Detailed comparison of *Candida albicans* and *Candida glabrata* biofilms under different conditions and their susceptibility to caspofungin and anidulafungin

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Candida biofilm development can be influenced by diverse factors such as substrate, culture medium, carbohydrate source and pH. We have analysed biofilm formation of *Candida albicans* SC5314 and *Candida glabrata* ATCC 2001 wild-type strains in the presence of different media (RPMI 1640 versus YNB) and using different pH values (pH 5.6 or 7.0). We determined adhesion and biofilm formation on polystyrene, changes in the expression of adhesin genes during these processes and the susceptibility of mature biofilms to echinocandins. Biofilms formed on polystyrene by both *Candida* species proved to be influenced strongly by the composition of the medium rather than pH. *C. albicans* and *C. glabrata* formed thicker biofilms in RPMI 1640 medium, whereas in YNB medium, both species manifested adhesion rather than characteristic multilayer biofilm architecture. The stimulated biofilm formation in RPMI 1640 medium at pH 7.0 corroborated positively with increased expression of adhesin genes, essential to biofilm formation in vitro, including ALS3 and EAP1 in *C. albicans* and EPA6 in *C. glabrata*. The thicker biofilms grown in RPMI 1640 medium were more tolerant to caspofungin and anidulafungin than YNB-grown biofilms. We also observed that mature *C. glabrata* biofilms were less susceptible in RPMI 1640 medium to echinocandins than *C. albicans* biofilms. Environmental conditions, i.e. medium and pH, can significantly affect not only biofilm architecture, but also the expression profile of several genes involved during the different stages of biofilm development. In addition, growth conditions may also influence the antifungal-susceptibility profile of fungal populations within biofilm structures. Therefore, before designing any experimental biofilm set-up, it is important to consider the potential influence of external environmental factors on *Candida* biofilm development.

INTRODUCTION

*Candida albicans* is considered the most commonly isolated human fungal species, followed by *Candida glabrata* (Segireddy et al., 2011). The importance of both species has increased dramatically because of their ability to form biofilms on different types of implant (Ramage et al., 2006). In comparison with the biofilm architecture of *C. albicans*, which is composed of yeast cells, hyphae and pseudohyphae, *C. glabrata* biofilms exist as a multilayer structure of yeast cells; in both cases, the biofilms are embedded in extracellular matrix. Despite their structural diversity, biofilm formation by both *Candida* species is influenced by the choice of biomaterial, medium and carbohydrate source and concentration. Additionally, pH plays an important role, as *Candida* spp. may colonize different niches with respect to the environmental pH (Biswas & Chaffin, 2005; Schmidt et al., 2008). To date, there is no strict recommendation for a specific medium...
suitable for in vitro biofilm experiments that would strictly resemble the composition of the human fluids surrounding the device in vivo. Recently, Uppuluri et al. (2009) characterized C. albicans biofilms in synthetic urine medium that mimics the physiological conditions during biofilm development on urinary catheters.

The development of C. albicans and C. glabrata biofilms involves the regulation of specific genes, expression of which can be affected by the environment. For instance, in vitro strains of C. albicans adhesins Als1, Als3 and Eap1 are highly expressed in biofilms in vitro (Li et al., 2007; Nailis et al., 2010) and in vivo (Nett et al., 2009; Ricicova et al., 2010). In C. glabrata, Epa6 has been shown to be active against mature C. glabrata biofilms formed in vitro (Iqai et al., 2005).

This study provides a detailed characterization of the influence of two different culture media (RPMI 1640 and YNB) at two different pHs (pH 5.6 and 7.0) on C. albicans SC5314 and C. glabrata ATCC 2001 adhesion, biofilm development and susceptibility to echinocandins.

**METHODS**

**Strains.** For this study, C. albicans SC5314 (Gillum et al., 1984) and C. glabrata ATCC 2001 (American Type Culture Collection) were used. The strains were routinely cultivated on YPD medium (1% yeast extract, 2% Bacto-peptone and 2% d-glucose, supplemented with 2% agar) at 37 °C.

