AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF MICAFUNGIN AND ANTI-BACTERIAL AGENTS ON *PSEUDOMONAS AERUGINOSA* BIOFILM FORMATION IN BALB/C MICE

by
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AN ABSTRACT OF THE THESIS OF

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Title: The Effect of Micafungin and Anti-Bacterial Agents on *Pseudomonas aeruginosa* Biofilm Formation in BALB/c Mice

Background: *Pseudomonas aeruginosa* is notorious for its biofilm forming capacity, which reduces the accessibility of antibacterial agents and renders the host defenses ineffective in clearing such infections. 1,3-β-D-glucan is a key component in the fungal cell wall and extracellular matrix (ECM) of *Candida albicans* biofilms. 1,3-β-D-glucan is discovered to be present as a periplasmic glucan and within the Extra-Cellular Matrix (ECM) of the *P. aeruginosa* biofilm. Micafungin, an anti-fungal drug, is known to inhibit the synthesis of β-D-glucans. Previous *in-vitro* experiments assessed the inhibitory effect of micafungin on biofilm formation and survival rates in BALB/c mice. This project aims at evaluating the effect of micafungin, singly or in combination with levofloxacin or ceftazidime on *P. aeruginosa* biofilm formation, by 1) determining the transcription levels of biofilm forming encoding genes (*pelC, algC,and ndvB*) in treated and untreated BALB/c mice, 2) measuring the thickness of biofilms in treated and untreated samples from BALB/c mice by confocal-scanning-laser-microscopy (CSLM),.

Methods: The effect of micafungin along with levofloxacin and ceftazidime on *P. aeruginosa* was assessed *in-vitro* on biofilms grown on microtiter plates and spectrophotometry. The relative gene transcription levels of *P. aeruginosa* biofilm-encoding *pelC*, *algC*, and *ndvB* genes, for pellicles, alginate and cell wall 1,3- β -D-glucan, respectively, were performed on RNA extracted samples from *in-vivo* experiments, in the presence of micafungin and/or levofloxacin or ceftazidime, by Quantitative Reverse Transcription PCR (RT-qPCR) experiments. Visualization and thickness calculation by Z-stacking of micafungin treated and untreated *P. aeruginosa* biofilms obtained from *in-vitro* and *in-vivo* samples, as determined by CSLM after staining with ethidium bromide and calcofluor-white.

Results: *In-vitro* results of treated-biofilms grown on microtiter plates showed phenotypic inhibition of biofilm formation by micafungin in combination with levofloxacin. Samples from micafungin-treated mice have shown a decrease in transcription levels in *pelC*, *ndvB*, and *algC* genes with values of 260, 74, and 2-fold decrease respectively. This indicates that micafungin inhibited the biofilm-encoding-genes. Physical reduction in biofilms was further confirmed with Z-stacking using CSLM that revealed a 16.8 % reduction in the

thickness of biofilms after treatment with micafungin *in-vitro*, and a 64% reduction in the thickness of biofilms post treatment with micafungin in lung tissue. No reduction in biofilm thickness was observed in samples treated with levofloxacin and ceftazidime.

Conclusion: Our data have shown that micafungin appeared to inhibit biofilm formation as reflected by a reduction in transcription levels of biofilm encoding genes as well as a decrease in biofilm thickness as demonstrated by confocal microscopy. This translates what could happen in the course of an acute infection with *P. aeruginosa*, whereby the administration of micafungin would inhibit subsequent biofilm formation and enhance entry and bactericidal efficacy of levofloxacin.

Keywords: *Pseudomonas aeruginosa*, micafungin, biofilm, ceftazidime, levofloxacin

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To the memory of my beloved brother, Shadi

CHAPTER I
INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, opportunistic pathogen capable of inhabiting in and adapting to many environments. In addition to its innate resistance to a wide variety of drugs, *P.aeruginosa* is capable of producing a complex, organized and highly structured biofilm that yields to unsuccessful treatment with antibacterial agents. *P.aeruginosa* is a common bacterium that is ubiquitously present in the environment (water, plant, soil, hospitals, sewage, animals and humans). It can cause severe infections in debilitated, immunosuppressed, and immunocompetent individuals. This organism is a major problem in burn wounds, cystic fibrosis, diabetic patients, chronic instructive pulmonary disorders, transplants, patients on urinary catheters and ventilators, intravenous drug users, and surface growth on medical devices as well as within hospital surface and water supplies.

P. aeruginosa can occur in the environment either as free-planktonic cells or as sessile-cells attached to a surface and forming a biofilm. The latter is a group of slow-growing microbial cells that are embedded in a self-produced extracellular matrix. The biofilm is composed of exopolysaccharides (Pel, Psl, and alginate) penetrated by minuscule water channels, extracellular DNA (eDNA) and proteins. *P. aeruginosa* can form biofilms outside the body on indwelling medical devices, thus presenting a major problem for patients on catheters and ventilators, and it can grow biofilms within the human body, at the site of burn wounds or in the lungs of patients with cystic fibrosis.

Similar to *Pseudomonas aeruginosa*, some fungal cells such as *Candida albicans* have the ability to grow biofilms. 1,3-β-D-glucan is a major component of the cell wall of *C. albicans* and is thought to be a major component of the biofilm-matrix produced by this organism. 1,3 glucans are synthesized by a plasma membrane-bound glucan synthase complex. Micafungin belongs to the echinocandin class of antifungal agents that acts by inhibition of the synthesis of 1,3-β-D-glucan.

P. aeruginosa produces 1,3-β-D-glucan similar to that of *C. albicans*. As a result, Micafungin acts as a potential agent in inhibiting the synthesis of 1,3-β-D-glucan in *P. aeruginosa* by inhibiting the 1,3-β-D-glucan synthase enzyme.

Levofloxacin is a fluoroquinolone antibiotic. Its mode of action is by inhibition of bacterial DNA gyrase, a type II topoisomerase. Fluoroquinolones have bactericidal activities toward non-growing *P. aeruginosa* cells and are able to eradicate biofilms formed *in vitro*. Ceftazidime is a cephalosporin antibiotic. The mechanism of action is through interfering with bacterial cell wall synthesis. Studies have shown that ceftazidime causes cell lysis of *E.coli, S. aureus and P. aeruginosa* due to its activity against penicillin-binding proteins (PBP).

Preliminary work at the Department of Experimental Pathology, Immunology and Microbiology revealed *in-vitro* the inhibitory effect of micafungin on *P.aeruginosa* biofilm formation.

This project aims at assessing *in-vitro* and *in-vivo* the effect of micafungin independently or in dual therapy with either levofloxacin or ceftazidime on the biofilm-forming isolate of *P. aeruginosa* (PAN14), by a) determining the level of biofilm synthesis using relative gene transcription levels of biofilm-encoding genes for pellicles and alginate (*pelC* and *algC*)

and cell wall 1,3- β -D-glucan encoding gene (*ndvB*) and b) determining the thickness of biofilm *in-vitro* and *in-vivo* samples, by confocal-scanning-laser-microscopy (CSLM), in micafungin, levofloxacin and ceftazidime treated and untreated *P. aeruginosa.*

CHAPTER II
LITERATURE REVIEW

A. General Properties of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative, rod-shaped, motile, non-fermenting, opportunistic pathogen (1). Historically, *P. aeruginosa* is characterized as a human pathogen, and this goes back to 1850 when Sédillot observed the presence of a blue-green pus coupled with infection in surgical wound dressings (1,2). The bacterium's hallmark feature is the production of a water-soluble blue green phenazine compound; pyocyanin pigment (3). Moreover, *P. aeruginosa* produces a sweet grape-like odor when cultured on agar plates. *P. aeruginosa* is a ubiquitous organism capable of inhabiting many inert surfaces and environments including water, soil, plants, and tissues of animals and humans (1,4). *P. aeruginosa* is an important human pathogen that causes clinical diseases when immune defenses are suppressed (5). Immunocompromised population such as patients on antibiotic therapy, in surgery, chemotherapy, transplants, diabetes, and leukemia. These are all factors that aid in the acquisition of *P. aeruginosa* infections in medical care centers (1,2). Furthermore, this bacterium is among the leading causes of nosocomial infections due to its possession on indwelling medical devices such as ventilators and catheters. It is the major source of bacteremia in burn wounds, urinary tract infections (UTI) in catheterized patients, and ventilator acquired pneumonia (VAP) in patients on respirators (4). *P. aeruginosa* has the largest bacterial genome sequenced with 6.3 million base pairs (Mbp) in 5570 predicted genes, 8.4 % of which are involved in gene regulation (2,4,6). This large genome encodes for a number of genes that confer intrinsic resistance to a vast

range of antibacterial agents, as well as the capability of *P. aeruginosa* to adapt to versatile environments (6).

P. aeruginosa can occur in the environment either as free-planktonic cells or as sessile-cells attached to a surface; biofilm (7,8). Highly structured biofilms add on to the bacterial innate resistance and as a result renders the treatment with antibacterial agents and host defense unsuccessful in clearing bacterial infections (1,9).

According to a review of surveillance data collected and reported by hospitals participating in the CDC National Nosocomial Infections Surveillance (NNIS) System from October 1986 to April 1998, *P. aeruginosa* was identified as: i) the second leading cause of nosocomial pneumonia ii) third most common cause of urinary tract infections iii) fourth most frequently isolated pathogen in surgical site infections, iv) fifth most frequently isolated nosocomial pathogen v) and seventh leading contributor to bloodstream infections (10,11).

B. Pathogenesis and Virulence Factors of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is equipped with a wide variety of virulence factors that attribute to its invasion, tissue damage and dissemination (1,2,9). The virulence factors include pili and flagella, in addition to the endotoxin lipopolysaccharide (LPS) and secreted toxins (1,6). Although there is no general vaccine for pseudomonas infections, several virulence factors have been studied as potential vaccines candidate (2).

1. Lipopolysaccharide

Lipopolysaccharide (LPS) is an important constituent of Gram-negative bacteria. It plays an important role in activation of innate and adaptive immune responses (2). Moreover,

LPS has a role in outer membrane permeability barrier (12). It consists of a hydrophobic basic domain known as lipid A, a conserved-non repeating core oligosaccharide, and a distal-variable polysaccharide O-antigen (1,6,13). The latter is responsible for the basic antigenic identification of P. aeruginosa serotypes (6) and to date there are 20 serotypes based on serological reactivity of O-antigen (2). On the other hand, lipid A component of LPS activates many pro-inflammatory pathways (14).

2. Type IV Pili

Pili are small filamentous surface appendages (6) that play an important role in adhesion to many cell types and tissues (tropism) (1). Type IV pili are associated with the twitching motility of *P. aeruginosa* (15) that allow the bacterium to spread along a surface and initiate biofilm formation (1,6). In addition, studies have shown that bacteria deficient in either pili or flagella have reduced virulence and aren't able to persist at wound site or spread throughout the host (16).

3. Flagella

P. aeruginosa has a single, polar, unsheathed flagellum that aids in the swimming motility of the bacterium. The so-called-swarming motility is due to the propulsion of bacterial flagellum (17). Aside from motility, flagella play a critical role in attachment and invasion (2,6). Furthermore, flagella are important in biofilm formation (1) and they elicit an NF-κB dependent inflammatory response through their interaction with Toll-like-receptors TLR2 and TLR5 (6).

4. Secretion Systems

P. aeruginosa has a diversity of secretion systems that serve as a mechanism to allow the bacterium to interrelate with its surrounding, and with other bacteria (18). At least four secretion systems play a role in virulence of *P. aeruginosa* (1).

