytes constitute the majority of epithelial cells in human skin (Pivarcsi et al. 2005). These cells are responsible for the formation of a natural barrier against physical, chemical, and microbial aggressions. Keratinocytes detect and respond to these stimuli producing immune-inflammatory mediators that in turn evoke a more specific response by cells of the immune system, some of which are recruited to the site where the aggression was detected (Brasch 2009). They also play an important role in wound healing (Shaw & Martin 2016). Here it was explored whether caffeine could interfere with the damage of keratinocytes during the infection of *T. mentagrophytes*. It was observed that the infection of a HaCaT cell culture, a lineage of human keratinocytes, with spores of *T. mentagrophytes* during 12 h leads to a decrease in cell viability to 11% of the control cells, not infected. The introduction of 10 mM caffeine during this 12 h-interaction period preserves the viability of keratinocytes. Surprisingly, caffeine alone leads to a decrease in keratinocyte viability.

It was already described that 5 mM caffeine reduces HaCaT cells viability and therefore promotes apoptosis upon exposure to Ultraviolet B radiation (Han et al. 2011)

which goes in accord with what was observed for MTT control assays. Apart from that, it was also reported that the drug at this concentration prevents human epidermal keratinocytes (HEK cell line) proliferation and migration, therefore suggesting it may have an inhibitory effect in wound closure and epithelisation during *in vitro* wound assays. Authors hypothesized that this delay caused by caffeine in cell migration and epithelisation might likely due to cytoskeleton changes caused by the drug, however this mechanism remains to be elucidated (Ojeh et al. 2014).

Nevertheless, during the course of an infection by *T. mentagrophytes* the presence of caffeine protects the keratinocytes from death. This effect was not observed for another agent of skin infection, *Malassezia furfur* (Annex 2). Moreover under these assay conditions, i. e. infection of keratinocytes with a dermatophyte in the presence of caffeine, there is a decay in the germination of fungal spores. Undoubtfully, these two aspects together indicate an amelioration of the infection process, since the ability of a fungal infection such as tinea to proceed and success depends on the germination of spores. On the other hand, the increase in eATP, also observed, will account for a more efficient immune response (Trautmann 2009).

Chapter 5

Conclusions & Final Remarks

5. Conclusions & Final Remarks

The main goal of this work was to unravel caffeine's anti-dermatomycotic properties, and to discover how the presence of this drug impacts the progression of infections caused by dermatophytes. To achieve this goal, this work was focused on *T. mentagrophytes*, one of the most common agents of these diseases and throughout this work, the impact of the compound on fungus growth and morphology was explored as well as its effect on keratinocytes (target skin cells of these diseases) and the pathogenic fungus.

The most important conclusions that were revealed throughout this thesis are that caffeine, especially in the concentration of 10 mM, a fifteen times lower concentration than the one found in most commercially available topical solutions (Herman and Herman, 2012), inhibits *T. mentagrophytes* growth, and alters not only its colonial and cellular morphology, but also the normal levels of both β-(1,3)-glucan and chitin, two important structural components of the fungal cell wall required for the robustness of the fungus, for an efficient recognition of this pathogenic microorganism by target host cells and for the triggering of an efficient immune response to promote its clearance during an infection. Besides this, it was also proven that caffeine has a rescuing effect on keratinocytes viability during an *in vitro* infection insult and that its presence delays microconidia germination during this environments that is required for an efficient adherence, colonization and infection of the skin by *T. mentagrophytes*.

A preliminary study showed that when caffeine is added during infection, the extracellular ATP concentration increases, indicating that it promotes a more efficient inflammation, even though these assays need further studies.

Even though the obtained results are relevant and show some of caffeine's potential as a therapeutic tool to treat this diseases, there are however other topics that need to be explored and other questions to be elucidated regarding this theme:

- The impact on this compound was only mainly tested in one of the many agents of dermatomycosis, but are this effects also verified for not only other

dermatophyte species, but also for other filamentous fungi and yeasts that can cause this skin diseases?

- Regarding the caffeine concentrations tested, it is a clear that the drug has a fungistatic effect on *T. mentagrophytes*, but if higher concentrations are tested, will the compound show antifungal effects, effectively killing the fungus and not only delaying its proliferation?
- β-(1,3)-glucan and chitin contents on the fungal cell wall are modulated by this drug, but what about other structural components and what are the molecular mechanisms that lead to this modulation?
- How and why do the alterations of the fungus cellular ultrastructure by caffeine influences its normal physiological processes?
- Why do high concentrations of caffeine like 10 mM tendentiously lower keratinocyte cellular viability upon stimulation with the drug alone, but rescues it when used in infection environments with *T. mentagrophytes*? Why is this phenomenon not verified for *M. furfur*?
- What are the molecular processes that are altered by caffeine that lead to a delay on microconidia germination?
- Does caffeine alters the secretion of other inflammatory mediators like cytokines or chemokines? What about antimicrobial peptides (Nestle et al. 2009)?
- The effect of this compound during infection was only tested *in vitro* using cellular monolayers of keratinocytes, but is this effect also verified when using tissue cultures? And what about in *in vivo* models?