**Biofilm formation.** Biofilms were formed in 96-well polystyrene plates according to Ramage et al. (2001). Briefly, C. albicans and C. glabrata were incubated at 37 °C overnight on YPD plates. Cell suspensions of 1 × 10^6 cells ml^-1 were prepared for both species by counting in RPMI 1640 medium (with glutamine and phenol red, without bicarbonate) buffered with MOPS (Sigma), or in YNB (Yeast Nitrogen Base with amino acids and ammonium sulfate; Difco) buffered with 0.05 M KH_2PO_4/Na_2HPO_4. Both media were supplemented with d-glucose (2% final). Of note, RPMI 1640 medium itself contains 0.2% d-glucose and therefore this medium was supplemented with d-glucose up to 2% as a final concentration. A supplementary table showing detailed composition of RPMI 1640 and YNB media used in this study is available in JMM Online. The pH was adjusted to 5.6 or 7.0. In RPMI 1640 medium, pH 7.0 was achieved by adjusting to 5.6 with a few drops of 1 M HCl. Inoculum of each strain (100 µl) was added to the flat-bottom 96-well polystyrene plate in quadruplicate. After a period of adhesion (90 min) at 37 °C, non-attached Candida cells were removed by two rounds of washing with 1× PBS. One set of plates was analysed immediately after the period of adhesion. A second set of plates was incubated at 37 °C for an additional 48 h in fresh medium. Mature biofilms (48 h) were washed twice with 1× PBS. Remaining cells were evaluated for their metabolic activity by the reduction of XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide], as described by Ramage et al. (2001). Each assay in quadruplicate was reproduced five times.

The mature (48 h) C. albicans and C. glabrata biofilms used for fluorescence microscopy (FM) and confocal scanning laser microscopy (CSLM) were developed on highly adhesive, round tissue-culture coverslips (diameter 13 mm; Sarstedt). One millilitre of cell suspension (1 × 10^6 cells ml^-1) prepared in RPMI 1640 or YNB medium at pH 5.6 or 7.0 was added to the wells, each containing a coverslip, and incubated at 37 °C for 90 min. Then, the coverslips were gently washed twice with 1 ml 1× PBS, placed into a clean 24-well tissue-culture plate and covered with fresh medium for an additional 48 h. The mature biofilms were further washed twice with 1× PBS before visualization.

**FM.** Visualization of the attached biofilms and of the morphological composition of the biofilm top layer was carried out by FM. Intact coverslips with attached biofilms were transferred to clean 24-well tissue-culture plates. Each well contained 500 µl 1× PBS with 20 µl Calcofluor White (0.05%, v/v) (Sigma). Biofilm structures were observed with a Zeiss Axiosplan 2 fluorescence microscope. Images were acquired with a Zeiss AxioCam HRm camera using Axiovision 3.0 software (Carl Zeiss). FM was performed three times independently. Each time, two coverslips were used per condition.

**CSLM.** The three-dimensional (3-D) structure of mature C. albicans and C. glabrata biofilms grown under different conditions was determined by CSLM. The biofilms formed on coverslips were transferred gently to a clean 24-well tissue-culture plate and stained with 50 µg Concanavalin A–Alexa Fluor 488 conjugate ml^-1 (C-11252; Molecular Probes) in PBS. The samples were incubated for 1 h at 37 °C in the dark with gentle agitation. Confocal images were acquired according to Ricicova et al. (2010). The vertical (xz) sections or side views of the 3-D reconstructed images were used to determine biofilm thickness and architecture. The thickness was estimated from the outer edges of the area where fluorescent signal gain intensity was above half of its maximum. CSLM was performed twice independently. Each time, two coverslips were used per condition.

**Quantitative real-time PCR.** Mature (48 h) Candida biofilms were formed in six-well tissue-culture plates to obtain the necessary amount of biofilm biomass. The biofilms were scraped off the bottom of the wells by using sterile cell scrapers and washed in DEPC-treated water. Total RNA from biofilms was extracted by using a RiboPure Yeast kit (AM1926; Ambion). Then, 1 µg RNA was treated with amplification-grade DNase I and used for cDNA synthesis using a Reverse Transcription kit (A3500; Promega). Quantitative PCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using a Kapa SYBR Fast kit (Kapabiosystems) according to the manufacturer's instructions. Data for each target gene were calculated and expressed as fold regulation in comparison to the reference gene ACT1 for each condition tested, using the standard curve quantification method (Livak & Schmittgen, 2001). The following primers were used for C. albicans analyses: those for ACT1 and EAPI were described previously (Ricicova et al., 2010). ALS1 and ALS3 primers were designed as described by Green et al. (2005). The sequence of the C. glabrata ACT1 forward primer was 5'- CGGTTTGGACTTCGAACAAGAA-3' and that of the reverse primer was 5'- GTTACCGATAGTGATGACTTGAC-3'. For C. glabrata Epa6, the sequence of the forward primer was 5'- CCAAATGTGACTTGAC-3' and that of the reverse primer was 5'- AGATCGAAACCAGACTTTGAT-3' (K. De Brucker, J. Serneels, E. Boons, T. Schwarzmueller, J. Quintin, M. Henrigues, K. Kuchler, P. Van Dijck & H. Tournu, unpublished data).