Type III secretions T3SS is among the most important secretion systems in *P. aeruginosa.* It involves a needle-like-basal-body for delivering the toxin directly into the adjacent host cell cytoplasm (19,20). Four effector proteins (cytotoxins) are secreted via T3SS: ExoS, ExoT, ExoU, and ExoY (20,21). ExoS and ExoT cytotoxins can inhibit phagocytosis, signal transduction, and adhesion (1,2). Rapid host cell lysis is related to ExoU secretions (1), whereas, ExoY secretions lead to disruption of actin cytoskeleton and inhibition of bacterial uptake by host cells (20).

Type II secretions T2SS require the release of toxins and enzymes into the extracellular environment via a pilus-like apparatus (1,22,23). T2SS include the release of exotoxin A involved in tissue damage and invasion (6), phospholipase C implicated in inflammation, elastase related in the rupture of respiratory epithelium (6), lipase, and alkaline phosphatase.

Type I secretions T1SS include proteases that play an important role in acute lung infections and wound infections (1) as well as corneal infections (6).

C. Antibacterial Resistance of *P. aeruginosa*

P. aeruginosa is categorized as one of the leading causes of nosocomial infections with high morbidity and mortality rates in cystic fibrosis patients (24). At 6.3 million base pairs, *P. aeruginosa* genome encodes for a vast number of genes that present innate resistance to

treatment with a wide range of antibacterial agents. Beside its intrinsic resistance, *P. aeruginosa* can acquire plasmids that harbor resistance genes (1,25). Moreover, this challenging organism can adapt and mutate, increasing its antibacterial tolerance.

1. Intrinsic Resistance

It is widely known that *P. aeruginosa* presents important levels of intrinsic resistance to a wide number of antibacterial agents including most β lactams, tetracyclines, chloramphenicol, and fluoroquinolones (24,26). Low permeability of the outer membrane of *P. aeruginosa* is one of the major causes of reduced susceptibility (1,24). The outer membrane by itself is inadequate in maintaining resistance, as a result other factors such as degenerative enzyme like periplasmic β-lactamases (AmpC β-lactamase) that cleave the lactam ring of penicillins, carbapenems, and cephalosporins are present. Moreover, multidrug resistant (MDR) efflux pumps build on to the ability of the bacterium to mediate resistance (26). Four resistance-nodulation-division (RND) MDR efflux systems have been described in *P. aeruginosa* so far: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (2,27).

2. Acquired Resistance

P. aeruginosa through horizontal gene transfer can acquire plasmids that harbor resistant genes, and can undergo mutations that increase their resistance. Usually, AmpC β-lactamases are expressed in low levels in *P. aeruginosa.* However, regulatory mutations can lead to the over production of AmpC β-lactamases (28). On the other hand, mutation in the *ampC* repressor gene *ampD* results in the overproduction of *ampC*. Furthermore, point mutations in the transcriptional regulator, *ampR* cause an overexpression of *amp C* (1,28).

A broad variety of β-lactamases, including Pseudomonas specific enzymes (PSE), OXA-type β-lactamases, ESBLs and metallo-carbapenemases are encoded within plasmids and can add to the resistance of *P. aeruginosa* to β-lactams (1,29). Additionally, mutations causing loss of specific porins (30) and up regulation of MDR efflux systems (26) add up to the resistance profile of *P. aeruginosa*.

D. *P. aeruginosa* Biofilm

1. Definition and General Characteristics

A biofilm is a collection of slow growing (31) microbial cells attached to a surface and embedded within a self-produced matrix (32). This mode of growth adopted by some bacteria is a survival strategy (8) that acts as a shield against chemical and mechanical stresses (33). Bacterial biofilms are defined as matrix-enclosed masses of bacteria benefiting from some of the multicellular life (34). Only 10-20% of the biofilm volume is bacteria (8,34), the rest is composed of polysaccharides, penetrated by minute water channels (8), extracellular DNA (eDNA) and proteins (35). *P. aeruginosa* biofilms form on medical devices (36) such as urethral catheters, ureteric and prostatic stents, testicular implants, artificial urinary sphincters, and hip and knee replacements (37). In addition, biofilms can grow within the host organism; in the lungs of patients with cystic fibrosis and site of burn wounds (8,38). Over 80% of microbial infections are due to biofilms (39).

2. P. aeruginosa Biofilm Matrix Components

Extracellular polymeric substance (EPS) or extracellular matrix of biofilms play an essential role in attachment to surfaces, as well as providing a barrier to protect core

bacterial cells (40). The EPS is composed of exopolysaccharides (Pel, Psl, and alginate), eDNA, proteins, and surface appendages (type IV pili, flagellum, and fimbriae) (41).

i. *Psl polysaccharide*

Polysaccharide synthesis locus (Psl) is responsible for the synthesis of Psl polysaccharide (42). Out of the 15 psl genes (pslA to pslO), only 11 are essential for the synthesis of Psl-dependent biofilm (41,42). Psl is composed of repeating pentasaccharide consisting of *D*-mannose, *D*-glucose, and *L*-rhamnose (42). Psl polysaccharide is important for cell-attachment to surfaces and maintenance of biofilm architecture (43). Apart from acting as a scaffold in biofilm formation, Psl plays a protective role against the immune system (41). Psl indirectly stimulates NF-κB activity and stimulates flagellin-mediated proinflammatory signaling (44). Moreover, it was shown that Psl inhibits efficient opsonization, resulting in reduction of production of reactive oxygen species (ROS) by neutrophils (45). Furthermore, Psl plays a role in antibiotic resistance (46).

ii. *Pel polysaccharide*

Pel polysaccharide is a glucose-rich cellulose-sensitive polysaccharide matrix component (41). Friedman et al. found that *pel* mutants were not capable of forming pellicles or mature solid-surface-associated (SSA) biofilms (47). Pel polysaccharide plays a crucial role in cell-cell interaction in *P. aeruginosa* biofilms, thus serving as a primary structural scaffold for the microcolony of cells in a biofilm (48). On the other hand, Pel enhances resistance to aminoglycoside antibiotics (41).

iii. *Alginate*

Alginate is frequently produced by *P. aeruginosa* isolates in cystic fibrosis (CF) patients (49).it is a linear unbranched polymer composed of D-mannuronic acid and L-guluronic

acid (9,50). Alginate is required for protection, stability, water and nutrient retention in biofilms (51). Leid et al. suggested that alginate plays an important role in defending mucoid *P. aeruginosa* biofilm from the human immune system although it may not play a role in bacterial attachment, biofilm development, and formation (52).

iv. *Extracellular DNA (eDNA)*

In *P. aeruginosa* eDNA is the result of bacterial subpopulations lysis and it presents an important factor in horizontal gene transfer (53). eDNA acts as a nutrient source for bacteria during starvation (41). In addition, it recognized by the innate immune system by the Toll-like Receptor (TLR) family of pattern recognition receptors (PRRs) (53). Interestingly, Fuxman et al. reported that matrix eDNA is a key pro-inflammatory component of *P. aeruginosa* biofilms (54).

E. *P. aeruginosa* Biofilm Lifestyle cycle

Biofilm formation is a dynamic (32) process in which organized bacterial microcolonies are embedded in a self-produced matrix (9). This cycle is divided into five main steps. First, bacterial cells adhere to an abiotic surface. This attachment is reversible during first hours (8). Second, the attachment becomes irreversible due to the pili and glycocalyx. Third, microcolnies form within EPS matrix. Fourth, the biofilm matures, expands and grows. Finally, bacteria are released from the biofilm and re-enter the planktonic stage to colonize other surfaces (9).

F. *Candida albicans* Biofilms

Candida albicans is the third leading cause of urinary tract infection and the fourth leading cause of nosocomial bloodstream infections (55). Similar to *P. aeruoginosa*, *C. albicans*

grow as a resistant biofilm (56). *C. albicans* colonizes and forms biofilms on inert surfaces including catheters, dental equipments, indwelling medical devices, joint replacements (57), and human heart valves (58,59). Furthermore, *Candida* inhabits and grows in biofilms in tissues such as vaginal and oral epithelia (59) and increases mortality rates in patients whose immune system is compromised (57). The formation of biofilm in *Candida albicans* requires three stages; first, attachment of the yeast cell to a surface, followed by matrix production and switching from yeast to hyphal form, finally maturation of the matrix taking on a three-dimensional architecture (60). A fully mature biofilm consists of yeast, hyphae, and pseudohyphae (61) enclosed within an extracellular matrix composed of proteins, carbohydrates, hexosamine, phosphorus and uronic acid (62). Like *P. aeruginosa*, the biofilm matrix of *C. albicans* is penetrated by water channels that act as a circulatory system to supply nutrients to the cells within the biofilm (63).

G. *Candida albicans* 1,3-β-D-Glucan

C. albicans cell wall is composed mainly of carbohydrates (80-90%), proteins (6-25%) and small amount of lipids (1-7%) (64). The major constituents of carbohydrates include: branched polymers of glucose containing β-1,3 and β-1,6 linkages (β-glucans), un-branched polymers of N-acetyl-D-glucosamine (GlcNAc) containing β-1,4 bonds (chitin) and polymers of mannose covalently associated with proteins (64,65).

Glucans are (1-3)-β-D-linked glucose polymers that constitute the cell wall of fungi and certain bacteria (66). 1,3-β-D glucans are also found in the fungal biofilm matrix and are linked to antifungal drug resistance (67). Glucan synthase complex is a membrane-bound enzyme that uses UDP-glucose as a substrate and synthesizes 1,3-β-D-glucan (55). The

latter released in the blood of patients infected with fungi and since humans are not capable of producing it, their presence is an indicative of infection (57). Among the diagnostic assays for the detection of 1,3-β-D glucans in serum is the Fungitell test that was approved in 2003 by the U.S. Food and Drug Administration for the presumptive diagnosis of incidence of invasive fungal infection (IFI) (68).

On a side note, synergistic and antagonistic interactions occur between bacteria and eukaryotic organs in diverse environments (69). An example of these interactions includes that of *P. aeruginosa* and *C. albicans* (70). Hogan et al. described an antagonistic relationship between *P. aeruginosa* and *C. albicans.* It was revealed through their work, that *P.aeruginosa* formed a biofilm on *C. albicans* hyphae and killed the fungus. Death was due to the secretion of a number of virulence factors by the bacteria. On the other side, *P.aeruginosa* was not able to bind to, or kill, yeast-form *C.albicans* (61).

H. *P. aeruginosa* and 1,3-β-D-Glucan

Surprisingly, 1,3-β-D glucan was detected in the serum of patients infected with *P. aeruginosa*. This was confirmed by the reactivity of Fungitell assay with 1,3- β-D glucan in patients with bacteremia due to *P. aeruginosa* (71). According to a study conducted by Mennink-Kersten et al., *P. aeruginosa* isolated from blood of immunocompromised patients showed 1,3-β-D glucan reactivity with Fungitell test. On the other hand, this reactivity decreased when the clinical isolates were treated with 1,3-β-D glucanase. Moreover, Sadovskaya et al., demonstrated that the product of the *P. aeruginosa ndvB* gene is involved in the synthesis of cyclic 1,3-β-D glucan (72). β-D glucans are present within the bacterial extracellular matrix and aid in antibiotic resistance (7,73).

I. Micafungin

Micafungin is a semi-synthetic lipopeptide echinocandin that acts as a noncompetitive inhibitor for 1,3-β-D-glucan synthase (74). By inhibition of the synthesis of 1,3-β-D glucan, fungal cells will no longer be able to maintain their shape and rigidity, thereby lyse due to increased osmotic pressure (75). Micafungin is approved for the treatment of adult and pediatric patients with invasive candidiasis and for the treatment of adult patients with esophageal candidiasis. Moreover, it is used as a prophylaxis against *Candida* infection in pediatric patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) (74–76). Due to the fact that1,3-β-D glucan is found in the bacterial biofilm matrix of *P. aeruginisa*, Bazzi et al., reported that micafungin acts as a potential agent for disrupting the structure of *P. aeruginosa* biofilm by preventing the synthesis of 1,3-β-D glucan through the inhibition of glucan synthase enzyme (7). The approach of the study conducted by Bazzi et al. at the department of Experimental Pathology, Immunology and Microbiology at the American University of Beirut was to study the phenotypic and genotypic *in-vitro* effect of micafungin in inhibiting biofilm formation by *P. aeruginisa.* Significant reduction in biofilm was observed for the treated samples. Moreover, the level of gene expression for the genes involved in biofilm formation in *P. aeruginosa* (*pelC* and *algC*) in addition to the gene involved in 1,3-β-D glucan synthesis (*ndvB)* decreased drastically upon treatment with micafungin (7).

