Title: In vitro analyses of mild heat stress in combination with antifungal agents against Aspergillus fumigatus biofilm

Running title: mild heat stress against A. fumigatus biofilm

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Abstract

Aspergillus fumigatus biofilms still present a challenge for effective treatment in clinical settings. While mild heat stress has been introduced as a treatment for infectious diseases, the effectiveness of mild heat stress on A. fumigatus biofilm formation and antifungal susceptibility is still unknown. In the present study, confocal laser scanning microscopy (CLSM) was used to image and quantify Aspergillus fumigatus biofilm formation under three different regimens of continuous mild heat stress: at 37, 39, and 41°C respectively. Furthermore, fungal growth has been investigated under the above conditions in combination with antifungal drugs (Amphotericin B (AMB), Micafungin (MCF), Voriconazole (VOC)) at early and late stages. CLSM analysis showed that higher temperatures induce earlier germination and greater hyphal elongation, but poorer polar growth and reduced biofilm thickness. In the early stage of biofilm formation, the combination of 39 or 41°C treatment with MCF or VOC, produced no visible difference in biomass formation from similar treatments at 37°C with the same drug. Interestingly, AMB treatment at 37°C inhibited early stage biofilm formation to a much greater extent than at 39 and 41°C. At the late stage of biofilm formation, the mild heat treatments at 39 and 41°C with AMB, MCF, and VOC inhibited biomass formation compared to 37°C. The present data shows that mild heat stress has a negative regulation effect on biofilm formation in vitro, and antifungal drug improvement with mild heat treatment at late stage biofilm formation provides useful indications of possible effective strategies in clinical management of
aspergillosis.

**Introduction**

The frequency of systemic mycoses has dramatically increased mainly because of the increased use of invasive medical devices, immunosuppressive therapy, and transplantation. *Aspergillus fumigatus* is the second most common invasive fungal pathogen after *Candida albicans* (1). In some patient populations, *A. fumigatus* is associated with mortality rates ranging from 30% to 90% (2-5). The significance of biofilm formation in fungal infections caused by *Aspergillus* spp. has been noted in bronchopulmonary lavage samples under microscopic examination (6, 7).

Biofilms are structured microbial communities attached to surfaces and encased within a matrix of extracellular polymeric substances (8, 9). The Center For Disease Control and Prevention reported that 65% of human infections are related to the biofilms (10). Compared with planktonic morphologies, this lower-dimensional morphology has increased resistance to antimicrobial agents (11, 12) and enhanced pathogenicity for the host (13, 14). The biofilm morphology of *A. fumigatus* has been demonstrated *in vitro* (9, 15) and its susceptibility to azoles, polyenes, and echinocandins according to CLSI M38-A methodology is almost 1000 times lower than that of planktonic cells (16-18). Combinations of some antifungal drugs themselves or new potential antifungal agents against *A. fumigates* biofilms *in vitro* have shown that synergistic antifungal activity occurred for most common antifungal drugs, but that the susceptibility was lower compared to planktonic cell
morphologies (19-21). With the greater prevalence of antifungal agent resistant strains, the treatment of invasive *A. fumigates* has become more difficult. Therefore, new therapeutic strategies against biofilm-associated mycoses are urgently needed.

Continuous mild heat stress has been introduced in the treatment of muscle injury (22) and infectious diseases (23, 24). Cowen and Lindquist (25) found that the susceptibility of planktonic *C. albicans* cells from clinical isolates to fluconazole is increased by mild heat stress. Cho (26) believes that continuous mild heat stress increases the susceptibility of *C. albicans* biofilm to antifungal drugs such as fluconazole, micafungin (MCF), and amphotericin B (AMB). Given the fact that *A. fumigatus* differs greatly from *C. albicans* with regard to genotype and phenotype, leading to its superior adaptability and more favorable entry, it is reasonable to determine whether antifungal drug susceptibilities are effected by mild heat stress in this pathogenic fungi. To address such a matter, we used confocal laser scanning microscopy (CLSM) to analyze the effectiveness of mild heat stress on biofilm formation in *A. fumigates* for the present study. The particular significance of temperature adaptation for drug effectiveness in *A. fumigatus* is further investigated in vitro by testing antifungal drug susceptibility at early and late stages of biofilm formation after mild heat stress treatment. Our results demonstrate that the antifungal drugs susceptibilities at either stage differ with the application of persistent mild heat treatment. The increased susceptibility to AMB, MCF and
VOC at late stage under high temperature suggests a potentially novel strategy of combining mild heat stress with antifungal agents.