The quantitative RT-PCR assay was performed twice independently. RNA isolation and cDNA synthesis were done twice from different biofilms.
Biofilm antifungal-susceptibility testing. Caspofungin (Merck) and anidulafungin (Pfizer) were used in this study. The stock solution of caspofungin was prepared in sterile water. Anidulafungin was prepared in 100% DMSO and kept at −20 °C before use. Prior to the experiment, both antifungals were dissolved in corresponding sterile medium according to the NCCLS M27-A3 protocol (NCCLS, 2008).

Susceptibility testing of planktonic cells to caspofungin and anidulafungin was performed according to the NCCLS M27-A3 protocol (NCCLS, 2008). Data were determined as minimal inhibitory concentrations of the drug that inhibit fungal growth by 50% or 90% (MIC50 or MIC90, respectively).

Mature Candida biofilms (24 h) were formed in 96-well polystyrene plates in RPMI 1640 medium, pH 5.6 or 7.0, or YNB medium, pH 5.6 or 7.0, as described above. Solutions of caspofungin (8–0.01562 μg ml−1) and anidulafungin (16-0.03125 μg ml−1) were added to preformed mature biofilms and incubated for an additional 24 h. Biofilms treated only with fresh medium served as untreated controls. Measurement of biofilm cell metabolic activity using the XTT-reduction assay was performed as described above. Antifungal effect was determined by comparing the reduction in the mean ΔA590 of the antifungal-challenged biofilm condition to the unchallenged controls. The results are shown as sessile MIC50 and MIC90 values (SMIC50 and SMIC90, respectively). These values determine the minimal inhibitory concentration of the drug that inhibits the growth of sessile cells by 50 and 90%, respectively. Each assay was performed in quadruplicate each time and repeated three times.

Statistical analyses. For the statistical analyses, Student’s t-test was used. Results were considered to be statistically significant when P<0.05.

RESULTS

Medium composition modulates adherence and biofilm formation more than pH in C. glabrata and C. albicans

Candida biofilm development in the host is affected by variable and sometimes changing environmental conditions, depending on the infection site. Many studies in vitro have reported the use of a multitude of media, substrates and techniques to study biofilms (Biswa & Chaffin, 2005; Schmidt et al., 2008; Uppuluri et al., 2009). Here, we compared the effect of two commonly used media, namely RPMI 1640 and YNB, at two different pH values (pH 5.6 and 7.0) on C. glabrata and C. albicans adhesion and mature biofilm development. Biofilms were formed on polystyrene and assessed by the XTT-reduction assay measured at 490 nm. The results are shown in Fig. 1 and are determined as the mean ± SD of the metabolic activity of adhering or biofilm-forming cells. C. glabrata adhesion properties were independent of the choice of medium or pH (P>0.05) (Fig. 1a), whereas the effect of growth conditions on the ability of C. albicans to adhere was more pronounced (Fig. 1c). C. albicans manifested significantly better adhesion properties when grown in RPMI 1640 medium, compared with its adhesion in YNB medium (P<0.05). Moreover, in RPMI 1640 medium at pH 7.0, C. albicans had significantly enhanced adhesion capabilities compared with those at pH 5.6 (A490=0.566 ± 0.108 and 0.924 ± 0.209, respectively) (P<0.05). However, the pH did not have a significant effect on C. albicans adhesion in YNB (P>0.05). The metabolic activity of C. albicans mature biofilm-forming cells was significantly reduced in YNB medium (A490=0.530 ± 0.072 and 0.660 ± 0.031, respectively) compared with RPMI 1640 medium (A490=1.240 ± 0.188 and 1.410 ± 0.086, respectively) (P<0.05) (Fig. 1d). Despite the fact that pH 7.0 enhanced the yeast-to-hyphae transition, it was not shown to have a significant effect on a mature C. albicans biofilm (P>0.05). Considering C. glabrata biofilm formation (Fig. 1b), the medium is a crucial factor during its maturation. C. glabrata biofilm-forming cells demonstrated significantly less metabolic activity when grown in YNB medium (A490=0.360 ± 0.075 and 0.453 ± 0.044, respectively) compared with the results obtained after growth in RPMI 1640 medium (A490=1.310 ± 0.272 and 1.483 ± 0.274, respectively) (P<0.05). Similar to adhesion, C. glabrata biofilm development was not influenced by the pH tested (P>0.05).