J. Antibacterial Therapy

The minimal inhibitory concentration (MIC) of antibacterial agents to biofilm-growing bacteria may be up to 100-1000-fold higher than that of free-living-planktonic ones (77).

As a result, treatment of biofilm-associated infections is challenging by using the classical antibacterial agents (78).There are many reasons that explain the antibacterial resistance of biofilms (79). One of which is alginate; a major constituent of the biofilm matrix. Alginate presents a physical barrier that prevents the access of antibacterial agents to the infecting cells (80) . Another reason is that the subpopulations of cells within the biofilm known as persister cells have reduced growth rates, thus making eradication by antibiotics very hard (81). Levofloxacin is a fluoroquinolone that inhibits DNA gyrase, rapidly killing target bacterial cells (82). Additionally, fluoroquinolones affect non-growing cells of *P. aeruginosa* and are capable of eradicating biofilms formed *in-vitro* (83). Levofloxacin has a powerful activity against Cystic fibrosis (CF) causing *P. aeruginosa* where oral and parenteral fluoroquinolones are broadly used in patients with CF for airway infections (84). Also, studies concluded that biofilms didn't increase the MIC of Levofloxacin (85).

Ceftazidime , a cephalosporin , interferes with bacterial cell wall synthesis (86). A study carried out by Bagge et al. showed that *P. aeruginosa*-growing biofilms can tolerate increased concentrations of ceftazidime due to the production of β lactamase combined with penetration inhibition by alginate (87). On the other hand, Permin et al. recommended that Ceftazidime is an effective and safe drug in the treatment of *P. aeruginosa* bronchopulmonary infection in CF patients, although it cannot eradicate the bacterial cells (88). Besides, Ishida et al. reported that diffusion rate of ceftazidime through the alginate layer was higher than that of levofloxacin (79).

National Healthcare Safety Network (NHSN) reported that highest resistance rate among nosocomial isolates of *P. aeruginosa* was for fluoroquinolones (< 30%) followed by

carbapenems (25%), after which comes piperacillin-tazobactam (18%), and cefepine (11%). Aminoglycoside amikacin has the lowest rate of resistance (6%). (1,89)

K. Visualization and Measurement of Biofilms

Microscopic detection of microorganisms dates back to 1675, when Leeuwenhoek was able to visualize them in water and saliva (90). Few years later, and ever since that date, biofilms are being studied using different methods. A number of techniques have been adopted to visualize and measure biofilms including light microscopy, electron microscopy (scanning and transmission), staining assays such as microtiter plate assay (using safranin or crystal violet), direct bacterial staining (usingDAPI, Propidum iodide or ethidium bromide) followed by measurement of absorbance, fluorescence *in-situ* hybridization (FISH), in addition to different fluorescent dyes that stain different biofilm components (concanavalin A, SYTO9, SYTO17, and calcofluor white) (91). Confocal scanning laser microscopy (CSLM) has become widely used nowadays in imaging of living biofilms and characterizing their architecture (92). Unlike traditional imaging techniques, CSLM provides a nondestructive, detailed visualization of thick samples, and allows horizontal and vertical optical sectioning of samples (93,94). the use of CSLM offers a number of advantages over the electron microscopy techniques that are difficult, can produce artifacts, and limit the three dimensional reconstitution of biofilms (93). The advantages of CSLM include three-dimensional reconstitution of samples such as biofilms, higher resolution of digital images with up to 1024 x 1024 pixels, procedures are not laborious and allow the elimination of out-of-focus haze (93,95). Moreover, images can be quantitatively analyzed by using computerized image data processing (95). Laser scanning microscopy is being

used frequently as a mean to visualize and analyze biofilms (94). CSLM uses filters with relatively broad bandwidth to detect fluorescent emissions from stained samples (92). Moreover, confocal scanning is characterized by the optical sectioning property that is used to record thin (~1 μm) sections of a specimen without the need for mechanical sectioning. Furthermore, a stack of consecutive sections can be automatically recorded by using computer-control to adjust the focus of the microscope (96).

CHAPTER III
MATERIALS AND METHODS

A. Source of Bacterial Isolates

P. aeruginosa isolates were previously obtained from clinical specimens of patients with nosocomial infections (97). PAN14; a strong- biofilm-forming isolate of *P. aeruginosa* obtained from deep tracheal aspirate was used in this study (7). The isolate was identified using colonial morphology, Gram staining, sweet-grape smell, API20 NE kits (bioMerieux. SA 69820, Marcy l'Etoile-France) and tested for susceptibility by the disc diffusion method against a panel of antibacterial agents according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (98).

Disc diffusion method revealed that *P. aeruginosa* is susceptible to several antibacterial agents including: ciprofloxacin (5 mcg), levofloxacin (5 µg), tobramycin (10 µg), ceftazidime (30 µg), imipenem (10 µg), piperacillin-tazobactam (110 µg), aztreonam (30 µg), gentamycin (10 µg), and oxacillin (1 µg).

B. Source of antimicrobial agents

1. Micafungin Sodium 50mg vials (Hikma Pharmaceuticals PLC, and Astellas Pharma Inc., Japan).
2. Levofloxacin (Sigma-Aldrich, Inc.,St Louis, MO)
3. Ceftazidime (GlaxoSmithKline, Verona, Italy)

C. Assessment of Biofilm Formation using the Microtiter Plate Assay

The protocol used was adopted from the work of Bazzi et al. with some modifications (7)

1. The biofilm-strong-forming isolate PAN14 was cultured on MacConkey agar (Becton-Dickinson, Le Pont de Claix, France) plate and incubated for 24 hours at 37°C.
2. Few bacterial colonies were transferred from MacConkey agar plate and inoculated into LB broth (Luria-Bertani, Becton-Dickinson, Le Pont de Claix, France) then incubated for 22 hours at 37°C.
3. The LB broth culture suspension was adjusted to 10^8 bacterial cells/ml or 0.5 McFarland using the DENSIMAT (bioMerieux. SA 69820, Marcy l'Etoile-France). Then the bacterial suspension was diluted 100-fold with LB broth.
4. The wells of the 96-well Polysterene microtiter plate (Costar 3788, Corning Incorporated, NY) were divided according to the design below and the experimental procedure was performed in sextuplicate.

    100 µl/well LB broth (negative control)

    100 µl/well bacterial suspension (untreated positive control)

    100 µl/well bacterial suspension and 100 µl/well micafungin (10 mg/ml)

    100 µl/well bacterial suspension and 100 µl/well levofloxacin (MIC=0.2 µg/ml)

    100 µl/well bacterial suspension and 100 µl/well micafungin (10 mg/ml) and 100 µl/well levofloxacin (MIC=0.2 µg/ml)

100 µl/well bacterial suspension and 100 µl/well ceftazidime (MIC=0.2 µg/ml)

100 µl/well bacterial suspension and 100 µl/well micafungin (10 mg/ml) and 100 µl/well ceftazidime (MIC=0.2 µg/ml)

5. The plate was incubated for 24 hours at 37°C.
6. Following incubation, planktonic cells were removed. Using a multichannel pipette, the wells of the 96-well Polysterene microtiter plate were rinsed with distilled water.
7. 150 µl of 1% crystal violet were added to each well. Crystal violet stains bacterial cells.
8. The plate was incubated for 15 minutes at room temperature. After which the wells were washed with distilled water to remove excess crystal violet.

   The plate was left to dry and then 200 µl of 95% ethanol were added to the stained wells in order to dissolve crystal violet.
9. Once again, the plate was incubated at room temperature for 20 minutes.

   125 µl were transferred from each well into a new Polysterene microtiter plate.
10. Absorbance was measured with a BIO-TEK ELx800 Automated Microplate Reader at 630 nm.
11. The mean average for each of the six wells with different conditions was calculated. Standard deviations were also calculated to determine statistically significant results using the "Student's t-Test". P-value < 0.05 were considered significant results.

D. *In-vivo* Experimental Approach for Quantitative RT-PCR

The *in-vivo* work was approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut. Fifty four BALB/c mice were used in this study. The BALB/c mice were divided into six groups with 9 mice/group, with different treatment conditions to determine the relative gene expression of biofilm-encoding genes for alginate and pellicles (*pelC* and *algC*) and cell wall 1,3- β -D-glucan-encoding gene (*ndvB)*.

i. Reagents and Solutions

Bacterial dose:

2LD 50 = 6 x 10^7 CFU/ml

Each mouse received a volume of 0.1 ml, so 2LD 50 = 6 x 10^8CFU/ml

Micafungin:

Dose: 2mg/Kg/day

Per mouse: 2mg x 0.03Kg = 0.06 mg/day

V=0.1 ml

C=0.06 mg/ 0.1 ml = 0.6mg/ml

Levofloxacin and ceftazidime :

In vitro broth dilution method MIC = 2 µg/ml =0.002 µg/µl

Antimicrobial agent in vivo MIC dose (µg) = [Antimicrobial agent in vitro MIC (µg/µl) x in vitro MIC broth volume (µl) x Bacterial CFU administered in vivo] / Bacterial CFU per in vitro MIC reaction

Antimicrobial agent in vivo MIC dose (μg) = [0.002 μg/μl x 1000 μl x ($6x10^7$ CFU/ml) / $5x10^5$ CFU/ml] = 240 μg

V= 0.1 ml/mouse

C= 240 μg/0.1ml = 2.4mg/ml

ii. *Protocol:*

Six groups of 9 mice/group, received different injections according to the below experimental design:

Group 1: Intraperitoneal injection of 2 LD50 of PAN14 (positive control)

Group 2: Intraperitoneal injection of 2 LD50 of PAN14 and 0.1 ml micafungin (0.6 mg/ml)

Group 3: Intraperitoneal injection of 2 LD50 of PAN14 and 0.1 ml levofloxacin (2.4 mg/ml)

Group 4: Intraperitoneal injection of 2 LD50 of PAN14 and 0.1 ml ceftazidime (2.4 mg/ml)

Group 5: Intraperitoneal injection of 2 LD50 of PAN14 and 0.1 ml micafungin (0.6 mg/ml) and 0.1 ml levofloxacin (2.4 mg/ml)

Group 6: Intraperitoneal injection of 2 LD50 of PAN14 and 0.1 ml micafungin (0.6 mg/ml) and 0.1 ml ceftazidime (2.4 mg/ml)

For all the protocols, bacterial injections along with micafungin were given at time t=0hrs while the antibacterial agents were given at t=3hrs. Relative size, weight, and physiological state of each mouse was observed on daily basis. Dead mice were dissected, blood and organs collected, and cultured on MacConkey agar (Becton-Dickinson, Le Pont de Claix,

France) plates for 24 hours at 37°C to make sure that the cause of death was due to bacterial infection.

In addition, monitoring of BALB/c mice survival over the three-day was recorded.

