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Real-time monitoring of biofilm growth identifies andrographolide as a potent antifungal compound eradicating *Candida* biofilms

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ABSTRACT

Candida species cause life-threatening infections with high morbidity and mortality rates and their resistance to conventional therapy is closely linked to biofilm formation. Thus, the development of new approaches to study *Candida* biofilms and the identification of novel therapeutic strategies could yield improved clinical outcomes. In the current study, we have set up an impedance-based *in vitro* system to study *Candida* spp. biofilms in real-time and to evaluate their sensitivity to two conventional antifungal groups used in clinical practice - azoles and echinocandins. Both fluconazole and voriconazole were unable to inhibit biofilm formation in most strains tested, while echinocandins showed biofilm inhibitory capacity at relatively low concentrations (starting from 0.625 mg/L). However, assays performed on 24 h *Candida albicans* and *C. glabrata* biofilms revealed that micafungin and caspofungin failed to eradicate mature biofilms at all tested concentrations, evidencing that once formed, *Candida* spp. biofilms are extremely difficult to eliminate using currently available antifungals. We then evaluated the antifungal and anti-biofilm effect of andrographolide, a natural compound isolated from the plant *Andrographis paniculata* with known antibiofilm activity on Gram-positive and Gram-negative bacteria. Optical density measures, impedance evaluation, CFU counts, and electron microscopy data showed that andrographolide strongly inhibits planktonic *Candida* spp. growth and halts *Candida* spp. biofilm formation in a dose-dependent manner in all tested strains. Moreover, andrographolide was capable of eliminating mature biofilms and viable cell numbers by up to 99.9% in the *C. albicans* and *C. glabrata* strains tested, suggesting its potential as a new approach to treat multi-resistant *Candida* spp. biofilm-related infections.

1. Introduction

Candida species (*C. albicans, C. krusei, C. parapsilosis, C. glabrata,* and others) are the most common opportunistic fungal pathogens colonizing skin, oral cavity, gastrointestinal, respiratory and urinary tracts in humans [1–3]. The adaptation capacity and colonization of these pathogens are linked to their growth on different surfaces, where they are encased in a self-produced biopolymeric matrix forming biofilms. This increases more than 1000-fold their resistance to first-line antifungal agents compared to planktonic growth of the same strains [4–6]. In addition, *Candida* spp. show high resistance to disinfectants and antiseptics repeatedly used in hospitals, being able to survive and persist in healthcare facilities [7,8]. For this reason, biofilm-related antifungal resistance contributes enormously to implantable medical device-related infections and healthcare-associated outbreaks [9–11].

The *in vitro* activity of antifungals is a key parameter for the good evolution of patients. Currently, amphotericin B, 5-fluorocytosine, azoles, and echinocandins are the four groups of drugs that can be used in the treatment of invasive candidiasis, but the last two families are the most effective and the best tolerated, which is why they are the most used in clinical practice [9,12,13]. While fungistatic azoles inhibit ergosterol synthesis, leading to cell membrane damage, fungicidal echinocandins interfere with fungal cell wall biosynthesis [14,15]. Recent studies have demonstrated that the resistance to these antifungal agents is highly associated with biofilm formation and the metabolic state of *Candida* spp. cells embedded in biofilms [16–18]. In addition, it has been observed that biofilm-grown *Candida* spp. can upregulate efflux pumps to survive high concentrations of drugs [19–21], and the

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use of high dosages of conventional antifungals can lead to both multi-resistance and severe health complications such as arrhythmias, renal failure, and kidney damage among others [5,15]. Recent studies also underline the high rate of strains resistant to antifungals in patients with COVID [22]. Therefore, there is a need to investigate novel, effective and rapid approaches to treat fungal biofilm-related infections [5,16]. This could be reached using already known antifungal combinations and/or searching for novel natural or synthetic compounds that attribute anti-biofilm properties alone or in combination with conventional antifungals. For example, various polyphenols, including flavonoids, terpenoids and others, have been shown to interfere with *Candida* ssp. biofilm formation and proliferation *in vitro* [23]. However, most of these compounds cannot eradicate mature biofilms [16,23].

Andrographolide is a natural diterpene lactone isolated from *Andrographis paniculata* plant, commonly used in oriental medicine [24,25]. This compound was proposed as effective in treating fever, influenza, and other life-threatening diseases, including dysentery, malaria, various forms of cancer and respiratory infections [26–28]. Andrographolide was shown to have *in vitro* inhibitory effect against staphylococcal biofilms, including both *Staphylococcus epidermidis* and methicillin-resistant *S. aureus,* suppressing quorum sensing (QS) systems [28]. Similar effect on the inhibition of different genes involved in elastase and pyocyanin synthesis was also observed in *Pseudomonas aeruginosa* [29–31]. However, the antifungal effect of this compound against fungal biofilms remains elusive.