RPMI 1640 medium promotes the development of mature biofilms and induces the yeast-to-hyphae transition in C. albicans

C. albicans biofilms are known to be composed of yeast and hyphal cells, building a layered, attached structure (Ramage et al., 2006). Therefore, we examined the morphology of the cells within a mature biofilm (48 h) by FM and the biofilm thickness with CSLM for the four conditions tested. The biofilm architecture of C. albicans and C. glabrata is illustrated in Fig. 2. The measurements of biofilm thickness are displayed in Table 1. The choice of the different media influenced the biofilm thickness and, additionally, the pH contributed to the yeast-to-hyphae transition in C. albicans. In comparison with the biofilms formed in RPMI 1640 medium (Fig. 2c, d), C. glabrata cells manifested adhesion rather than multilayer biofilm architecture in YNB medium, regardless of the pH (Fig. 2a, b). Therefore, in this particular case, the thickness could not be estimated reliably. On the other hand, in RPMI 1640 medium at pH 5.6, C. glabrata cells formed scattered biofilm layers, with some areas of the substrate remaining uncovered (Fig. 2c). The thickness of the mature biofilms on covered places was approximately 20 μm. Despite this, in the same medium, but at pH 7.0, the polystyrene surface was fully covered with yeast cells and the thickness of the mature biofilm increased to approximately 25 μm (Fig. 2d). Similarly to C. glabrata, C. albicans manifested adhesion rather than biofilm architecture when grown in YNB medium at both pHs (Fig. 2e). Although pH 7.0 enhanced the yeast-to-hyphae transition, which is an important factor for C. albicans multilayer biofilm development, the polystyrene remained unevenly covered (Fig. 2f). These patches of Candida cells were measured and their thickness was revealed to be approximately 50 μm. On the other hand, C. albicans biofilms formed in RPMI 1640 medium at pH 5.6 or 7.0 were composed mainly of hyphae (Fig. 2g, h). During biofilm development in RPMI 1640
**Fig. 1.** RPMI 1640 medium significantly promotes biofilm formation in *C. glabrata* ATCC 2001 and *C. albicans* SC5314. An XTT-reduction assay, determined at $A_{490}$, was used as a measure of metabolic activity of *C. glabrata* ATCC 2001 (a, b) and *C. albicans* SC5314 (c, d) cells during adhesion and biofilm formation on 96-well polystyrene plates in RPMI 1640–MOPS and YNB media, at pH 5.6 or 7.0. The XTT measurements were performed after a period of adhesion (90 min at 37 °C), whilst biofilms were quantified after 48 h at 37 °C. The results were considered as statistically significant when $P<0.05$. SD was calculated from five independent experiments. Each condition was tested in quadruplicate.

**Fig. 2.** Growth medium and pH influence cell morphology and biofilm thickness. FM images display the top layers of 48 h-old biofilms developed on polystyrene coverslips. Mature *C. glabrata* ATCC 2001 (a–d) and mature *C. albicans* SC5314 (e–h) biofilms were stained with Calcofluor White prior to visualization. Magnification, ×40. FM was performed three times on two coverslips used per condition.
medium at pH 5.6, the biofilm thickness was approximately 60 µm (Fig. 2g), whereas in the same medium, but at pH 7.0, the thickness reached approximately 100 µm (Fig. 2h). It is noteworthy to mention that the observations of hazy material covering C. albicans or C. glabrata biofilms formed in RPMI 1640-MOPS medium (Fig. 2c, d, g, f) suggest the presence of extracellular matrix.

These results demonstrate that RPMI 1640 medium induces the yeast-to-hyphae transition in C. albicans, resulting in thicker biofilms compared with growth in YNB medium. In addition, we showed that C. glabrata forms multilayer biofilms on polystyrene only in the presence of RPMI 1640 medium at pH 7.0.