E. RNA Extraction for *in-vivo* Samples:

RNA extraction was done using the Illustra RNAspin Mini RNA Isolation Kit (GE healthcare, UK) according to the manufacturer's specifications for bacterial cells.

i. *Reagents and Solutions:*

1. RNase free water (with kit)
2. 70% Ethanol
3. TE buffer (Amresco,USA)
4. Lysozyme (USB, USA)
5. Buffer RA1 (with kit)
6. β-mercaptoethanol
7. Membrane Desalting Buffer (MDB) (with kit)
8. DNase I
9. DNase Reaction Buffer (with kit)
10. Buffer RA2 (with kit)
11. Buffer RA3 (with kit)
12. Ribolock RNase inhibitor (Fermentas, USA)

The six groups of mice were subjected to the following procedure: 3 mice/group were euthanized after 24 hours post bacterial injection, dissected, heart punctured, blood collected in EDTA tubes, centrifuged at 1500xg for 30 minutes at 4°C, and serum collected.

The remaining mice continued to receive treatment for the remainder of the experiment. Extraction was run according to the manufacturer's specifications for bacterial cells.

ii. *RNA Extraction Protocol:*

a. Cell lysis and Homogenization:

1. 500 µl of serum from different subgroups of mice at different days were taken and added to 100 µl TE buffer containing 0.2 mg/ml lysozyme, the mixture was vortexed and incubated for 10 minutes at 37℃.
2. 350 µl buffer RA1 buffer and 3.5 µl β-mercaptoethanol were added to each mixture. viscosity of the suspensions was reduced by vortexing the samples immediately and vigorously.

b. Filtartion of lystae:

The mixture in each tube was transferred to a corresponding violet RNAspin Minifilter unit placed in a collection tube; the solution was centrifuged for 1 min at 11,000 x g (12,800 rpm).

c. RNA binding conditions adjustments:

1. 350 µl 70% ethanol were added to the mixture.
2. The mixture was pipetted up and down and then transferred to a Blue RNAspin Mini column placed in a collection tube.
3. Centrifugation for 30 seconds at 8,000 x g was performed and the column was placed in a new collection tube.

d. Desalt silica membrane and DNA digestion:

1. 350 µl of Membrane Desalting Buffer (MDB) were added to each column. The samples were then centrifuged for 1min at 11,000 x g to dry the membrane.

2. The filtrate was discarded and the column was returned to the same collection tube.
3. 10 µl/sample of reconstituted DNase I were added to 90 µl DNase reaction buffer and the solution was mixed by flicking the tube
4. 95 µl of the DNase reaction mixture was added directly to the center of the silica (for each sample). The samples were then incubated at room temperature for 30 minutes.

e. Washing and Drying

1. 200 µl of buffer RA2 were added to an each RNA spin Mini column, the samples were centrifuged for 1 min at 11,000 x g
2. The column of each sample was placed into a new collection tube.
3. 600 µl of buffer RA3 were added to each RNA spin Mini column, the samples were centrifuged for 1 min at 11,000 x g.
4. The filtrate was disposed and the column of each sample was placed back into the same collection tube.
5. 250 µl of buffer RA3 was added to each RNA spin Mini column, the samples were centrifuged for 2 min at 11,000 xg. The column of each sample was transferred into a nuclease free 1.5 ml microcentrifuge tube.

f. Elution and Aliquoting:

1. RNA was eluted in 60 µl RNase free water and the samples were centrifuged at 11,000 x g for 1 min.
2. Eluted RNA was directly placed on ice.
3. 1µl Ribolock RNase inhibitor was added to each sample. The latter was stored at -80℃.

g. Calculation of RNA concentration:

The concentration of the RNA was determined using a spectrophotomer (NanoDrop ND®-1000) at an absorbance of 260 nm.

F. Reverse Transcription and cDNA Synthesis:

After RNA extraction, cDNA was produced using QuantiTect® Reverse Transcription Kit (Qiagen, Germany) according to manufacturer's procedures.

i. *Reagents and Solutions*

1. RNase free water
2. Extracted RNA
3. gDNA Wipeout Buffer (with kit)
4. Quantiscript Reverse Transcriptase containing RNase inhibitor (with kit)
5. Quantiscript RT Buffer containing dNTPs and Mg^{2+} (with kit)
6. RT primer Mix containing oligo-dT and dissolved in water (with kit)

ii. *Protocol:*

RNA samples were thawed on ice while the reagents were melted at room temperature. The reagents were then mixed evenly by flicking and the reagents were stored on ice while working on the procedure.

a. Elimination of genomic DNA (gDNA):

1. Based on the protocol, the RNA used to prepare the cDNA can range between 10 pg and 1 µg. Therefore, 0.1 µg of the RNA was used to prepare the cDNA.
2. 2 µl of gDNA wipeout buffer were added to each sample.
3. The samples were incubated at 42 ºC for 2 minutes after which they were placed on ice.
4. 14 µl were taken from each sample to be used in reverse transcription reaction.

b. Reverse Transcription Reaction

1. Master mix was prepared on ice using the following measurements: 1 µl of Quantiscript reverse Transcriptase (per sample), 4 µl of Quantiscript RT buffer (per sample), and 1 µl of RT Primer Mix (per sample).
2. 6 µl of the master mix was mixed with the 14 µl mixture (per sample) prepared from the "elimination of genomic DNA (gDNA)" step. This yielded a total reaction volume of 20 µl (per sample).
3. The samples were then mixed and placed on ice.
4. The samples were later placed in a thermal cycler (PCR Sprint ThermalCycler, Thermo Fisher Scientific, Waltham, MA, USA) accorsding to the following incubation conditions: 15 minutes at 42 ºC and 3 minutes at 95 ºC.
5. Aliquots of 20 µl of cDNA were prepared and stored at -20 ºC.

G. Quantitative Reverse Transcription (RT-qPCR)

RT-qPCR was performed on the synthesized cDNA of 14 samples from mice of group I that were treated with different therapy compared to those who did not receive any treatment. RT-qPCR was performed to evaluate the efficiency of therapy *in-vivo* by determining the level of biofilm synthesis by assessing the relative gene transcription level of biofilm-encoding genes for pellicles and alginate (*pelC* and *algC*) and cell wall 1,3-β-D-glucan encoding gene for *P. aeruginosa* (*ndvB*). *rpoD* gene encoding the housekeeping sigma factor σ^{70} of *P. fluorescens that shows 9*6.1% similarity with *P. aeruginosa* was used as a house-keeping gene (99). The primers for the genes used in RT-qPCR are described in Table I (7)**.**

i. *Reagents and Solutions*

1. cDNA prepared in section L
2. QuantiFast SYBR Green PCR master mix (with kit) including:
3. dNTP mix
4. SYBR Green I
5. HotStar Taq® Plus DNA polymerase
6. QuantiFast SYBR Green PCR buffer containing Tris-Cl, KCl, NH_4Cl, $MgCl_2$, and additives
7. RNase free water
8. Real time primers with a concentration of 5 µM

ii. *Protocol:*

1. The samples and reagents were thawed, mixed and kept on ice until use.
2. Four separate Master Mixes per qRT-PCR run were prepared for the samples, three for the genes in question *(ndvB*, *algC*, and *pelC* genes) and another for the housekeeping gene (*rpoD*).
3. Each master mix included: 10 µl per sample QuantiFast SYBR Green PCR master mix, 4 µl per sample RNase free water, 2 µl per sample 5 µM Forward primer, 2 µl per sample 5 µM Reverse primer.Tthe total volume of master mix per sample was 18 µl.
4. Two- 96 well plates were used for each run and 18 µl of the master mix corresponding to either the genes of interest or the housekeeping gene were distributed into the respective wells.

5. This was followed by the addition of 2 µl cDNA samples into their corresponding wells. As a total, each well contained a volume of 20 µl reaction mixture. Each sample was run in duplicate for the genes in inquiry and the housekeeping gene.
6. The wells were sealed and the plate was tapped to make sure no bubbles were present.
7. Real time runs were carried out in a Bio-Rad CFX96 Real Time System C1000 Thermal Cycler (Bio-Rad, Germany) and the cycling condition steps for each primer were as follows (7):
    a. Pre-incubation and enzyme activation at 95°C for 15min.
    b. The amplification step consisted of a 3-step cycle repeated 45 times as follows:
        - Denaturation at 95°C for 10 seconds.
        - Annealing at 65°C for 10 seconds.
        - Extension at 72°C for 20 seconds.
    c. Melting curve analysis consisted of 3 segments as follows:
        - Segment 1 at 95°C for 5 seconds.
        - Segment 2 at 40°C for 30 seconds.
        - Segment 3 at 95°C for 10 seconds.
    d. Cooling at 40°C for 30 seconds.

Melting and amplification curves were calculated using the Bio-Rad CFX Manager software (BioRad).The latter calculated the transcription levels of the gene in inquiry in the

samples treated micafungin and/or the antibacterial agents compared to samples left untreated, employing the reference gene *rpoD* as a standard.

H. Preparation of *in-vitro* and *in-vivo* Samples for Confocal Microscopy

1. Growth of Biofilm on Membrane Filter

Biofilms were grown on a membrane filter. The protocol used was adopted from Bardon et al. with modifications (33).

i. *Protocol*

1. PAN14 obtained from culture and from mice in addition to E. coli, were grown on LB agar and MacConkey agar plates respectively. E. coli was used a negative control since it is deficient in biofilm formation.
2. A single colony of PAN14 and E. coli was taken from each culture, inoculated in 10 ml LB broth each, and incubated overnight at 37°C without shaking.
3. 10mg/ml of micafungin was added to one of the PAN14 suspensions. For the in-vivo samples, PAN14 previously injected in mice (2LD50) was cultured from the blood of micafungin-treated (0.6mg/ml) and untreated mice.
4. Optical Density (OD) was adjusted to 0.5 McFarland ($1x10^8$ cells/ml).
5. Membrane filter (0.4 μm pore size, diameter 4.6 cm, isopore membrane filters) were prewetted with distilled water and transferred using a sterile forceps to the filtration apparatus.
6. The 10 ml suspension were deposited on the membrane filter under negative pressure.

7. Excess medium was removed from the membrane filter by washing with 50 ml distilled water.
8. The membrane filter was transferred onto LB agar plate and incubated for 48 hours at 37°C for the biofilm to grow on it.

2. Fixation and Staining of Biofilm-Covered Membrane

i. Reagents and Solutions:

1. 3.7% formaldehyde
2. PBS (Phospahte phosphate-buffered saline): 5mM K_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.0)
3. Ethidium bromide (1mg/ml)
4. Calcofluor white (20 µl/ml PBS)

ii. Protocol:

1. Biofilm-covered membranes were cut and transferred into glass slides. Biofilms were fixed by depositing the membrane filter in 3.7% (w/v) formaldehyde and then stored for 17 hours at 4°C.
2. Formaldehyde was removed by washing the membrane with sterile phosphate-buffered saline (PBS), and the biofilms were submerged in ethidium bromide (1 mg/ml) to stain the bacterial cell for 1 hour at room temperature, protected from light.
3. After removal of ethidium bromide by sterile PBS, biofilms were incubated with calcofluor white (20 µl/ml, 3.8 mM) to stain extracellular-polymeric matrix substances for 1 hour at room temperature, protected from light.

4. Calcofluor white was removed, and the biofilms were washed with 50 ml sterile PBS and kept in darkness until imaging with Confocal Scanning Laser Microscopy (CSLM).

I. Visualization and Measurement of Biofilms Using Confocal Scanning Laser Microscopy (CSLM)

Using CSLM, stained membrane-bound-biofilms were visualized and the two-day old biofilm thickness was assessed using focus stacking or Z-stacking. The latter is a method used to process digital images (100). The thickness of biofilms in treated compared to untreated samples was processed by this technique through combining multiple images taken at different focal distances to provide a composite image. Eight different fields from various locations within the biofilm-covered membrane were taken. Z-stacking was performed, the average of stacks was calculated per biofilm, and the relative thickness of biofilms was compared between treated and untreated samples.