Materials and methods

Strains and conidial preparation

*A. fumigatus* Af293 (ATCC MYA-4609, CBS 101355) was used throughout this study. The isolate was maintained at 4°C on sabouraud dextrose agar (SDA) slopes, and grown on SDA plate at 37°C for 72 h. The cell suspension at $1 \times 10^5$ conidia ml$^{-1}$ in RPMI 1640 was prepared according to the method as previously described (11). All manipulations of cells were carried out in a purifier biological safety cabinet (ESCO).

Antifungal drug preparations

All antifungal agents were purchased in powder form: voriconazole (VOC, Sigma), AMB (Sigma), and MCF (Fujisawa). VOC and AMB were diluted in 100% dimethyl sulfoxide (DMSO) to make a stock solution of 1,280µg/ml. MCF was diluted in sterile distilled water as a 1,280µg/ml stock solution. Each drug was tested for antifungal susceptibility in RPMI 1640 medium, buffered to pH 7.0 with 0.165 M MOPS, and serially diluted twofold into final concentrations ranging from 0.06 to 2.0µg/ml. Cultures without antifungal agents at each temperature in RPMI 1640 medium were used as controls.
Biofilm visualization and thickness measurement

*A. fumigatus* A293 biofilms were formed on coverslip in polystyrene, flat-bottomed, 24-well tissue culture plate (Corning). Biofilm phenotypes observed at 39°C and 41°C, respectively, are compared to the phenotypes at 37°C. A 13 mm-diameter sterile plastic cell culture coverslip (Fisher) was used as a growing surface for the biofilm formation that was inserted into the 24-well tissue culture plate. Approximately 500μl of the *A. fumigates* cell suspension (total of 1.2×10⁵ cells) described above was added to each well. Subsequently, the plates were incubated at 37, 39, and 41°C respectively. The biofilms were checked at the following time points of 2, 4, 6, 8, 10, 16, 18, 24, 48, and 72 h. The temperature stabilization of the culture was guaranteed by continuous monitoring the incubator.

The biofilms on the coverslips were washed by PBS at each indicated time point and then stained with FUN-1 (Invitrogen), thereby binding the intravacuolar structures of the fungal cells according to the manufacturers’ instructions. For biofilm visualization, 200μl of FUN-1 solution in concentration of 25μmol/L was added to the surface of each biofilm and then incubated at 37°C in dark for an additional 20 min. The biofilm was washed again with PBS and mounted on a slide. CLSM (OLYMPUS FV1000) was used to examine the fluorescent filamentous biomass. An excitation wavelength of 488 nm using an argon ion laser at 200× magnification was used to examine the biofilms. The hyphal length was measured by means of software provided by the manufacturer. The thickness of the biofilm was measured at 1μm intervals along the z-axis of the sections taken parallel to the x-y plane.
Three-dimensional images were assembled using software (Olympus fluoview ver.3.1 viewer) provided by CLSM. All experiments were conducted in triplicate.

**Drug susceptibility at early stage of biofilm formation**

*A. fumigatus Af293* biofilm was carried out according to a partially modified method by Pierce et al (27). Approximately, 200μl of *A. fumigatus* cell suspension (4.8×10⁴ cells in total) was added to the 96-well tissue culture plates. The cells were allowed to adhere for 4 h at 37°C. After 4 h, the medium was removed, and the wells were washed three times with PBS. The RPMI 1640 medium, with and without antifungal agents (AMB, MCF and VOC), was added to the wells at concentrations ranging from 0.06 to 2.00μg/ml. The plates were then incubated at three temperatures (37, 39, and 41°C) for 24 h, individually. The temperature was monitored consistently. After 24 h of incubation, Biofilm biomass was assessed by XTT assay and CV assay as previously described by Pierce et al. (27) and Li et al. (28). The optical densities (OD) measured by micro-ELISA plate reader at 490 nm for XTT assay and 570nm for CV assay were recorded. All experiments were conducted in quintuplicate.