Chapter 6

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6. References

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Annexes

Annex 1

Effect of caffeine in *Trichophyton rubrum* radial growth

Aim: To understand if caffeine inhibits the radial growth of another dermatophyte, *Trichophyton rubrum* as found with *Trichophyton mentagrophytes*.

Materials & Methods

Fungal strains

Trichophyton rubrum was kindly provided by Professor Carmen Lisboa of the Laboratory of Microbiology, Faculty of Medicine, University of Porto. Its identification was confirmed using the ITS-5.8 S fragment of the 18S rDNA (Paulo et al. 2009).

Fungi maintenance and culture conditions

T. rubrum was cultured and maintained in an incubator at 30°C in Potato Dextrose Agar medium (PDA) (0.4% potato starch (w/v), 2% dextrose (w/v), 1.5% agar (w/v)) (BD Biosciences®, San Jose, California, USA) sterilized by autoclaving at 121°C for 20 min under a pressure of 1.2 atm. To obtain an optimal spore yield, fungi were cultured in small petri dishes (with approximately 10 mL of culture medium per dish) for a period of 21 days minimum and 42 days maximum prior to microconidia harvest for further use in *in vitro* assays.

Microconidia harvest

To harvest *T. rubrum* spores and obtain purified microconidia suspensions for further use in all experimental assays, two to four petri dishes of PDA inoculated with the dermatophyte were removed from the incubator and fungal aerial mycelia from all plates were rinsed twice with the same volume of 2 mL 0.1% Tween 80 solution (v/v) per dish (Sigma-Aldrich®, St. Louis, Missouri, USA) to help detach conidia from hypha. After proper washing, all the volume of 0.1% Tween 80 solution containing the harvested conidia were collected from the rinsed dishes and filtered through sterile handmade sacred linen filtration systems to remove media remains and hyphae fragments. The filtered suspension was then centrifuged twice at 16.060 g for 10 min at

4°C, supernatants were discarded, pellets were ressuspended first in 1 mL cold Phosphate Buffered Saline (PBS) (10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl (w/v), pH 7.3) and after the final centrifugation, pellet resuspension was done in 1 mL cold PBS for posterior use in radial growth inhibition assays.

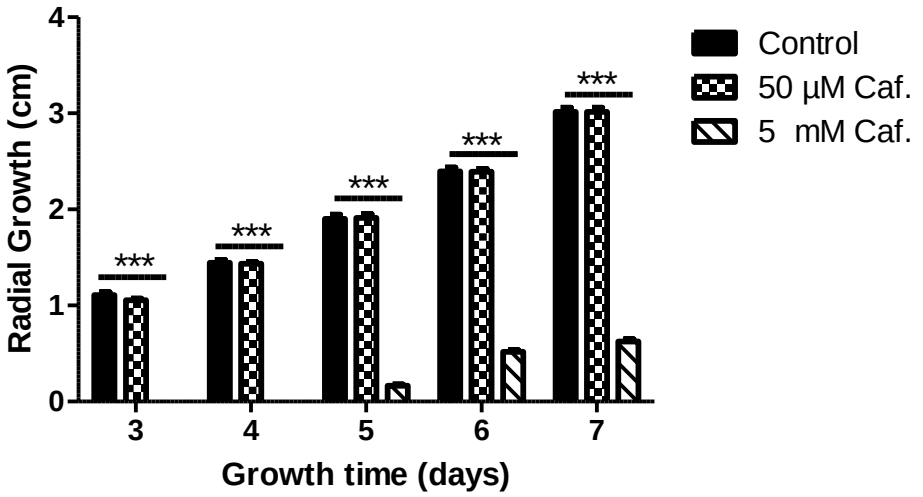
Spore concentrations of the purified suspensions were estimated in microconidia/mL using a haemocytometer.

Radial growth inhibition assays

Radial growth inhibition assays were performed by inoculating 1.25 x 10^4 *T. rubrum* microconidia in regular petri dishes with 20 mL of PDA supplemented with different caffeine concentrations (50 µM, 1 mM, 5 mM and 12 mM). Spores were aseptically inoculated in the centre of the dishes, and fungal radial growth was monitored from the third until the seventh day of incubation at 30°C by measuring the aerial mycelium length in four different axis centred on the inoculation site in each dish. Mycelia daily measurements were performed roughly at the same time in each day of the assay, using the same regular office ruler.