Standard protocols to study fungal biofilms and evaluate antifungal sensitivity are based on end-point measures where biofilm growth is quantified by crystal violet staining [32]. However, these methods have limited reproducibility due to human manipulation, are influenced by the point at which biofilm growth is stopped and lack information about biofilm growth dynamics [32,33]. This has been solved in bacterial biofilms using real-time measurements derived from time-lapse confocal microscopy or electric impedance measurements [34,35]. The latter is based on the fact that bacteria growing attached to electrodes impede an electrical flow, and this impedance has been shown to the proportional to biofilm mass and to correlate well with other classical measures [36,37]. In the current manuscript, we first set up an impedance-based method to monitor fungal biofilm formation in real-time. We have also evaluated the efficacy of commonly used azole and echinocandin antifungals groups (fluconazole and voriconazole; and micafungin and caspofungin, respectively) in the prevention of *Candida* spp. biofilm formation, as well as the echinocandins effect on pre-formed, mature 24 h biofilms. Finally, we assessed the antifungal properties of andrographolide on planktonic *Candida* spp. growth and on their biofilm formation and eradication, both alone and in combination with micafungin. Obtained results were confirmed using viable cell counting and scanning electron microscopy, in order to establish the efficacy of the different compounds and their joined potential as treatment for *Candida* spp. biofilms-associated infections.

2. Materials and methods

2.1. Strains and growth conditions

Fungal strains used in the current manuscript are listed in Table S1. The strains were isolated from vaginal exudates at the Microbiology Department of Alicante General Hospital (Alicante, Spain) and cultivated on YPDA (Yeast Peptone Dextrose Agar) plates. For biofilm assays, *Candida* spp. strains were grown in YPD (Yeast Peptone Dextrose), YNB (Yeast Nitrogenase Base) or RPMI1640 with 165 mM MOPS liquid media alone or supplemented with additional 2% of glucose when needed, at 30 °C at 120 rpm overnight.

2.2. In vitro real-time yeast biofilm growth monitoring

Real-time *Candida* spp. biofilm growth analysis was performed in 96-well E-plates using an xCELLigence SP equipment (Agilent) according to the manufacturer's instructions [38]. To set up the system for yeasts, three different growth media were tested: YPD (Yeast Peptone Dextrose), YNB (Yeast Nitrogenase Base), and RPMI1640 with 165 mM MOPS. All three growth media were used without additional glucose or were enriched with 2% of additional glucose when needed. Briefly, 100 μL of growth media (with or without additional glucose) were added into the E-plates wells in triplicate for background measurements. Subsequently, overnight *Candida* spp. cultures were diluted in fresh growth media and 100 μL were added into corresponding wells reaching a final optical density of $OD_{600} = 0.1$ (approximately 1×10^5 cells/mL, depending on the strain). Appropriate negative controls (growth media minus fungal inoculum) were included in each experiment in triplicate. The plates were introduced into the device and incubated at 37 °C and fungal biofilm growth was measured for 72 h by cell impedance measurements which were registered every 10 min. The biofilm growth was expressed as Cellular Index (CI), which directly correlates to total fungal biofilm mass, and CI data were normalized by subtracting corresponding negative control values [35,37,39,40].

2.3. Biofilm quantification with crystal violet staining

To quantify the biofilm formation capacity of different *C. albicans* and *C. glabrata* isolates in microtiter plates, 24 h and 48 h biofilms were stained using Crystal Violet (CV) as previously described [41]. Briefly, each strain was grown overnight in YPD broth as described above. Overnight cultures were diluted to $OD_{600} = 0.1$ and 300 μl of fungal suspensions were inoculated into 96-well flat-bottom Ibidi Treat plates 89626 (Ibidi, Germany) for 24 h and 48 h, respectively. After that, the culture supernatant was discarded, biofilms were gently washed using Phosphate Buffer Saline (PBS pH = 7.4) and attached biomass was stained for 30 min using 0.4% CV. Subsequently, CV was removed, biofilms were washed with PBS to remove residual CV and resuspended using 30% acetic acid. After that, the absorbance of the released CV was measured by an absorbance plate reader Infinite M200 (Tecan, Durham NC) at 610 nm. Each strain was tested for biofilm production in triplicates and the assay was repeated three times (biological replicates).

2.4. Antifungal susceptibility assay in Candida spp. Biofilms

In order to evaluate the effect of conventional antifungals on the prevention of *Candida* spp. biofilm formation (minimal biofilm inhibitory concentrations - MBICs), different antifungals: azoles – fluconazole (TEVA) and voriconazole (TEVA) – and echinocandins – caspofungin (Sandoz) and micafungin (Astelas), were tested. Shortly, 100 μl of each antifungal diluted in YPD media without additional glucose (two-fold serial dilutions to final concentrations from 128 mg/L to 0.0625 mg/L) were used for background measurements [35,36]. Next, 100 μl of fungal suspensions were added into the corresponding wells in E-plates, reaching a final concentration of $OD_{600} = 0.1$. After that, biofilm was grown at 37 °C for 48 h registering impedance values every 10 min.

For the study of established biofilm eradication, 100 μl of fungal suspensions ($OD_{600} = 0.233$) were used for background measurements. Afterwards, 75 μl of YPD medium were added into the corresponding wells and biofilm growth was monitored for 24 h [35,37]. Next, 25 μl of antifungals were added, reaching final concentrations from 128 to 0.0625 mg/L (two-fold serial dilutions) for each tested antifungal. Biofilm growth was measured for an additional 48 h. Each experiment included two replicates of each condition and two negative controls.

2.5. MIC and MBIC determination

Minimum Inhibitory Concentrations (MICs) of fluconazole, voriconazole, micafungin and caspofungin was evaluated using an E-test assay (bioMérieux) according to the Clinical and Laboratory Standards Institute (CLSI) [42]. Given that EUCAST breakpoints have been not yet

established for caspofungin, strains susceptible to micafungin were also considered susceptible to caspofungin as suggested by European Committee on Antimicrobial Susceptibility testing [42].