Transcriptional expression of adhesin genes correlates positively with biofilm formation

We anticipated that the expression profile of Candida biofilm-associated genes may change due to exposure to different environmental conditions. We therefore analysed the changes in expression of genes participating significantly in adhesion and biofilm formation, such as C. glabrata EPA6 (Iraqui et al., 2005) and C. albicans ALS1, ALS3 and EAP1 (Nailis et al., 2010; Nobile et al., 2006). The results are displayed in Figs 3 and 4(a, b, c), respectively. The transcript profile of EPA6 was induced significantly in cells growing in RPMI 1640–MOPS medium compared with YNB medium, regardless of the pH used (P<0.05) (Fig. 3). Additionally, significant upregulation was observed in the same medium at pH 7.0 (P<0.05) (Fig. 3). For comparison, we examined the EPA6 expression profile from 48 h-old planktonic cultures (stationary phase) in RPMI 1640 or YNB medium at pH 5.6 or 7.0. Neither RPMI 1640 medium nor the pH promoted EPA6 expression, compared with YNB medium when cells were grown in liquid cultures (data not shown), indicating that the results obtained from sessile populations are biofilm-specific.

Further, we examined the changes in the expression profile of C. albicans adhesion-associated genes such as ALS1, ALS3 and EAP1. The results are displayed in Fig. 4(a, b, c), respectively. None of the three genes analysed was regulated in YNB-grown biofilms, regardless of the pH. On the contrary, RPMI 1640 medium influenced the expression of these adhesins in a pH-dependent manner. The ALS3 gene was upregulated significantly at pH 7.0 compared with pH 5.6 and in YNB-grown biofilms (P<0.05) (Fig. 4b). A similar pattern of expression was observed for the EAP1 gene (P<0.05) (Fig. 4c). In contrast, ALS1 expression was not promoted by the conditions tested (Fig. 4a). It is noteworthy to mention that the data demonstrated above were normalized to ACT1; it is known that the expression of this gene may change during morphogenesis (Debrecik & Ernst, 1993; Michán & Pueyo, 2009), which is characteristic for C. albicans during biofilm development. Because of this, in addition, the data were also normalized to TEF1. Regardless of the housekeeping gene used, the expression levels of ALS1, ALS3 and EAP1 were identical to those observed above (data not shown). This observation excludes the potential influence of housekeeping-gene expression on the evaluation of the expression of tested genes.

RPMI 1640-grown biofilms are less susceptible to echinocandins than YNB-grown biofilms in C. albicans, but not in C. glabrata

The results mentioned above led us to the assumption that the increased biofilm thickness observed for C. albicans and C. glabrata grown in RPMI 1640 medium at pH 5.6 and 7.0 might have an influence on the efficiency of antifungal drugs against biofilms. Hence, we tested the effect of two echinocandins, caspofungin and anidulafungin, against biofilms formed under the different environmental conditions. C. albicans and C. glabrata planktonic cell cultures were susceptible to both echinocandins (Table 2). Following antifungal treatment, biofilms were assessed by XTT measurements, and the data are displayed in Table 3. Both caspofungin and anidulafungin were shown to be very

Table 1. Thickness of mature C. glabrata ATCC 2001 and C. albicans SC5314 biofilms as determined by CSLM

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<tr>
<th>Growth conditions</th>
<th>C. glabrata ATCC 2001</th>
<th>C. albicans SC5314</th>
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<tr>
<td>YNB, pH 5.6</td>
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<td>29–34</td>
</tr>
<tr>
<td>YNB, pH 7.0</td>
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</tr>
<tr>
<td>RPMI 1640, pH 5.6</td>
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</tr>
<tr>
<td>RPMI 1640, pH 7.0</td>
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<td>100</td>
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Fig. 3. EPA6 in C. glabrata ATCC 2001 is upregulated significantly in RPMI 1640 medium. Expression analyses of the adhesion-encoding gene in mature (48 h) C. glabrata biofilms developed in RPMI 1640 or YNB medium at pH 5.6 or 7.0 are shown. The data were normalized to ACT1 using the standard curve quantification method. The increased expression levels of EPA6 were considered as statistically significant when P<0.05. SD was calculated from two independent experiments on different occasions.