CHAPTER IV
RESULTS

A. Assessment of Biofilm Formation Using the Microtiter Plate Assay

According to the Microtiter Plate Assay, and after measuring the Optical Density OD using BIO-TEK ELx800 Automated Microplate Reader, we found out that OD decreased significantly upon using levofloxacin (OD 1.019) compared to the positive control (OD 2.978). On the other hand, there was a drop in OD after adding micafungin to levofloxacin (OD 1.989) but not as significant as that observed with levofloxacin alone.

Moreover, the addition of micafungin alone induced a decrease in OD (2.6857) but not as significantly as with levofloxacin. Furthermore, when used alone, ceftazidime provoked a decrease in OD (1.705), which was more substantial than the decrease in OD when micafungin was combined with ceftazidime (OD 2.544). The data is summarized in Table 2 and Figure 1.

B. Quantitative Reverse Transcription (qRT-PCR)

The results showed a remarkable decrease in gene transcription level for the samples treated with micafungin alone for *algC*, *pelC* and *ndvB* genes. The most significant decrease in transcription level was that for *pelC* gene upon treatment with micafungin. The data revealed around 260-fold decrease on day 2 in group of mice treated with micafungin compared to those kept untreated. Similarly, significant results on day 2 were for groups of BALB/c mice treated with ceftazdime (11.4409 fold decrease). This decrease in *pelC* gene transcription level further declined upon combining micafungin with ceftazidime (16.5837

fold decrease on day 2). A dual therapy of micafungin along with levofloxacin yielded a decrease in transcription level expression monitored for three consecutive days (from 0.00803 fold decrease on day 1, to 0.9645 on day 2, to 6.48508 on day 3). Treatment with levofloxacin alone did not cause a drop in transcription level of *pelC* gene compared to a combination with micafungin (0.56991 fold decrease for levofloxacin alone on day 2 to 0.9645 with a dual therapy).

There was a two-fold decrease in gene transcription level of *algC* gene after 2 days from treatment with micafungin. Moreover, therapy with micafungin combined with ceftazidime revealed around one fold decrease in *algC* transcription level. On the other hand, there was no such significant fold decrease upon treatment with ceftazidime alone (0.1169 fold decrease). Although there was no notable decrease in *algC* gene transcription level upon treatment with levofloxacin alone (0.0298 fold decrease on day 3), there was a higher decrease in the transcription level upon combining micafungin with levofloxacin (0.11681 fold decrease on day 3).

For *ndvB* gene, there was a remarkable decrease in gene transcription level with micafungin alone after 1 day of treatment (5.4704 fold decrease) and further decrease after 2 days of therapy (74.4047 fold decrease). Additionally, there was a significant decrease in gene expression upon treatment with ceftazidime (7.9872 fold decrease on day 2) and with levofloxacin (7.4349 fold decrease on day 1). The data is summarized Figure 2, Figure 3 and Table 3.

During the course of rt-qpcr it was noted that 72 hours post injection with 2ld50 of the biofilm forming pan14 strain along with either micafungin and levofloxacin or levofloxacin

alone, 100 percent survival of balb/c mice was seen. our data revealed that death was observed in 55% of mice 24 hours after treatment with ceftazidime or micafungin and ceftazidime.

Blood from dead mice cultured on MacConkey agar plates and incubated for 24 hours at 37°C showed growth of *P. aeruginosa* colonies. This is indicative that the mice death was due to infection. Moreover, the weight of mice from each subgroup was monitored and showed no remarkable drop over the course of treatment.

C. Visualization and Measurement of Biofilms Using Confocal Scanning Laser Microscopy (CSLM)

Z-stacking revealed a significant decrease in the thickness of biofilms in micafungin-treated samples compared to untreated ones. There was a drop from 13.75 µm to 11.125 µm in micafungin-treated *in-vitro* samples. On the other side, there was a drop from 12.125 µm to 4.375 µm in micafungin-treated lung samples. Physical reduction in biofilms was confirmed with Z-stacking using CSLM that revealed a 16.8 % reduction in the thickness of biofilms after treatment with micafungin *in-vitro*. Moreover, a 64% reduction in the thickness of biofilms post treatment with micafungin in lung tissue. The data is summarized in Table 4 and Figure 4 to Figure 9.

Table 1: Primers of ndvB, algC, PelC and rpoD genes

Primer	Primer sequence	Product size (bp)	Reference
ndvB-F	5'-GGCCTGAACATCTTCTTCACC -3'	138	(101)
ndvB-R	5'-GATCTTGCCGACCTTGAAGAC -3		
algC-F	5'-CTACTTCAAGCAGATCCGC-3'	204	(97)
algC-R	5'-AGGTCCTTCAGGTTCTCC-3'		
pelC-F	5'-TGCTCCAGCTTCACCAG-3'	192	(97)
pelC-R	5'-CAGTTGCAGGTCGCCTT-3'		
rpoD-F	5'-AGGTGGCGTAGGTGGAGAA-3'	177	(7)
rpoD-R	5'-GGGCGAAGAAGGAAATGGTC-3’		

Table 2: Average absorbance, p-values and statistical significance of different treatments of pan14

Sample	Average OD	P value	Statistical significance (P value < 0.05)
LB broth (NC)	1.7668	-	-
PAN14 (PC)	2.978	-	-
PAN14 + Micafungin	2.6857	0.0598	Not quite significant
PAN14 + levofloxacin	1.0198	<0.0001	Extremely Significant
PAN14 + micafungin + levofloxacin	1.9894	<0.0001	Extremely Significant
PAN14 + ceftazidime	1.705	<0.0001	Extremely Significant
PAN14 + micafungin + ceftazidime	2.54467	0.00444	Significant

NC: Negative control PC: Positive control

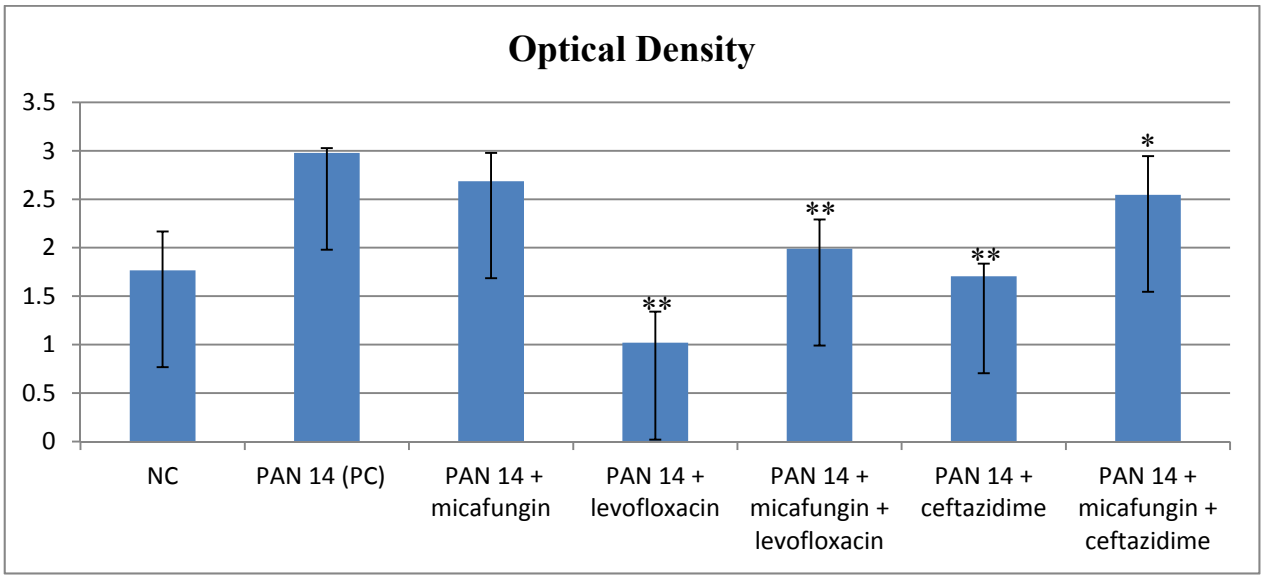
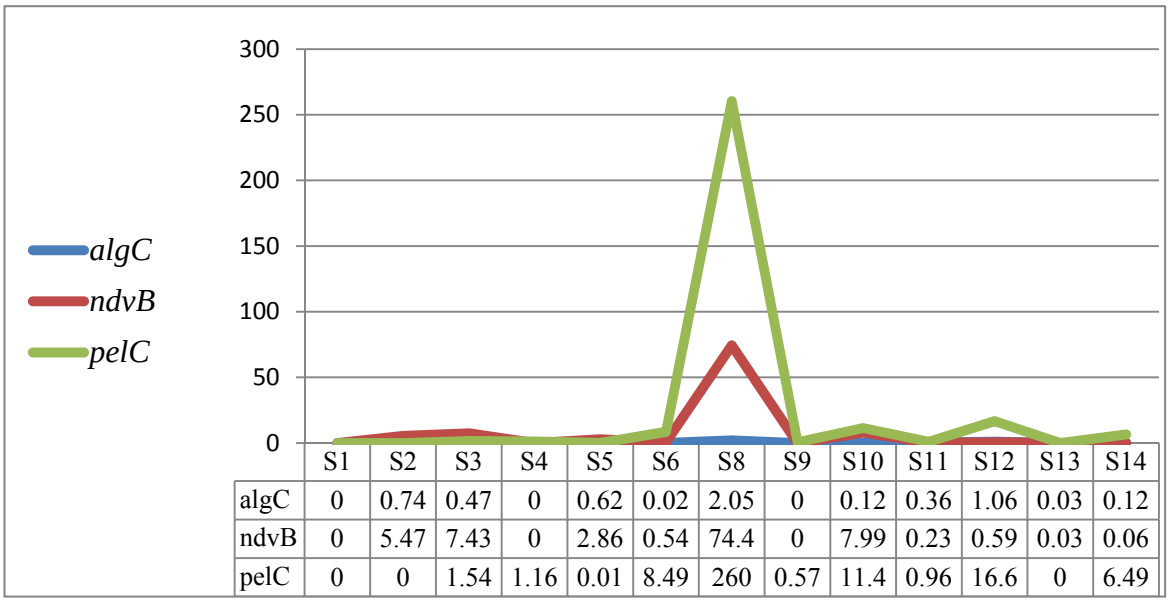
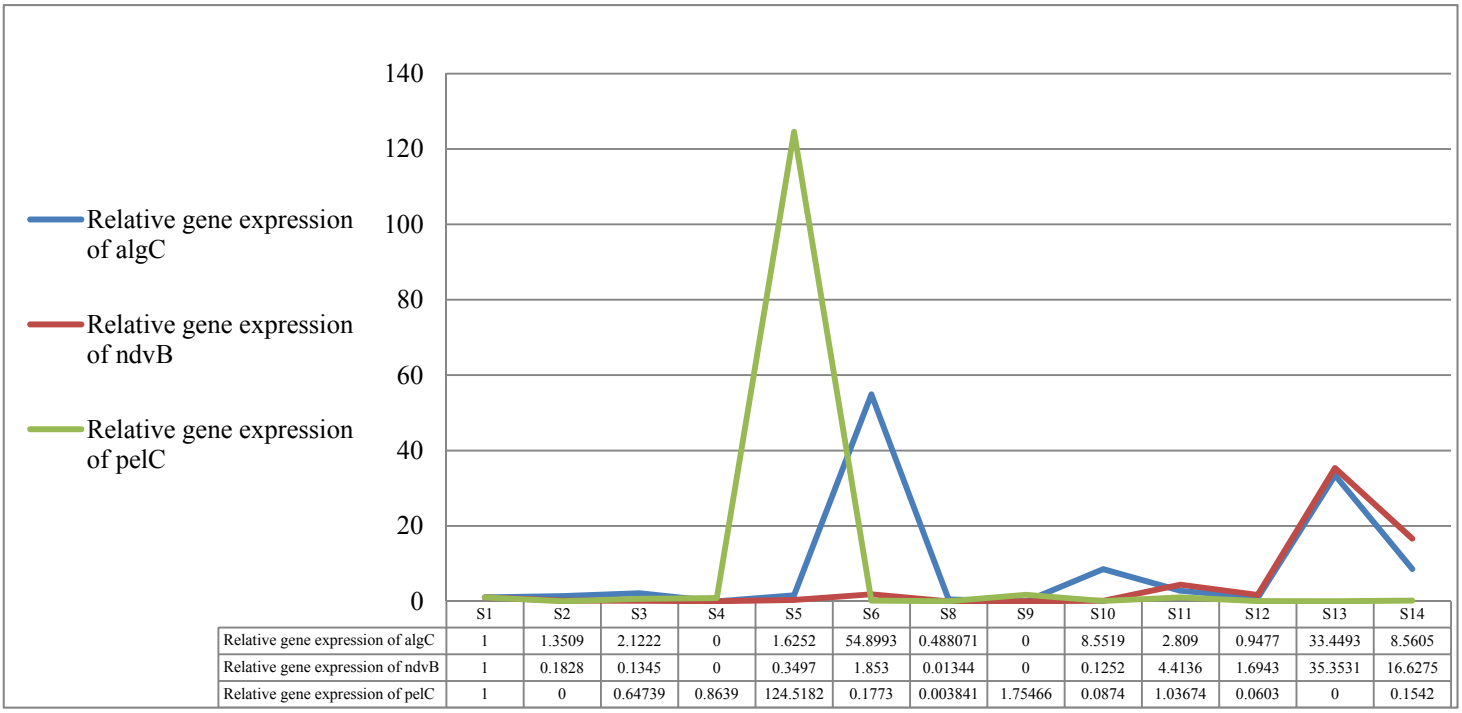