**Drug susceptibility at late stage of biofilm formation**

Drug susceptibility testing of *A. fumigatus Af293* biofilm was carried out following the method of Pierce et al (27). About 200μl of cell suspension of *A. fumigatus* (4.5×10⁴ cells in total) was added to 96-well tissue culture plates (Corning), and then all
of the plates were incubated at 37°C for 24 h. After 24 h the medium was removed and the biofilms were washed three times with sterile PBS without touching the biofilms. Fresh RPMI 1640 medium with and without the antifungal agents was added as described above. The plates were then incubated at 37, 39, and 41°C, respectively, for an additional 48 h. Then the medium was discarded and the plates were washed three times by PBS. Biofilm biomass was assessed by XTT assay and CV assay as described above. All experiments were conducted in quintuplicate.

**Statistical analysis**

All assays were performed on at least three independent occasions. Statistical analyses were performed as mean ± standard deviations (SD). Differences between groups were tested using a Student’s *t*-test with values of *P* < 0.05 considered to be statistically significant.

**RESULTS**

**The dynamic of biofilm production under different temperatures**

To better understand whether mild heat stress has the potential to affect biofilm formation and interfere with drug susceptibility in *A. fumigatus*, we first monitored the process of biofilm formation at three different temperatures. A host temperature of 37°C was assigned as the control for entire study, and a persistent mild heat treatment was set up at 39°C or 41°C. The thickness of biofilm formed by *A.*
*fumigatus* was measured quantitatively by using CLSM after staining with FUN-1. As shown in Figure 1, the thickness of biofilm produced at the higher temperatures exceeded that of the control temperature, with 82.4\(\mu\)m\(\pm\)7.1\(\mu\)m \((P < 0.05)\) at 39°C and 118.8\(\mu\)m\(\pm\)3.5\(\mu\)m \((P < 0.05)\) at 41°C after 16 h. The difference in biofilm production between 39°C and 41°C was also significant \((P < 0.05)\) at 16 h (Fig. 1). Conversely, in the time period from 24 h to 72 h, the thickness of biofilm produced at higher temperatures lagged behind that which was formed at 37°C, with 160\(\mu\)m\(\pm\)6.7\(\mu\)m \((P < 0.05)\) at 39°C and 131\(\mu\)m\(\pm\)5.8\(\mu\)m \((P < 0.05)\) at 41°C (Fig. 1). Moreover, the difference in biofilm thickness between 39°C and 41°C was statistically significant \((P < 0.05)\) (Fig. 1). These results indicate that mild heat stress accelerates biofilm production at early stages \(< 16\) h), but this early faster growth will not remain at 24 h. Therefore, while 37°C is optimal temperature for maximal biofilm development, the mild heat treatment alleviated this process effectively at 24 h.

Apparently, the dynamics of biofilm formation in *A. fumigatus* were affected differently with different temperature in CLSM studies as observed above, especially at time period from 16 to 24 h. To understand roles of the early events on the biofilm development, we then examine the adherence rate and morphologic features at early stage of biofilm formation. At 4 h, we found that the conidia began to adhere to the supporter regardless of the set temperature as shown in Figure 2A. Compared to 37°C, the adhesion rate of conidia to a coverslip at 39°C or 41°C is not
significantly different from a statistical point of view ($P > 0.05$) (Fig. 3A). Compared to 37°C, the conidial concentration on a coverslip at 39°C or 41°C is not significantly different from a statistical point of view ($P > 0.05$) (Fig. 3B). At 8 h, adhered conidia began to germinate for all 3 temperatures. However, the germination rates of the conidia were increased if the temperature was elevated. In Figure 3C, the germination rates at 8 h were shown as 8.56±1.04%, 13.2±1.4% and 19.88±1.9% for 37 °C, 39°C and 41°C, respectively. At same time period (8 h – 10 h), the hyphal length formed at 39°C or at 41°C in Figure 3D was much longer than the one at 37°C. The higher germination rate and longer germination tube under mild heat treatment at early stage of biofilm development is consistent with a thicker biofilm at 16 h as we mentioned above. However, after 10 h (Fig. 2C), the polar growth of hyphal was clearly disrupted by mild heat stress (39°C or at 41°C) which correlated to a less compact biofilm structure after 24 hours observation in Figure 2F. Taken together, these data indicate that the polar growth of conidia at 37°C optimizes biofilm thickness and density although the higher temperature leads to faster germination rates and adherence at an earlier stage of biofilm formation.