Fig. 4. Expression analysis of ALS1, ALS3 and EAP1 in planktonic cells and biofilms. The expression level was standardized to TEF1 and ACT1. The data were normalized to TEF1 and ACT1. The expression level was standardized to TEF1 and ACT1. The data were normalized to TEF1 and ACT1.
on biofilms grown in RPMI 1640 medium at pH 5.6, but to a lower extent. In conclusion, YNB-grown biofilms seem more susceptible to echinocandins than RPMI 1640-grown biofilms.

DISCUSSION

This study gives a detailed comparison between the biofilms formed by *C. albicans* SC5314 and *C. glabrata* ATCC 2001 grown in RPMI 1640 and YNB media. We also elucidated the effect of pH 5.6 and 7.0 during biofilm development, because adaptation to changes in pH during biofilm formation is crucial not only for its development, but also for survival of microbial communities at colonized host sites (Schmidt et al., 2008) as well as on medical devices (Ramage et al., 2006). The two media tested, YNB and RPMI 1640, have remained favourite choices for *in vitro* biofilm formation (Biswas & Chaffin, 2005; Cateau et al., 2008; Chandra et al., 2001). YNB medium is less nutrient-rich for growth of planktonic cells than RPMI 1640 medium, which mimics the composition of human fluids (Chandra et al., 2005). This medium was used previously for *C. albicans* attachment on polyurethane prior to subcutaneous catheter implantation *in vivo* (Ríčiová et al., 2010). Moreover, RPMI 1640 is the standard medium for *in vitro* susceptibility testing of planktonic cells to antifungals according to the NCCLS M27-A3 protocol (NCCLS, 2008). However, there are no studies that have focused strictly on the testing of RPMI 1640 and YNB media on the different steps of biofilm development.

From our study, it is clear that the medium and pH tested did not have any effect on the *C. glabrata* adhesion process, whereas the ability of *C. albicans* to adhere was significantly higher in RPMI 1640 than in YNB medium. This observation could be explained by the induced *C. albicans* yeast-to-hyphae transition process, particularly in RPMI 1640 medium. The yeast-to-hyphae transition has been considered one of the crucial factors involved in *C. albicans* attachment to a surface (Blankenship & Mitchell, 2006). Neutral pH promotes the yeast-to-hyphae transformation and contributes to better *C. albicans* adhesion in our assay in RPMI 1640 medium. In contrast, *C. glabrata* is not able to switch from yeast to hyphae, but it was able to form a multilayer, highly potent biofilm, as described previously (Kucharíková et al., 2010b; Nikawa et al., 1997). In addition to cell morphology, the thickness of the biofilms also showed great variation. Both *C. glabrata* and *C. albicans* formed significantly thicker biofilms in RPMI 1640 medium, whereas in YNB medium, cells proliferated in an adherent manner rather than forming a multilayer biofilm. Strikingly, RPMI 1640 medium seems to be superior for biofilms formed by both species. In addition, pH 7.0 helped to promote a dense biofilm structure composed of a basal layer of yeast cells followed by hyphae in *C. albicans*, or a multilayer structure of the yeast cells in *C. glabrata*.

Because of the striking effect of the different media on biofilm formation and architecture in both *Candida*...
species, we investigated the expression profile of the C. glabrata EPA6 gene and the C. albicans ALS1, ALS3 and EAP1 genes, all involved directly or indirectly in biofilm formation. It was demonstrated previously that C. glabrata EPA6 is transcribed at the highest level during the stationary-growth phase, in contrast to other EPA genes, and that cells in the stationary phase adhered better than metabolically active cells (Iraqui et al., 2005). In all of our assays, C. glabrata cultures were grown up to the stationary phase to ensure that the medium contained totally adherent cell populations. We observed an upregulation of C. glabrata EPA6 during biofilm formation in RPMI 1640 medium at both pHs. This upregulation of EPA6 correlates well with the increased thickness and viability of the biofilms developed in the same medium.

During C. albicans biofilm development, ALS1, ALS3 and EAP1 play roles in different stages of biofilm formation. Yeater et al. (2007) demonstrated, by microarray analyses of biofilms, that the expression of ALS1 increases during the early stages of biofilm development (6–12 h) and declines at the later stages. This documented profile of expression could explain why we did not observe any regulation of ALS1 under any of the conditions tested, as biofilms were grown for 48 h before analyses. However, the data are controversial for that gene, as it is expressed differentially depending on growth in vitro (García-Sánchez et al., 2004; Nailis et al., 2009) or in vivo (Nett et al., 2009; Říčcová et al., 2010) and also depending on the growth conditions. In our assays, the C. albicans ALS3 and EAP1 genes were upregulated in an ascending manner from pH 5.6 to 7.0 in RPMI 1640 medium.