Figure 1: Effect of micafungin and combination therapy on pan14 biofilm synthesis as compared to un-treated pan14
(Significant ** Extremely Significant)*



	S1	S2	S3	S4	S5	S6	S8	S9	S10	S11	S12	S13	S14
algC	0	0.74	0.47	0	0.62	0.02	2.05	0	0.12	0.36	1.06	0.03	0.12
ndvB	0	5.47	7.43	0	2.86	0.54	74.4	0	7.99	0.23	0.59	0.03	0.06
pelC	0	0	1.54	1.16	0.01	8.49	260	0.57	11.4	0.96	16.6	0	6.49

Figure 2: Overall fold decrease in expression of algC, pelC and ndvB genes in treated and untreated groups of BALB/c mice



	S1	S2	S3	S4	S5	S6	S8	S9	S10	S11	S12	S13	S14
Relative gene expression of algC	1	1.3509	2.1222	0	1.6252	54.8993	0.488071	0	8.5519	2.809	0.9477	33.4493	8.5605
Relative gene expression of ndvB	1	0.1828	0.1345	0	0.3497	1.853	0.01344	0	0.1252	4.4136	1.6943	35.3531	16.6275
Relative gene expression of pelC	1	0	0.64739	0.8639	124.5182	0.1773	0.003841	1.75466	0.0874	1.03674	0.0603	0	0.1542

Figure 3: Overall relative gene transcription levels of algC, pelC and ndvB genes in treated and untreated groups of BALB/c mice

Table 3: Relative gene expression of algc, pelc, and ndvb genes in treated and untreated groups

Sample	Group	Duration of therapy (days)	Relative gene expression			Fold decrease			p-values		
			***algC*(+/-)**	***pelC*(+/-)**	***ndvB*(+/-)**	***algC***	***pelC***	***NdvB***	***algC***	***pelC***	***NdvB***
S1	1 (PC)	1	1	1	1	-	-	-	-	-	-
S2	2		1.3509 (+)	-	0.1828(-)	0.74024	-	5.4704	-	-	0.9
S3	3		2.1222 (+)	0.64739(-)	0.1345(-)	0.471209	1.5446	7.4349	0.55	0.99	0.65
S4	4		-	0.8639(-)	-	-	1.1575	-	-	0.99	-
S5	5		1.6252 (+)	124.5182(+)	0.3497(-)	0.61530	0.00803	2.8595	0.75	0.99	0.59
S6	6		54.8993 (+)	0.11773(-)	1.8530(+)	0.01821	8.4940	0.5396	0.85	0.99	0.59
S8	2	2	0.488071(-)	0.003841(-)	0.01344(-)	2.0488	260.3488	74.4047	0.04	0.99	0.434
S9	3		-	1.75466(+)	-	-	0.56991	-	-	0.99	-
S10	4		8.5519 (+)	0.08740(-)	0.1252(-)	0.1169	11.4409	7.9872	-	0.91	0.70
S11	5		2.8090 (+)	1.03674(+)	4.4136(+)	0.35599	0.9645	0.2265	0.78	1.0	0.95
S12	6		0.9477 (-)	0.06030(-)	1.6943(+)	1.0551	16.5837	0.5902	0.96	0.99	0.56
S13	3	3	33.4493 (+)	-	35.3531(+)	0.0298	-	0.0282	-	0.99	0.01
S14	5		8.5605 (+)	0.15420(-)	16.6275(+)	0.1168	6.48508	0.06014	0.44	0.99	0.6

Table 4: Biofilm thickness for in-vitro and in-vivo samples as calculated by Z-stacking

	Sample	Field thickness (µm)								Average thickness (µm)	Standard deviation (±)	Percentage decrease (%)
		1	2	3	4	5	6	7	8			
In-vitro	PAN14 biofilm (NC)	14	13	19	18	14	10	5	14	13.375	4.405	16.822
	PAN14 biofilm + micafungin	13	17	18	9	9	8	7	8	11.125	4.323	
In-vivo	PAN14 biofilm (NC)	10	12	12	13	13	14	11	12	12.125	1.246	63.917
	PAN14 biofilm + micafungin	3	2	7	5	4	5	4	5	4.375	1.505	

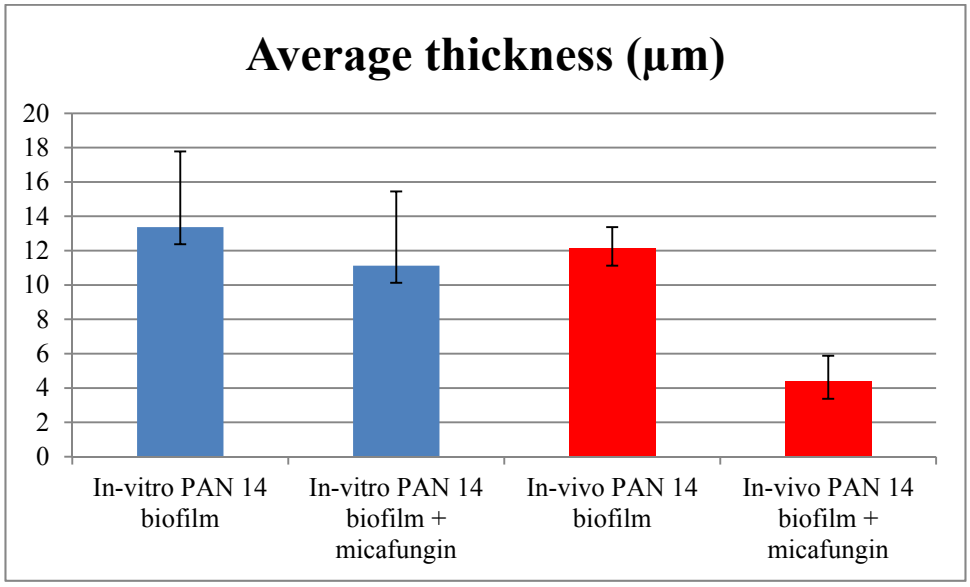


Figure 4: Biofilm thickness for in-vitro and in-vivo samples as calculated by Z-stacking

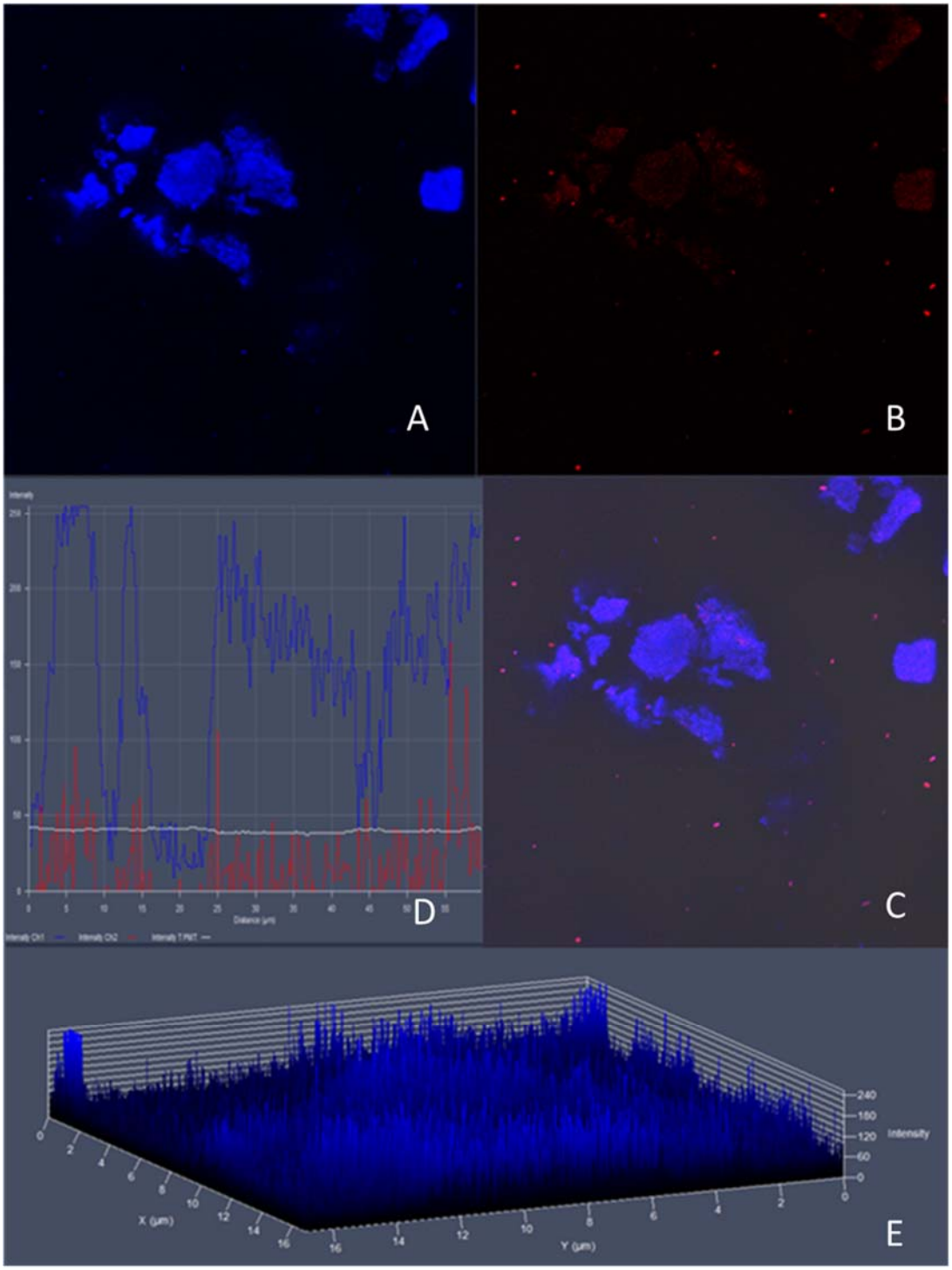


Figure 5: Confocal microscopy images for in-vitro PAN14 two-day old biofilms grown on membrane filters stained with calcofluor white (blue florescence of biofilm) (A), ethidium bromide (red florescence bacterial cells) (B), merged images of A and B (C), fluorescence intensity distribution profile (D), and 2.5D view of biofilm (E)