Minimum Biofilm Inhibitory Concentrations (MBICs) were determined using impedance-based graphs where CI threshold values ≤ 0.15 at 48 h of biofilm growth were considered as inhibitory.

2.6. Andrographolide effect on planktonic Candida spp. Growth

To describe andrographolide effect on planktonic growth, *Candida* spp. overnight cultures were adjusted to $OD_{600} = 0.2$ and 100 μL of these suspensions were transferred into 96 well-plates (Thermo Fisher Scientific). After that, 100 μL of andrographolide compound (Sigma Aldrich) were added into the corresponding wells reaching final concentrations from 5 g/L to 20 mg/L (serial two-fold dilutions). Subsequently, the plates were incubated at 37 °C with orbital and linear shaking at 120 rpm and fungal cell growth was monitored for 24 h every 30 min with an absorbance plate reader Infinite M200 (Tekan, Durham NC).

2.7. Andrographolide effect alone and in combination with micafungin on Candida spp. Biofilms

To assess andrographolide effect on biofilm formation, andrographolide was serially diluted in YPD broth (two-fold dilutions to concentrations ranging from 5 g/L to 165 mg/L) and 100 μL of these suspensions were used for background measurements in the xCELLigence system. Then, overnight cultures of *Candida* spp. strains were diluted using fresh YPD broth and 100 μL of these suspensions added to the corresponding E-plate wells, reaching a final $OD_{600} = 0.1$. After that, biofilm growth was monitored for 24 h in real-time. Each experiment included two replicates of each condition and two negative controls for each andrographolide concentration.

To evaluate whether andrographolide alone or in a combination with micafungin, could eradicate mature *Candida* spp. biofilms, the biofilms were growth and monitored in the impedance system for 24 h as described above. Later, 25 μL of andrographolide, micafungin or their combination were added on the biofilms to the corresponding E-plate wells reaching final concentrations of 5 and 2.5 g/L for andrographolide and 128 mg/L for micafungin, respectively, and biofilm growth was quantified in real-time for additional 24 h.

2.8. Viable cell counting

Established 24 h biofilms of *Candida* spp. treated with micafungin (128 mg/L), andrographolide (5 g/L and 2.5 g/L) or their combination as described above, were collected using 100 μL of PBS and suspensions were sonicated for 2 min in order to eliminate cell aggregates and released fungal cells embedded in the biofilm matrix. After sonication, serial dilutions were prepared and 100 μL of each condition suspension were plated in triplicate on YPD agar plates and incubated at 37 °C for approximately 48 h. Colony-forming units (CFUs) were then counted, averaged, and expressed as log10 CFU/ml. Each experiment included three technical replicates and was repeated three times.

2.9. Scanning electron microscopy

To assess the effect on the biofilm spatial structure after treatment with micafungin and andrographolide alone or their combination, scanning electron microscopy was used. Biofilms of *C. albicans* strain 86 and *C. glabrata* 96 were grown for 24 h in the xCELLigence system and then treated with micafungin, andrographolide or their combination. After additional 24 h, supernatants were discarded, and biofilms were gently washed using PBS to eliminate unattached cells. Prior to observations, samples were fixed using Karnovsky's fixative for 8 h (4 °C), rinsed with PBS three times, dehydrated using gradual ethanol series (30%-50%–70%) twice and dried using critical point drying with CO_2. Biofilm samples were observed with a Hitachi S-4800 high-resolution electron microscope (Electron Microscopy Service, University of Valencia Spain), applying an accelerating voltage range of 10 kV and a magnification of x1K.

2.10. Statistical analysis

Differences in biofilm CI values before and after treatment were evaluated by regression analysis using a linear model ((https://cran.r-project.org/web/packages/glmulti) function lm, library stats, accessed in April 2022) in the R statistical package at 24 h of biofilm growth. Viable cell counting experiments (CFUs counts) were performed in triplicate with three independent replicates in each experiment. Statistical significance was assessed using Student's t-test, where a *p-value* < 0.05 was considered significant.

3. Results

3.1. Influence of culture conditions on Candida spp. Biofilm formation

To evaluate the capacity of *Candida* spp. to attach on 96 well E-plate surfaces and to form biofilms, three different growth media (YPD, YNB and RPMI1640) alone or supplemented with 2% of glucose were used. Real-time measurements showed that the most suitable medium for biofilm growth of both *C. albicans* and *C. glabrata* strains was YPD (Fig. S1), and therefore this culture medium was selected for future experiments. In general, all tested strains showed the ability to form robust biofilms in both YPD and YNB medium, reaching at least two times higher CI values compared to those in RPMI1640 alone or with additional glucose. When YPD and YNB media were supplemented with glucose, the biofilm growth of most of the tested strains was not affected, suggesting that glucose might not be a crucial factor in the robust biofilm formation of these strains. Besides, the presence of additional glucose in YPD media decreased the biofilm formation capacity of laboratory strains CA86, CA94 and CG96, as a delay in biofilm growth and a decrease in final CI values were observed. A maximum CI value was observed in all cases, and reaching this biofilm stationary phase is probably the consequence of nutrient depletion.

In contrast, the weakest biofilm formation capacity of all tested strains was observed in RPMI1460 medium. In this case, the effect of glucose addition was strain dependent, causing a biofilm growth delay (CA95), or growth acceleration (CA86), and even no effect compared to when they were grown in RPMI1460 alone.