Both caspofungin and anidulafungin were effective against C. albicans mature biofilms, regardless of the environmental conditions used. Similarly to our study, the data of Jacobson et al. (2008) showed the SMIC$_{50}$ for anidulafungin to be $\leq 0.03125$ gm l$^{-1}$ in 28 of 30 C. albicans clinical isolates tested when the biofilm was formed in RPMI 1640 medium. In contrast to the work of Katragkou et al. (2008), we did not observe any indication of paradoxical growth during caspofungin activity against a mature C. albicans biofilm, even when high concentrations (4–8 gm l$^{-1}$) of the drug were administered to biofilms (data not shown). C. glabrata cells forming mature biofilm were less susceptible than the diploid species to caspofungin and anidulafungin in both media tested, albeit with up to half the thickness of C. albicans biofilms. In a previous study, C. glabrata bloodstream clinical isolates displayed a median caspofungin SMIC$_{90}$ of 1 gm l$^{-1}$ (Choi et al., 2007), similar to data that we obtained. Both C. albicans and C. glabrata showed reduced susceptibility to echinocandins when biofilms were formed in RPMI 1640 medium compared with YNB medium, which corresponded with denser biofilm structures. The pH was not shown to have a significant effect on antifungal susceptibility of the cells within the biofilm.

In conclusion, C. albicans and C. glabrata biofilms displayed heterogeneous architecture, expression profiles and susceptibility to caspofungin and anidulafungin when formed under diverse environmental conditions. The medium and its pH influenced adhesion and biofilm development in different manners. Therefore, it is important to clearly define growth conditions prior to and during the biofilm experimental set-up. It is also essential to mimic physiological conditions when performing biofilm formation in vitro. In particular, RPMI 1640 medium contributed to the development of thick biofilm structures in both C. albicans

### Table 2. Susceptibility testing of C. glabrata ATCC 2001 and C. albicans SC5314 planktonic cells to caspofungin and anidulafungin

<table>
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<th>Antifungal agent</th>
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<th>C. albicans SC5314</th>
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<td></td>
<td>MIC$_{50}$</td>
<td>MIC$_{90}$</td>
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<tr>
<td>Caspofungin</td>
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<tr>
<td>Anidulafungin</td>
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<td>0.5</td>
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SMIC$_{50}$ and SMIC$_{90}$ values are shown as gm l$^{-1}$.

### Table 3. Antifungal-susceptibility testing of C. glabrata ATCC 2001 and C. albicans SC5314 biofilms to caspofungin and anidulafungin

<table>
<thead>
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<th>Antifungal agent</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>SMIC$_{50}$</td>
<td>SMIC$_{90}$</td>
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<tr>
<td>Caspofungin</td>
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<td>Anidulafungin</td>
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<td>0.5</td>
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<tr>
<td></td>
<td>YNB, pH 7.0</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td></td>
<td>RPMI 1640, pH 5.6</td>
<td>2</td>
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<tr>
<td></td>
<td>RPMI 1640, pH 7.0</td>
<td>1</td>
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</tr>
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</table>
and C. glabrata. Moreover, during C. albicans biofilm development, the choice of a pH of 7.0 contributed to a faster yeast-to-hyphae transition, which resulted in denser biofilm structures compared with those formed in YNB medium. It is important to take into consideration that different environmental conditions will affect not only biofilm architecture, but also its susceptibility to antifungal agents, which underscores the need for standardized methods.

ACKNOWLEDGEMENTS

Authors’ contributions: S.K. and H.T. participated in the design of the study. S.K. performed all experimental procedures and carried out the data analyses, and drafted the manuscript. H.T. helped to correct the manuscript. K.L. provided pure substances of anidulafungin and corrected the manuscript. P.V. D. helped in the design of the study and corrected the manuscript. H.B. participated in the coordination of the study and helped to correct the drafted manuscript. All authors read and approved the manuscript. This work was supported by the Slovak Ministry of Education (grant VEGA 1/0396/10), the Fund for Scientific Research Flanders (WO.004.06N) and Pfizer (grant 8960). We thank Dr D. Chorvát, Jr. for assistance during CSLM and N. Van Vangoethem for assistance with the figures and tables.

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