**Early stage of biofilm is resistant to AMB at 39°C and 41°C**

Our next focus is on drug susceptibility in the biofilms developed at different temperatures. For the study of the early stages, AMB, MCF and VOC at concentration from 0.06 to 2.0 μg/ml was introduced at the early stage of biofilm formation (4 h) at all 3 set temperatures. Growth inhibition under drug treatment was
measured by both XTT assay and CV assay for each drug. As shown in Figure 4, mild heat stress at 39°C or 41°C shows no effect on biofilm formation with the antifungal drugs MCF or VOC within the range of concentrations tested (0.06 to 2.00µg/ml) in CV assay and XTT assay, \( P > 0.05 \) when compared to 37°C (Fig. 4B1, 4B2, 4C1 and 4C2). There was also no statistical difference \( (P > 0.05) \) in biofilm formation in the presence of MCF or VOC between 39°C and 41°C. However, the biomass of cells experiencing AMB concentrations ranging from 0.06 to 0.50µg/ml at 39°C or 41°C were not reduced in either the CV or the XTT assay, demonstrating statistical significance \( P < 0.05 \) when compared to the 37°C results in Figures 4A1 and 4A2. Within the same range of AMB concentrations, mild heat stress at 39°C produced a greater reduction in biofilm formation than at 41°C in the XTT assay \( (P < 0.05) \), but not in the CV assay \( (P > 0.05) \). The different effects seen with the two methods of assays here may be due to their difference in detecting principle (29). The XTT assay detect living cells only, and CV assay stains both the living and dead cell. Therefore, these subtle differences maybe come from the dead cells in CV assay.

With the higher concentration of antifungal drugs AMB (1.0 to 2µg/ml), mild heat stress at 39°C or 41°C shows no effects on biofilm formation compared to 37°C in both CV and XTT assays \( (P > 0.05) \). The AMB tolerance seen in the early stages of biofilm formation at 39°C or 41°C occurred only with the lower concentrations (0.06 to 0.50µg/ml) of the drug.
Late stage biofilm is sensitive to AMB, MCF and VOC at 39°C and 41°C

The drug sensitivities of AMB, MCF and VOC were also tested on late stage biofilm under mild heat circumstances. As shown in Figure 5, the *A. fumigatus* biofilm formed after 24 h of pre-grown at 37°C displayed different drug inhibitory patterns when subjected to mild heat environments (39°C and 41°C) after formation. Compared to persistence at 37°C, the inhibitory effects of AMB and MCF in the range of 0.06 to 2.0\( \mu g/ml \) were significantly higher in both CV (Fig. 5A1, B1) assay and XTT (Fig. 5A2, B2) assay when tested at 39°C or 41°C \((P < 0.05)\). This relation of hypersusceptibility to mild heat treatment was also observed with VOC in XTT assay (Figure 5C2) in a broad range of drug concentrations (0.06 to 2.0\( \mu g/ml \)), but was only seen in a high range of drug concentrations (0.25 to 2.0\( \mu g/ml \)) with CV assay for VOC testing (Figure 5C1). These results indicate that mild heat stress at 39°C or 41°C increases the sensitivity of late stage biofilm to antifungal drugs AMB, MCF and VOC.

DISCUSSION

*A. fumigatus* is a ubiquitous opportunistic fungus that causes the majority of human aspergillosis cases. *A. fumigatus* infections have undergone a dramatic upsurge recently. Although new antifungal drugs have been introduced, the mortality of *A. fumigatus* infections is still high. Thus, new alternatives to increase the antifungal susceptibility of *A. fumigatus* biofilms are urgently needed. Mild heat stress achieves...
satisfactory efficacy in some diseases. This study tests whether this satisfactory
effect can occur on *in vitro*. Our study is the first to determine the effects of mild
heat stress on *A. fumigatus* biofilms and is also the first to relate those effects to
antifungal susceptibility of biofilms *in vitro*.