We have also tested the most suitable initial optical density on *Candida* spp. biofilm growth in the impedance system and results showed that although higher optical density did have an influence on biofilm formation time, final CI values were similar in all tested cases (Fig. S2).

3.2. Comparison of CV staining and impedance-based measurements

We evaluated whether impedance-based measurements are comparable with the classical end point method of fungal biofilm staining with CV. Fig. 1 shows the biofilm formation dynamics of both *C. albicans* and *C. glabrata* isolates when grown in real time in the xCELLigence system using YPD growth media (panel a), while panel b represents biofilm biomass stained with CV at both 24 h and 48 h of biofilm growth of these strains, respectively. In accordance with impedance-based measurements, *C. albicans* isolates exhibited a very strong biofilm formation capacity, reaching high CI values at approximately 15 h of growth, while *C. glabrata* isolate (CG96) showed a lower capability to produce biofilm. Similar biofilm formation capacity results were observed for all tested strains between impedance and CV staining methods at both 24 h and 48 h of biofilm growth.

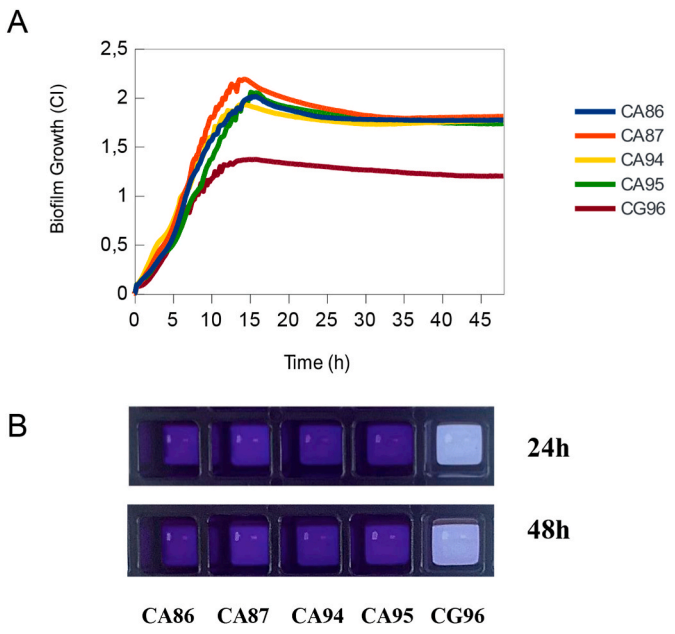

Fig. 1. a - Biofilm formation capacity of *Candida albicans* (CA) and *C. glabrata* (CG) isolates when grown in YPD medium quantified in real-time using impedance measurements during 48 h of biofilm growth. Data are means of triplicates. SDs are not shown for clarity. **b** - Quantification of biofilm biomass by classical CV staining in Ibidi 96-well plates at 24 h and 48 h of biofilm growth.

3.3. Impact of conventional antifungals on Candida spp. Biofilm formation

To evaluate the efficacy of conventional antifungals in preventing *Candida* spp. biofilm formation, four first-line antifungals – fluconazole, voriconazole, micafungin, and caspofungin were added together with fungal inoculum, and biofilm formation was monitored for 48 h in real time. Both tested antifungal groups showed a clear concentration and species-dependent effect (Figs. 2 and 3). Within the azole group (Fig. 2), both fluconazole and voriconazole provoked strain-dependent changes in biofilm growth dynamics. They showed only a slight biofilm inhibitory effect in the model strain CA86 and in the clinical isolate CA95, although none of the tested concentrations could halt biofilm formation completely. In addition, low concentrations of fluconazole (0.0625–1 mg/L) resulted in biofilm growth induction in both CA86 and CA95 strains reaching final CIs values higher than those observed in untreated controls (Fig. 2). In contrast, in strain CA94, the highest tested concentration of these antifungals was able to inhibit biofilm formation to a large extent. Regarding *C. glabrata* strain CG96, voriconazole showed a delay in biofilm growth in a concentration-dependent manner. However, although concentrations of 128 and 64 mg/L were efficient in preventing biofilm formation, subinhibitory concentrations produced an increase in the amount of biofilm formed (0.0625–4 mg/mL) (p-*value*<0.05). By contrast, no concentration of fluconazole tested was able to inhibit the biofilm formation of the *C. glabrata* strain. In fact, high concentrations (128-4 mg/L) of this antifungal resulted in biofilm biomass induction (Fig. 2). Contrary to azoles, both caspofungin and micafungin, which interfere with fungal cell wall biosynthesis (Fig. 3), fully prevented biofilm formation of almost all tested strains. Even at low concentrations both antifungals were able to prevent biofilm formation. For instance, the lowest concentrations (0.125 mg/L and 0.0625 mg/L) of micafungin inhibited biofilm formation up to 82% in strain CA86, 77% in both CA94 and CA95, and up to 69% in strain CG96 at 48 h of biofilm growth (p-*values* < 0.001). This inhibition degree was even stronger in the case of caspofungin, suggesting that this antifungal could be more efficient in preventing biofilm-related fungal infections.