Results

As stated before in this work, the radial growth inhibition assays were performed to understand if caffeine impacts on dermatophyte fungal growth, in this case *T. rubrum*. Supplementary figure 1 shows the obtained results for these assays.



Supplementary figure 1: Impact of caffeine on *T. rubrum* radial growth. Microconidia were inoculated in PDA plates in the presence of caffeine, and mycelia growth was monitored for 5 days. "Control" stands for the negative control, were the fungus was grown in plates containing no caffeine, "50 µM caf." represents plates inoculated with fungal microconidia in the presence of 50 µM caffeine and "5 mM caf." with 5 mM caffeine supplementation. Results were plotted as mean ± SEM from quadriplicates (n=3), repeated measures ANOVA, ***p<0.001 (Control vs 5 mM caf)

Whilst 50 µM caffeine does not seem to have a direct impact on mycelial growth since no significant differences were detected between control, it was strongly inhibited in the presence of 5 mM caffeine by comparison with data from control condition where no caffeine was added. 12 mM caffeine was also tested, and no mycelial growth could be detected in the days the assays were conducted using the same measurement methodology. Only at the eleventh day after inoculation, a minimal mycelial growth was detected.

Annex 2

Impact of caffeine on the viability of keratinocytes during the course of a *Malassezia furfur* infection

Aim: To understand if caffeine impacts in keratinocytes viability during the course of a *Malassezia furfur* infection, a pathogenic yeast agent of human dermatomycosis such as pityriasis versicolor, seborrheic dermatitis and folliculitis.

Materials & Methods

Fungal strains

Malassezia furfur was obtained from Professor Carmen Lisboa of the Laboratory of Microbiology, Faculty of Medicine, University of Porto.

Fungi maintenance and culture conditions

M. furfur was maintained in an incubator at 30°C, cultured in Sabouraud Agar medium (1% peptone (w/v), 4% glucose (w/v), 2% agar (w/v)) supplemented with 2% biologic olive oil (v/v) and 0.2% Tween 80 (v/v) sterilized through autoclaving at 121°C for 20 min under a pressure of 1.2 atm. Yeasts were cultured in regular size petri dishes (approximately 20 mL of culture medium per dish) for four days to be used in MTT assays.

Infection assays

Cell line maintenance and culture conditions

The HaCaT cell line of immortalized human keratinocytes was selected for this study. Cells were obtained from the German Cancer Research Centre (Heidelberg, Germany), seeded in 75 cm^2 cell culture flasks and maintained in Dulbecco's Modified Eagle Medium (DMEM D5645) (Sigma-Aldrich®, St. Louis, Missouri, USA) supplemented with 10% non-inactivated Fetal Bovine Serum (FBS), 10 mM HEPES, 12 mM sodium bicarbonate and 2 mM L-Glutamine at 37°C in a humidified atmosphere with 5% CO2.

Cell cultures were maintained from 3 to 4 days until keratinocytes reach a state of approximately 70% confluence. Upon reaching said confluence state, media contained in the culture flasks was rejected, cells were washed twice with PBS at 37°C to remove media residues and then detached from the bottom of the dish by incubating with 2 mL of trypsin-EDTA solution (Sigma-Aldrich®, St. Louis, Missouri, USA) for 10 min at 37°C with 5% CO_2. After incubation, flasks were lightly tapped to help cell dislodging and trypsin activity was neutralized by adding 8 mL of fresh DMEM (37°C) to each flask. Cell suspensions were properly homogenized, and to start cultures anew suspended keratinocytes were split 1:5 (2 mL of suspension) and seeded in new flasks with fresh media. All infection assays described in this work were performed using HaCaT cells from passages #35 to #55.

In vitro infection assays using HaCaT cells and *M. furfur* yeasts

For *in vitro* infection assays, HaCaT cells were grown and detached as previously described. After cell detaching and new cultures establishment, the remaining volume of cells suspended in medium-neutralized trypsin was collected to a single sterile conical centrifuge tube and posteriorly centrifuged for 4 min, 4°C at 500 g. Supernatant was discarded after centrifugation, the pellet was ressuspended and properly homogenized in DMEM and an aliquot (10 µL) of the suspension was collected to count cells and estimate keratinocyte concentration using a haemocytometer. Following cell counting, 10^5 keratinocytes were seeded in 12-well assay and incubated overnight in a cell incubator at 37°C in a humidified atmosphere with 5% CO_2 for 18 h to allow keratinocytes to settle, adhere to the bottom of the plate, and to duplicate. After the incubation period, it was expected that keratinocyte doubling was achieved, hence upon addition of conidia, it was assumed to have a density of 2 x 10^5 keratinocytes per seeded well. Plates were then incubated again at 37°C in a humidified atmosphere of 5% CO2 for 6 h to 24 h