Biofilm formation consists of initial adherence, conidial germination, filamentous
growth, hyphal intertwining and matrix growth. Our results show that the adherence
phase proceeds similarly no matter what temperature is used, indicating similar
adherent rates for 37°C, 39°C and 41°C, however the conidia behave differently at
the phase of morphogenetic conversion for the three temperatures, when conidia
switches to the filamentous phase. The germination times were much earlier and the
germination rates higher at 39°C and 41°C than that at the control temperature of
37°C. This result is similar to that described by Cho et al. in their study of *C. albicans*
biofilm formation (26). We also find that polar growth (i.e., coherent
directional growth) was noticeable only at 37°C. While it is clear that this coherent
polar growth was somehow disturbed at higher temperatures, suggesting that it may
reduce the pathogenicity of *Aspergillus fumigatus*, but the reason is still unclear.
Polarized growth is found mostly in filamentous fungi (30) and is highly correlated
with the pathogenicity of fungi (31). The conidia undergo a brief period of
symmetric growth after germination and continue to grow in this manner until they
form the hyphae and mycelia. Although much progress in investigating the
mechanisms for polar growth has been made, it is still not very clear for the complex
process of cell wall and cell membrane synthesis for polar growth. Among them, some researchers found that maintaining polar growth had a certain relationship with temperature. Zhang (32) reported that the temperature-sensitive \textit{A. fumigatus} strain $\text{Af}cwh41$ showed abnormalities of polarity comparing to wild type strain \textit{Aspergillus fumigatus} YJ-407 when incubating at $37^\circ C$ for 24 h. Similar results were found by Castillo-Lluva (33) for a temperature-sensitive \textit{cdk5} mutant from \textit{Ustilago maydis} in his study, and by Meyer (34) for a temperature-sensitive apical-branching ramose-1 mutant from \textit{Aspergillus nidulans}. However, Chen and Song (35) have found that polar growth at $42^\circ C$ was similar to polar growth at $37^\circ C$ on \textit{Aspergillus nidulans} GFP-cam strain. This result is quite different from ours. One explanation is that as an environmental fungus \textit{Aspergillus nidulans} must retain improved temperature adaptability, which is not needed for the human pathogenic fungi \textit{A. fumigatus}.

Somewhat related to the polar growth status (since more coherent growth can be expected to lead to denser formations), we found that the biofilm was thicker at $37^\circ C$ than those at $39^\circ C$ and at $41^\circ C$ after a 24 h period. Momany (36) claimed that highly polar growth allowed \textit{A. fumigates} to invade blood vessels and tissue. Scheffer (31) also found that polar growth was essential for the ergot fungus \textit{Claviceps purpurea} to penetrate the host. Given these results, it would appear that polar growth may have better growth in the vertical direction. Therefore, it can form a thicker biofilm.
With regard to drug interference in biofilm formation, our results of this study differ from those of Cho (26) when drug resistance is tested at the earliest stage of biofilm formation of 4 h (after spores adhering to the supporter). Cho concluded that mild heat stress increased the effectiveness of antifungal agents in low concentrations against \textit{C. albicans} biofilm formation at 1.5 h after spore application (37, 38).

However, in our study, mild heat stress did not significantly influence the biomass and metabolism of the biofilm formation when either MCF or VOC were used at concentrations ranging from 0.06 to 2.00 $\mu$g/ml. Ji and Yang (39) reported that mild heat stress increased AMB resistance, but reduced VOC resistance, and had no influence on echinocandin susceptibility in planktonic \textit{A. fumigatus} cells. This AMB resistance was also seen in our higher temperature treatment at an early stage of biofilm formation when AMB concentrations were in the range of 0.06 to 0.50 $\mu$g/ml. However, this correlation of AMB resistance with high temperature was not evident when AMB concentrations were higher than 1.00 $\mu$g/ml.

The observation of Ji and Yang (39) in planktonic \textit{A. fumigatus} cells may be due to the precise antifungal mechanism of AMB. At low concentrations, AMB prompts fungal cells to increase DNA and RNA production (40), and therefore may indirectly promote biofilm production. Increasing the drug concentration to 1.00 $\mu$g/ml increased the AMB antifungal effect sufficiently to become lethal against biofilms, suggesting that we may need to avoid situations in clinical settings when the drug dose is low and the host is suffering from fever. The antifungal effects of AMB, MCF and VOC were improved with higher temperature treatment once the biofilm
has been formed after 24 h in this study, indicating that the mild heat stress response of *A. fumigatus* favors drug susceptibility on well-formed biofilm.