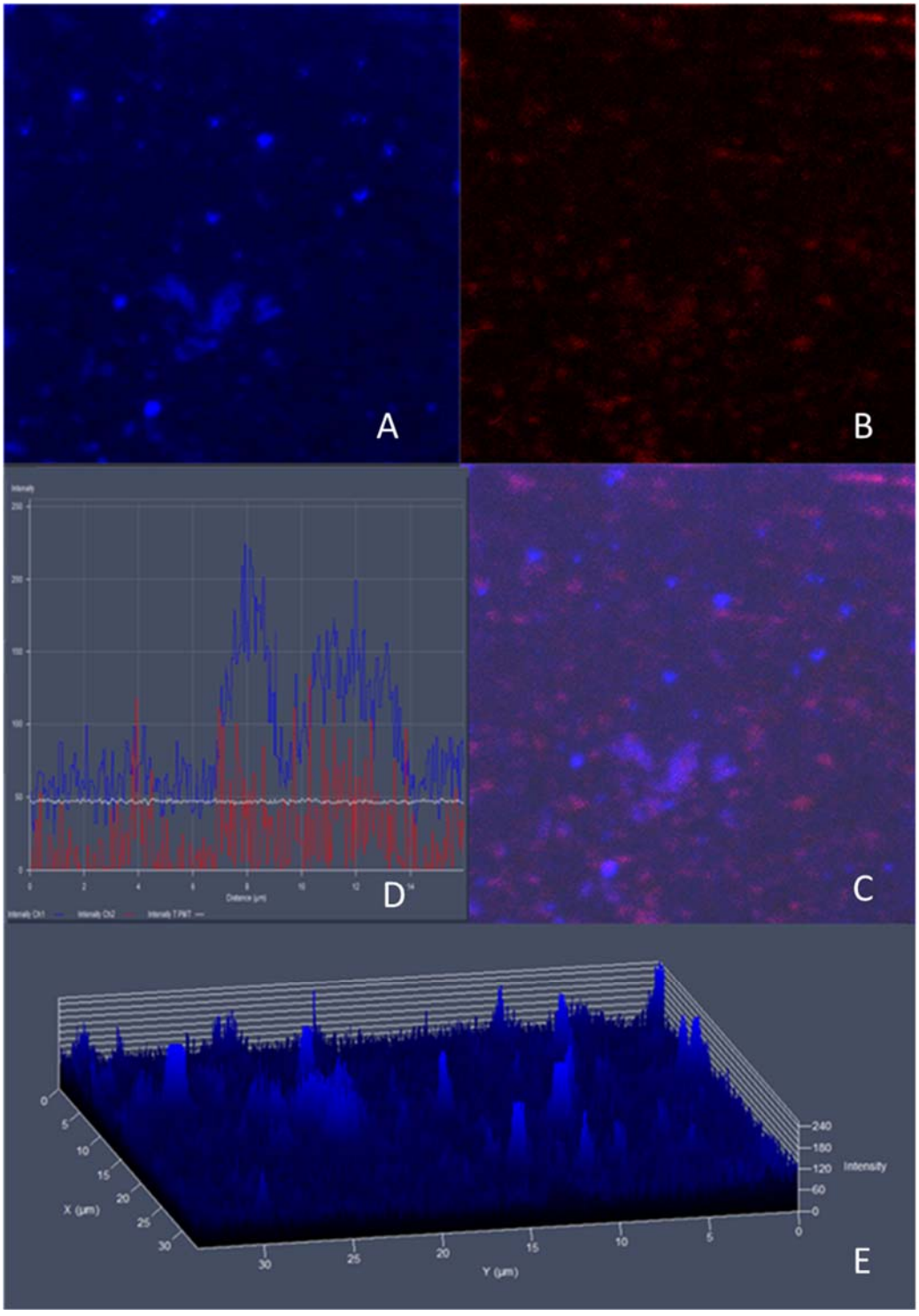


Figure 6: Confocal microscopy images for in-vitro PAN14 two-day old biofilms grown on membrane filters, treated with micafungin (10 mg/ml) and stained with calcofluor white (blue florescence) ***(A)****, ethidium bromide (red florescence)* ***(B)****, merged images of A and B* ***(C)****, fluoresce intensity distribution profile* ***(D)****, and 2.5D view of biofilm* ***(E)***

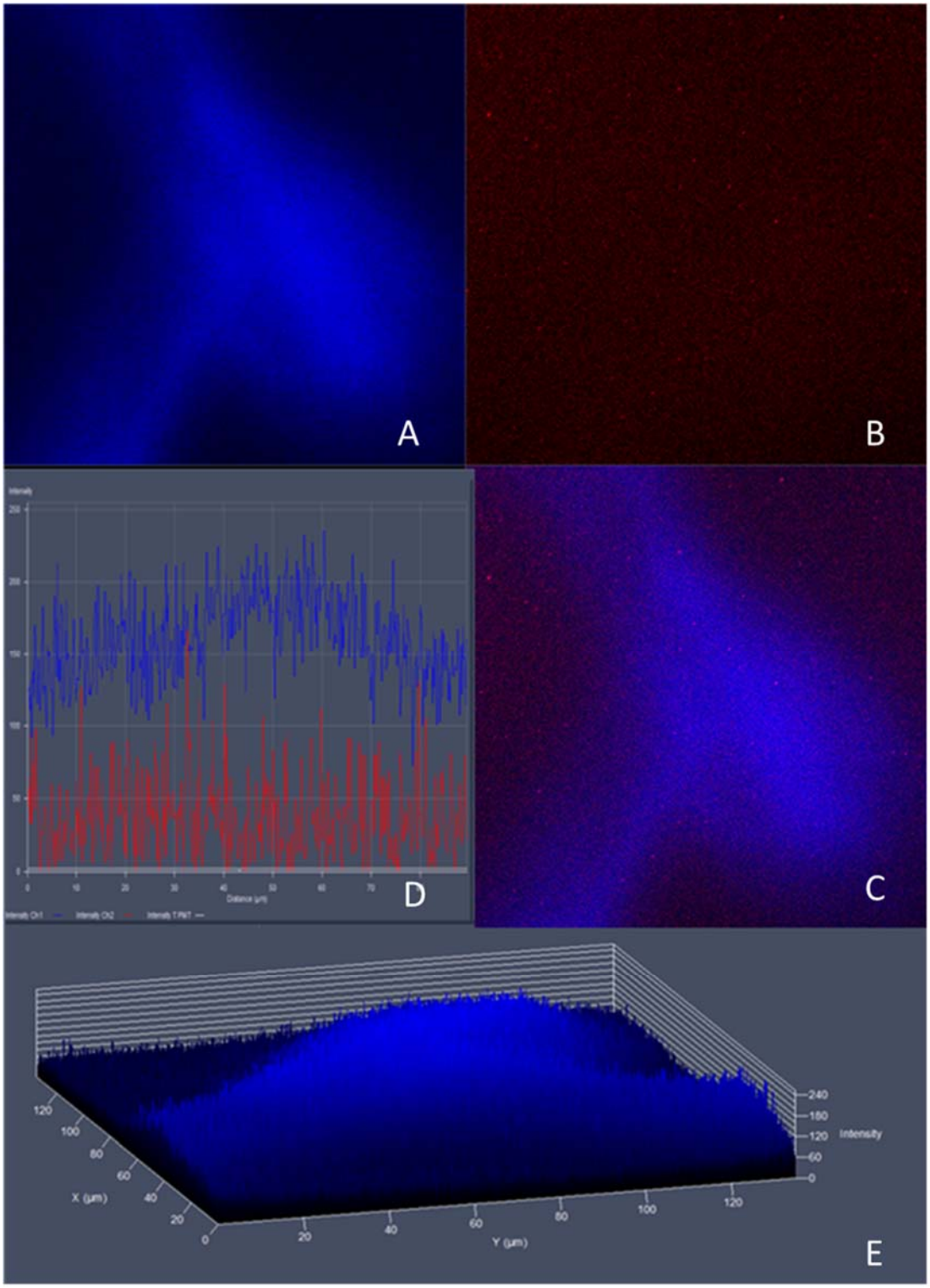


Figure 7: Confocal microscopy images for in-vivo (lung tissue) PAN14 two-day old biofilms grown on membrane filters, stained with calcofluor white (blue florescence of biofilm) ***(A)****, ethidium bromide (red florescence of bacterial cells)* ***(B)****, merged images of A and B* ***(C)****, fluorescence intensity distribution profile* ***(D)****, and 2.5D view of biofilm* ***(E)***

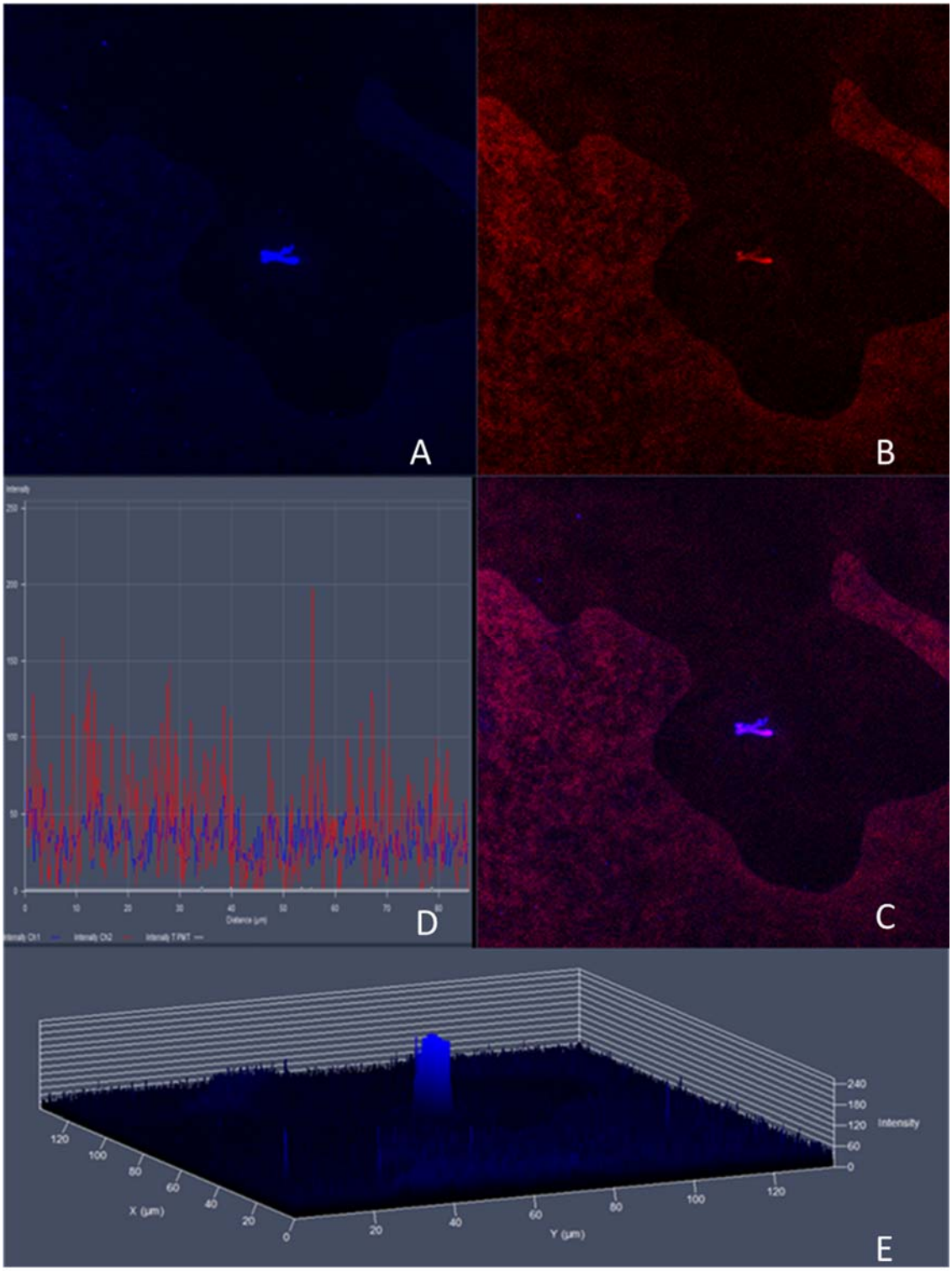


Figure 8: Confocal microscopy images for in-vivo (lung tissue) PAN14 two-day old biofilms, previously treated with micafungin (0.6 mg/ml), grown on membrane filters, and stained with calcofluor white (blue florescence of biofilm) ***(A)****, ethidium bromide (red florescence of bacterial cells)* ***(B)****, merged images of A and B* ***(C)****, fluorescence intensity distribution profile* ***(D)****, and 2.5D view of biofilm* ***(E)****.*