In contrast to these results, the standard E-test showed that minimum inhibitory concentrations (MICs) for some antifungals were at least 100 times lower compared to the minimum biofilm inhibitory concentrations (MBICs) obtained by impedance measurements (Table S2). In addition, both MICs and MBICs values were higher for azoles when compared to echinocandins, suggesting limited antifungal properties of azoles against the tested strains (Table S2).

3.4. Mature biofilm disruption by echinocandins

In contrast to azole group antifungals, echinocandins showed a strong capacity to inhibit *Candida* spp. biofilm formation when added together with the fungal inoculum. For this reason, we further investigated whether micafungin and caspofungin were able to disaggregate or eliminate mature *Candida* spp. biofilms. Dose-response experiments in real-time performed on 24 h biofilms of *C. albicans* and *C. glabrata* strains showed that both micafungin and caspofungin were unable toeradicate mature biofilms, and in most cases even resulted in an induction of new biofilm accumulation up to 30% (p-*value* < 0.001) when compared to untreated controls, confirming that established biofilms are extremely difficult to eradicate (Fig. 4).

3.5. Andrographolide effect on Candida spp. Planktonic and biofilm growth

As previous experiments showed that established *Candida* spp. biofilms were extremely resistant to tested conventional antifungals, we wanted to investigate whether andrographolide, with demonstrated activity on biofilms of Gram-positive and Gram-negative bacterial species, could also have an effect against *Candida* spp. Results in Fig. S3 show that 2.5 g/L and 5 g/L of andrographolide were able to completely suppress planktonic growth of all tested *C. albicans* and *C. glabrata* strains, while lower concentrations (1.25 g/L – 315 mg/L) resulted in concentration-dependent growth inhibition or delay.

Given that andrographolide showed antifungal properties on planktonic growth in all tested *Candida* strains, we further evaluated the potential capacity of this compound to prevent *Candida* biofilm formation. Impedance-based measurements showed that concentrations equal to 5 g/L of andrographolide (2x MIC value) completely inhibited biofilm formation in all tested strains, while lower andrographolide concentrations showed a concentration-dependent effect in *Candida* spp. biofilm inhibition (Fig. 5).

The next approach was to evaluate the ability of this natural compound to eliminate *Candida* mature biofilms. Similarly, when andrographolide was added on 24 h biofilms at the concentrations of 2.5 g/L and 5 g/L, it provoked a notable decrease in CI values in both *C. albicans* strains (up to more than 60% in biofilm reduction, p-value<0.001) and up to 100% in *C. glabrata*, suggesting that this compound besides the biofilm prevention properties is also capable to interfere with mature biofilm architecture and detach yeast cells embedded in 24 h-old biofilms (Fig. 6).

In order to assess whether andrographolide, besides the ability to detach established biofilms, also had fungicidal properties, we performed viable cell counts of the biofilms treated with andrographolide at 24 h of biofilm growth. The results showed a similar trend which was observed using impedance-based measures (Fig. 7). Andrographolide was able both to eradicate fungal biofilms and kill biofilm-embedded fungal cells up to 99.9% of *C. albicans* cells (three orders of magnitude) when administered at 5 g/L, suggesting the potential of this compound against fungal biofilm-related infections (Fig. 7). It is important to highlight that in *C. glabrata* isolate CG96, andrographolide provoked almost immediate and larger biofilm detachment as detected by the impedance system, while showing a lower effect on cell viability compared to all tested *C. albicans* isolates. This may suggest that biofilm architecture is different in *C. glabrata* (Figs. 6–7).