The activation of calcineurin and high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) cascade (39, 41) has also been associated with mild heat stress response. Both pathways promote FKS2 expression (42) which is known to reduce susceptibility to MCF in planktonic *A. fumigates* cells (43, 44). However, the precise mechanism for drug susceptibility of *A. fumigatus* biofilm under mild heat stress remains unclear. The growth of biofilm extracellular matrix (ECM) could be one explanation. At 24 h, the biofilm reaches its maximum thickness and then synthesizes the matrix in succeeding 24-h periods (45). The extracellular polymeric substances (EPS) of *A. fumigatus* biofilms usually contain glucose (74%), mannitol (18%), glycerol (5%), trehalose (3%), melanin, proteins (2%) (46). Extracellular DNA (eDNA) accounts for less than 1 % of the ECM composition of *Pseudomonas Aeruginosa* biofilms. It plays an important role in biofilm structural integrity and antifungal resistance (47-50) that can also be found in *A. fumigatus* biofilms (47, 48). The release of eDNA (which is genomic DNA coming from autolysis) of ECM in *A. fumigatus* biofilms was less with higher temperature treatment but phase dependently (8<12<24<48 h) (47) in other’s study. The reason for this situation may be that long term mild heat stress will force chitinase, a regulator of autolysis (51), to lose its activity (52). The evidence shown that the chitinase activity retained only 75±4%, 34±1% and 0%, respectively after 30 min at 40°C, 50°C and 60°C although
it can reach its maximum at 55-65°C (52). Thus, we presume that increasing temperature would decrease the activity of chitinase and the release of eDNA which may increase susceptibility of *A. fumigatus* biofilms to antifungal drugs.

Almost all of the results of the XTT assay agreed with the results of the CV biomass assay and both methods showed high repeatability in our investigation. In antifungal tests on mature biofilm, the CV biomass assay encountered difficulty in distinguishing live from dead cells and the matrix. However, even with these drawbacks the CV biomass assay method is still able to reveal the effect of mild heat stress on the biomass under the influence of antifungal drugs.

In summary, we conclude that mild heat stress affects *A. fumigatus* biofilm independently of any of the antifungal treatments studied here, and also on the antifungal susceptibility of mature biofilm. More animal tests and a greater amount of clinical experience are needed to determine whether this effect can be used in clinical treatment.

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**Legends**

**Figure 1.** The thickness of biofilm at different mild heat stress at 37, 39
and 41°C. The biofilm thickness of *A. fumigatus* was measured by confocal laser-scanning microscopy (CLSM) at different time point as indicated in the graph. Means of thickness from three independent experiments are shown. Bar represents standard deviations.

**Figure 2.** The images of *A. fumigates* Af293 biofilm formation at 37, 39 and 41°C obtained by CLSM. The cells were stained with FUN-1. Three-dimensional (3-D) images (F row) depict the differences of biofilm thickness with different heat stress treatment (37, 39 and 41°C). The original magnification, was ×200 and the scale bar = 100 μm on the images.

**Figure 3.** The dynamic of *A. fumigatus* cells in the process of biofilm formation at different mild heat stress. The results were quantified in triplicate using FUN-1 staining. The values that are significantly different by *Student's* t-test are indicated by a bracket with asterisks, as follow: *, *P* < 0.05; **, *P* < 0.01. (light bars represent for 37°C, light-fray bars for 39°C and dark-gray bars for 41°C). (A) The adherence rates; the rates were also normalized with the cells number observed in CLSM by the followings formula (amount of cells adhered to the coverslips) / (the total number of cells inoculated in the tested well). (B) The concentration of cells on the coverslips was calculated by the number of cells in CLSM assay by the following formula (amount of cells in the photographed area) / (the area size). (C) The germination
rate of *A. fumigatus* cells was quantified with CLSM by the following formula
(amount of germination cells)/ (the total number in the area). (D) The hyphal
lengths of each experimental condition at 8h and 10h were measured by aCLSM
software.

**Figure 4. The antifungal drug susceptibilities at early stage of biofilm formation.** The antifungal activities of AMB, MCF and VOC against early
stage of biofilm at different mild heat stress (37, 39 and 41°C) were tested in
RPMI-1640. The results were obtained by crystal violet assay at OD570nm (the
left image) and XTT assay at OD490nm (the right image). Bar represents
standard deviations. The data are shown as mean ±SD values for 5 samples.

**Figure 5. The antifungal drug susceptibilities at late stage of biofilm formation.** The antifungal activities of AMB, MCF and VOC against later stage
of biofilm at different mild heat stress (37, 39 and 41°C) were tested in
RPMI-1640. The results were obtained by crystal violet assay at OD570nm (the
left image) and XTT assay at OD490nm (the right image). Bar represents
standard deviations. The data are shown as mean±SD values for 5 samples.