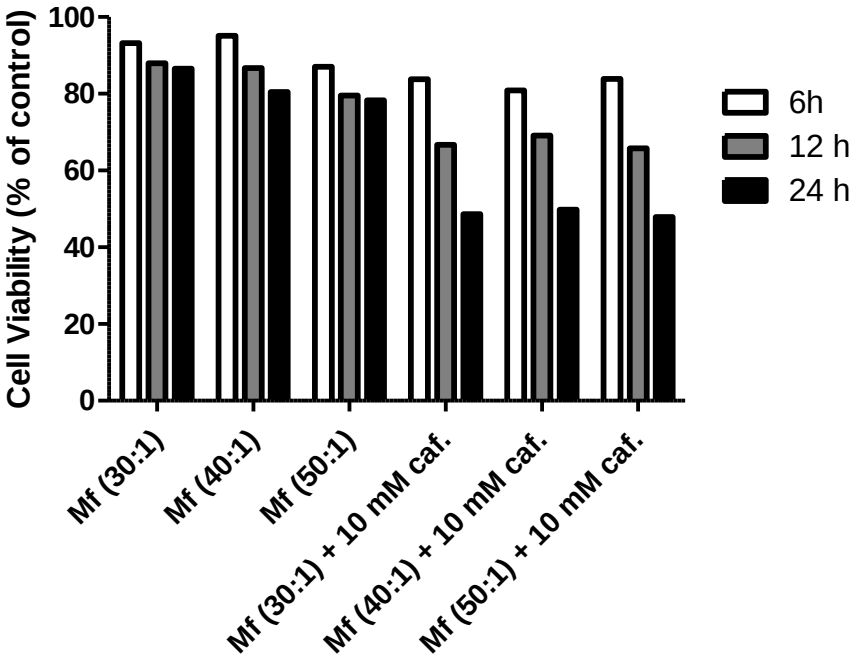
MTT assays

To perform MTT assays, 10^5 HaCaT cells were plated in 12-well assay plates as described before. *M. furfur* was added to cells in three different MOIs: 30:1, 40:1 and 50:1 with or without 10 mM caffeine, 18 h posterior to cell seeding and after culture

media renewal. Plates were then incubated at 37°C in a cell incubator with 5% CO_2 for 6, 12 and 24 h and after each incubation period has ended, media from each well was rejected and 900 µL of new media was added per well along with 100 µL of a 5 mg/mL MTT solution (Sigma-Aldrich®, St. Louis, Missouri, USA) in PBS (w/v) in order to achieve a final MTT concentration of 500 µg/mL per well. Plates were incubated again for 2 h to allow keratinocytes to metabolize the compound. Prior to this incubation period, media was discarded, 500 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich®, St. Louis, Missouri, USA) was added to each well, plates were wrapped in tin foil to avoid photo degradation and were spun at 120 rpm for 5 min in an orbital shaker to dissolve formazan crystals. Contents in each well were transferred to 96-well microtiter plates in 100 µL triplicates and absorbance at 570 nm was measured in a SpectraMax® PLUS 384 spectrophotometer.

Results

The quantification of keratinocyte viability was obtained as described before using the MTT viability assay, a colorimetric assay that allows the quantification of cells viability according to their metabolic activity. Supplementary Figure 2 shows the obtained results for this assay.



Supplementary figure 2: Impact of caffeine on HaCaT cells viability during an *in vitro* infection insult with *M. furfur*. Keratinocytes were stimulated with caffeine at different concentrations for 6 to 24 hours, and posteriorly incubated with an MTT solution for 2 h in order for cells to metabolize the compound. "Mf (30:1)" defines the condition where cell were incubated with M. fufur yeast in a MOI of 30:1, "Mf (40:1)" where cell were incubated with yeasts in a MOI of 40:1, "Mf (50:1)" represents cells incubated with yeasts in a MOI of 50:1, in the following conditions cells were incubated with yeast in a similar MOI but in the presence of 10 mM caffeine. (n=1)

For this experiments, three incubation time points were selected: 6 h, 12 h and 24 h. By analysing the graph it is possible to infer that there is a declining tendency on cell viability over time in all conditions set for this assay. However, in the conditions where 10 mM caffeine was added, there is a much more marked viability loss than those where no drug was added. This effect happens in the same way regardless of the tested MOI, meaning that tested yeast densities are apparently not directly involved in the loss of cellular viability.