Further experiments were undertaken to evaluate the possible

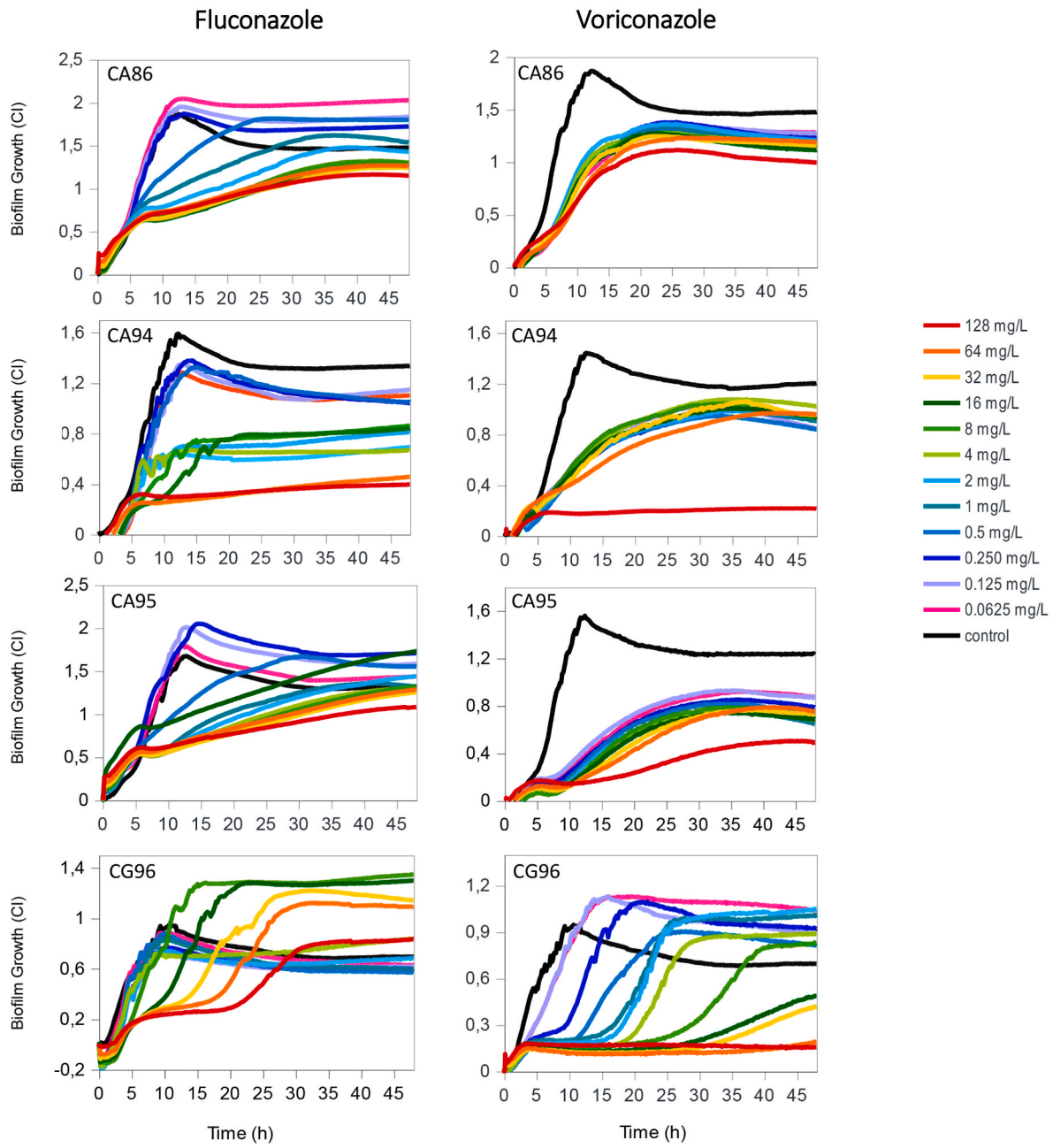

Fig. 2. The effect of fluconazole and voriconazole on *Candida albicans* (CA) and *C. glabrata* (CG) biofilm formation. Both antifungals were added at the beginning of biofilm formation together with the fungal inoculum at concentrations ranging from 128 to 0.0625 mg/L. Biofilm growth was monitored in real-time for 48 h at 37 °C in triplicates using impedance-based measurements. Black lines represent untreated controls. SDs are not shown for clarity.

synergistic effect between andrographolide and micafungin on established biofilms, given that the latter was efficient at preventing biofilm formation but had no effect on mature biofilms. The combination of micafungin (final concentration of 128 mg/L) with 5 g/L of andrographolide did not show any synergy either in detaching capacity (assessed by impedance) or in fungicidal properties (measured by colony counting). In fact, micafungin combination with andrographolide (both 2.5 g/L and 5 g/L, respectively) showed a mild antagonistic effect as indicated by biofilm viability counts (Fig. 7).

In addition, micafungin resulted in a nonsignificant decrease in viable cell number, when compared to untreated controls. Similar results were obtained using SEM microscopy of 24 h biofilms where andrographolide alone resulted in almost complete biofilm eradication in both of tested strains, validating results obtained by impedance measurements. On the contrary, micafungin alone did not affect biofilm architecture in *C. albicans* nor in *C. glabrata* established biofilms (Fig. 8B and Fig. S4B). Thus, the effect observed in the combined treatment of micafungin with andrographolide is due exclusively to the latter, which resulted in almost complete biofilm removal in both species (Fig. 8 and Fig. S4).

4. Discussion

Candida spp. cause different life-threatening infections with a high mortality rate [11,43]. Moreover, their ability to adopt a biofilm growth form allows *Candida* spp. strains to withstand conventional treatment and immune system attack [9,23]. For this reason, conventional antifungals usually fail to eradicate the infection and tend to only suppress it, despite high dosages and long-term treatments [6,44]. Although numerous *in vitro* studies, including microfluidics and time-lapse