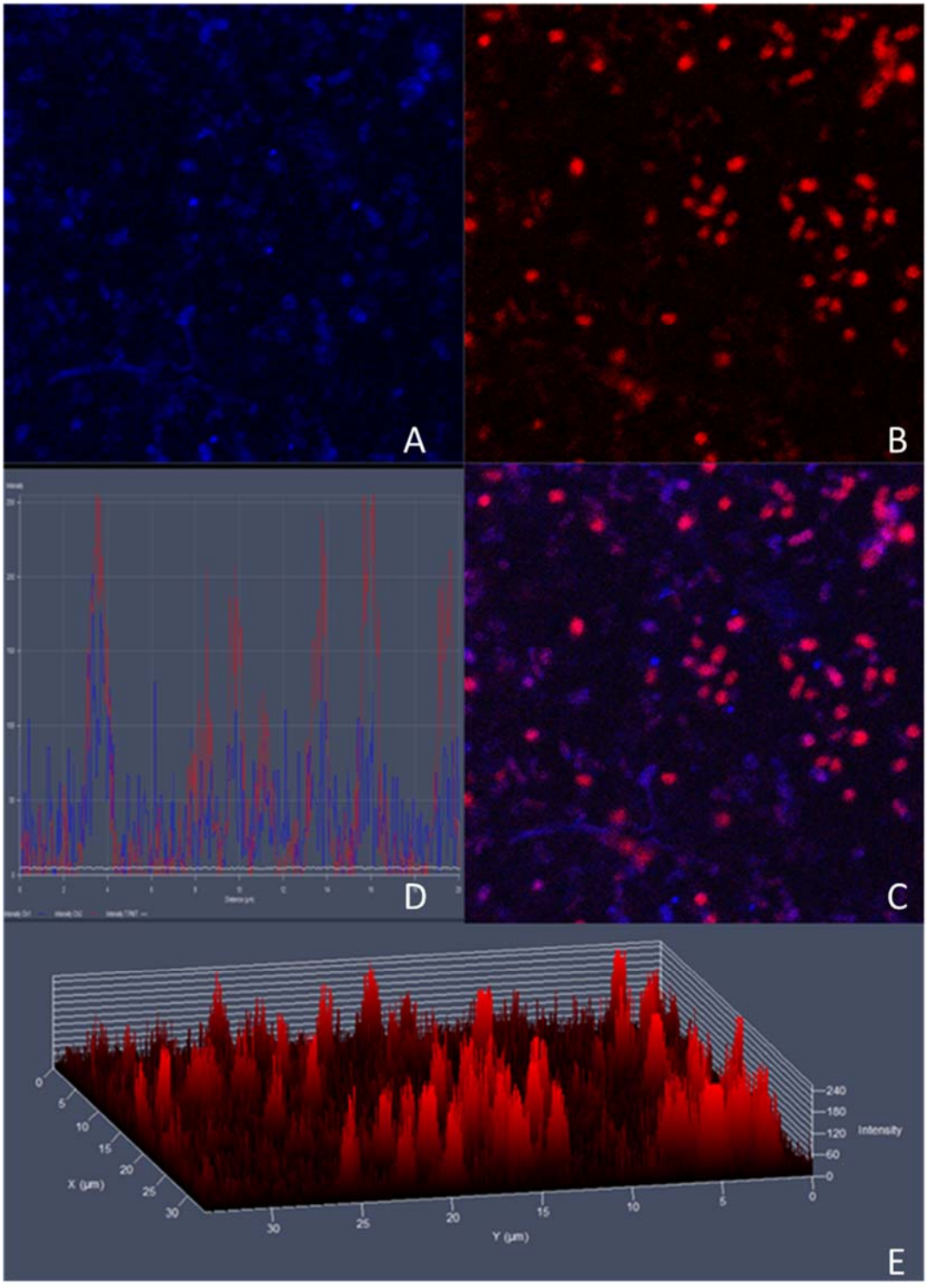


Figure 9: Confocal microscopy images for in-vitro non-biofilm forming E.coli grown on membrane filters, and stained with calcofluor white (blue florescence of biofilm) ***(A)****, ethidium bromide (red florescence of bacterial cells)* ***(B)****, merged images of A and B* ***(C)****, fluorescence intensity distribution profile* ***(D)****, and 2.5D view of biofilm* ***(E)****.*

CHAPTER V
DISCUSSION

In a previous study done by Bazzi et al., the *in-vitro* efficiency of micafungin as a potential agent to inhibit *P. aeruginosa* biofilm-synthesis, was demonstrated (7). Micafungin is an antifungal agent, known to inhibit the synthesis of 1,3-β-D-glucan; a main component of cell wall and biofilm-extracellular matrix in *C. albicans* (102). Since 1,3-β-D-glucan is found in the extracellular component of *P. aeruginosa* biofilm (71), micafungin can act on this component and serve as a potential treatment option for infections caused by *P. aeruginosa*.

In addition to the bacterial intrinsic and acquired resistance to antibacterial agents, Biofilms provide a protective shield to the core planktonic bacteria, and hence cause a penetration barrier for antibacterial agents. By inhibiting biofilm formation with micafungin, the accessibility of antibacterial agents will not be hindered by any physical barrier, and can be delivered to their target and initiate their bactericidal effect. To further dwell into the effectiveness of dual therapy of antifungal along with antibacterial agents on biofilm inhibition, a series of *in-vitro* and *in-vivo* experiments were conducted.

The effect of micafungin independently and in combination with levofloxacin or ceftazidime was studied *in-vitro* with the microtiter plate assay, on PAN14 strain of *P. aeruginosa* for being a highly biofilm producing organism. A decrease in Optical density was observed in samples treated with micafunginn combined with levofloxacin or ceftazidime.

Furthermore, the effect of micafungin on transcription levels of genes encoding for biofilm formation was evaluated *in-vivo*. Gene transcription levels of *pelC, algC* and *ndvB* encoding genes were assessed in treated and untreated BALB/c mice samples by RT-qPCR. Micafungin affected significantly the transcription level of *pelC* encoding gene, with a 270-fold decrease in transcription level, as compared to untreated samples. *Pel* operon includes seven genes *pelA* to *pelG* (103). *PelC* encodes for glycosyltransferases that is important in the formation of a solid-surface-associated-glucose-rich exopolysaccharide matrix (104). Due to down regulation of *pelC* gene, we hypothesize that micafungin inhibits the production of glycosyl transferases, thus preventing the formation of a thick-glucose rich biofilm.

Samples treated with micafungin showed a 74-fold decrease in *ndvB* gene transcription level. *ndvB* gene encodes a glucosyl transferase that is important in the synthesis of 1,3-β-D periplasmic glucan. The latter physically interacts with antibiotic increasing biofilm resistance to treatment (105). Since micafungin inhibits 1,3-β-D-glucan synthase enzyme *in C. albicans* that has a glucosyltransferase role, and given that *P.aeruginosa* has 1,3-β-D-glucan in its cell wall, these results suggest that micafungin inhibits the action of glucosyltransferase, essential in glucan production in *P. aeruginosa,* in a similar manner as in *C. albicans* via non-competitive inhibition.

Our data also showed a 2-fold decrease in transcription levels of *algC* gene which encodes for phosphomannomutase enzyme (PMM), important in the biosynthesis of the LPS and alginate (106). Our *in-vivo* RT-qPCR data correlates with previous work done by Bazzi et

al. (7), where a significant down regulation of transcription level for *pelC*, *ndvB* and *algC* genes was observed.

During the course of RT-qPCR it was noted that 72 hours post injection with 2LD50 of the biofilm forming PAN14 strain along with micafungin and levofloxacin or levofloxacin alone, 100 percent survival of BALB/c mice was seen. This is in concordance with previous data on survival rates with micafungin in combination with levofloxacin that showed a 60% survival rate (109). Based on these results, micafungin prevented the subsequent biofilm formation during the course of infection, and levofloxacin was capable of killing growing bacteria. Our data revealed that ceftazidime was not as efficient as levofloxacin, alone and in combination with micafungin, since 55% of mice died 24 hours after treatment with ceftazidime or micafungin and ceftazidime. The ineffectiveness of ceftazidime is due to either the slow-growing bacteria within biofilms, or the restriction in antibacterial penetration through the biofilm (79).

The survival of mice combined with in-vivo RT-qPCR showed that micafungin inhibits the biofilm-encoding genes (*pelC*, *ndvB* and *algC*), subsequently causing levofloxacin to reach its target and initiating its therapeutic efficacy against biofilm-related infection.

To further explore the *in-vitro* and *in-vivo* physical aspects of *P. aeruginosa* biofilms in treated and untreated samples, bacterial cells and exopolysacchride (EPS) components of biofilm stained with ethidium bromide (red fluorescence) and calcofluor white (blue fluorescence), in membrane –bound biofilm, were visualized. The results showed a decrease in the thickness of biofilms after comparing several Z-stacks from different fields in treated and untreated samples.

As phenotypically observed, treatment with micafungin reduced significantly biofilm thickness *in-vitro* and *in-vivo*. Physical reduction in biofilms by Z-stacking using CSLM revealed a 16.8 % reduction in the thickness of biofilms after treatment with micafungin *in-vitro*. While, a 64% reduction in the thickness of biofilms post treatment with micafungin in lung tissue and blood respectively. The observed reduction in *in-vivo* samples is significantly higher than *in-vitro*. This suggests that micafungin is diminishing the mechanical shield surrounding the bacteria and making the diffusion of antibacterial agents easier to access core planktonic and exerts its bactericidal effect on bacteria within a biofilm. This is in concordance with transcription level experiments of biofilm forming encoding genes.

Nutrient restrictions, poor antibiotic penetration, and slow bacterial growth, characterizes biofilm-infections. Moreover, bacterial biofilms are more resistant to killing by antibacterial agents than planktonic cells. This is due to the presence of tolerant persister cells(107), reduced metabolic rates (31), bacterial growth-arrest due to starvation, and limited diffusion of antibacterial agents (108).

As a conclusion, biofilm eradication requires an intensive research to select the proper antimicrobial agents that interfere with biofilm development and architecture. Our data showed that micafungin significantly inhibited biofilm-encoding genes, thus decreasing the biofilm thickness and enabling levofloxacin that showed an important therapeutic efficacy against *P. aeruginosa*, to reach growing bacteria and have an efficient bactericidal effect. Future approaches should focus more on tagging *P. aeruginosa* with GFP and visualizing the effect of combination therapy.

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