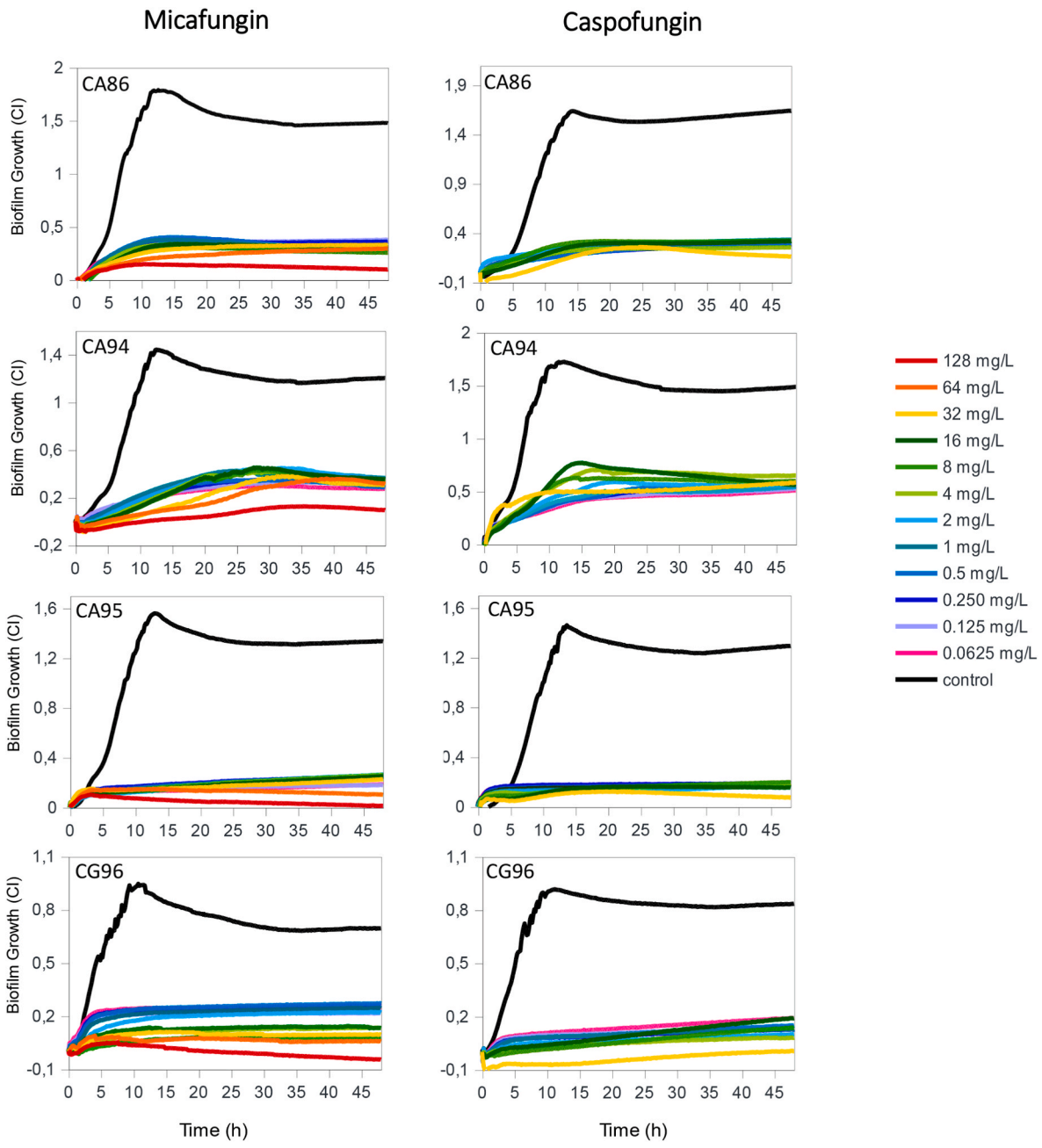

Fig. 3. The effect of micafungin and caspofungin on *Candida albicans* (CA) and *C. glabrata* (CG) biofilm formation. Both antifungals were added at the beginning of biofilm formation together with the fungal inoculum at concentrations ranging from 128 to 0.0625 mg/L. Biofilm growth was monitored in real-time for 48 h at 37 °C in triplicates using impedance-based measurements. Black lines represent untreated controls. SDs are not shown for clarity.

microscopy have been undertaken [10,45] to study fungal biofilm development, none of them describe *Candida* spp. biofilm growth dynamics and eradication patterns using impedance measurements. In the current manuscript, we evaluated the ability of *Candida* spp. to attach and form biofilms on electrodes-coated plates and assessed in real-time the influence of nutritional conditions such as growth media composition and additional glucose on the capacity of different *Candida* spp. strains to produce biofilms. In contrast to standard biofilm testing methods, this methodology permits to evaluate biofilm growth continuously (showing each phase of their growth), without previous labeling or manipulation [33,35].

Results from our study indicate that all of the tested *Candida* spp. isolates were able to form robust biofilms in YPD and YNB media rich in dextrose and suggest that RMPI1460 medium might favor planktonic, but not biofilm mode of growth, as observed CIs values were up to ten times lower in some of the tested strains when compared to YPD growth medium [46]. Moreover, the results indicate that glucose addition to the media delayed *C. albicans* biofilm formation, while *C. glabrata* biofilm growth was not affected, suggesting that *C. glabrata* strains might have some mechanisms to facilitate biofilm formation regardless of glucose levels. Our results also highlight the importance of standardizing experimental protocols for biofilm growth, such as the time of biofilm mass measurement, to accurately describe each isolate's capacity to form biofilms.

In addition, in this study we have also used the impedance system for dose-response experiments to test biofilm susceptibility to commonly used antifungals. Impedance measurements showed that azole group antifungals (fluconazole and voriconazole) were not able to fully

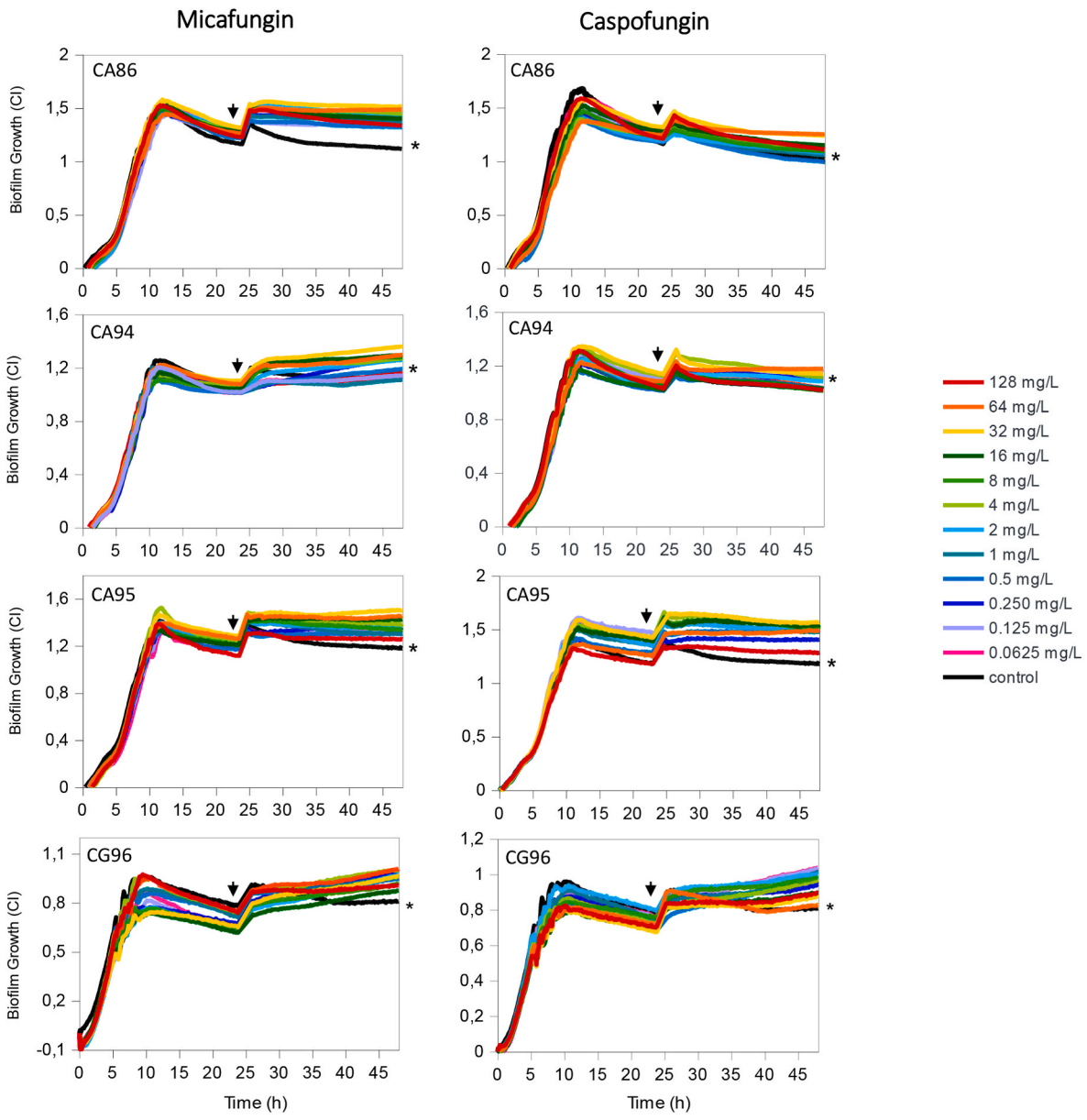

Fig. 4. The effect of micafungin and caspofungin on mature 24 h *Candida albicans* (CA) and *C. glabrata* (CG) biofilms. Both antifungals were added on the established biofilm at 24 h of growth (indicated by black arrows) at concentrations ranging from 128 to 0.0625 mg/L. After the addition of antifungals, biofilm growth was monitored for an additional 24 h period. Black lines represent untreated controls and are marked by asterisks. Data are means of three replicates. SDs are not shown for clarity.

prevent biofilm formation but resulted in biofilm growth delay. Moreover, both fluconazole and voriconazole provoked changes in biofilm growth dynamics in all tested strains, suggesting that these antifungals might interfere with some components in the EPS biofilm matrix and provoke changes in biofilm architecture. Similar findings have been reported by other authors who demonstrated that azole group antifungals can result in phenotypical changes in biofilm development and structure [47–49]. Contrary to azole group antifungals, echinocandins (micafungin and caspofungin) showed a strong biofilm formation inhibitory effect when added together with the fungal inoculum, as has been described elsewhere [10,12,14]. However, both micafungin and caspofungin lacked a biofilm eradication effect when added on 24 h biofilms, and at some concentrations even induced biofilm formation (Figs. 2–4). These findings are similar to those described by Kaneko and colleagues, who found that although micafungin was capable to suppress biofilm formation and development, fluconazole resulted in only partial biofilm inhibition, and they both failed to eradicate established fungal biofilms [10]. Thus, this emphasizes the need for new potent strategies to combat established fungal biofilms [7]. For this reason, an effort was undertaken to study the effect of the novel compound andrographolide, a natural component from the plant *Andrographis paniculate,* which was shown to have anti-inflammatory, anti-viral and antimicrobial properties against different bacteria [24,26,28,31,50]. Andrographolide not only showed the capacity to inhibit planktonic *Candida* spp. growth but also resulted in biofilm inhibition when added at concentrations equal to or higher than 2.5 g/L in all tested strains, suggesting that this compound could be used as a promising tool to prevent fungal biofilms formation and accumulation (Fig. 5 and S3). This anti-biofilm capacity against *C. albicans* and *C. glabrata* has not been shown before, and our data indicate that andrographolide was also effective against mature fungal biofilms, where the addition of this compound resulted in almost complete biofilm detachment (Fig. 6). Additionally, andrographolide reduced cell viability of up to 99.9% in *C. albicans*. Although andrographolide showed strong biofilm

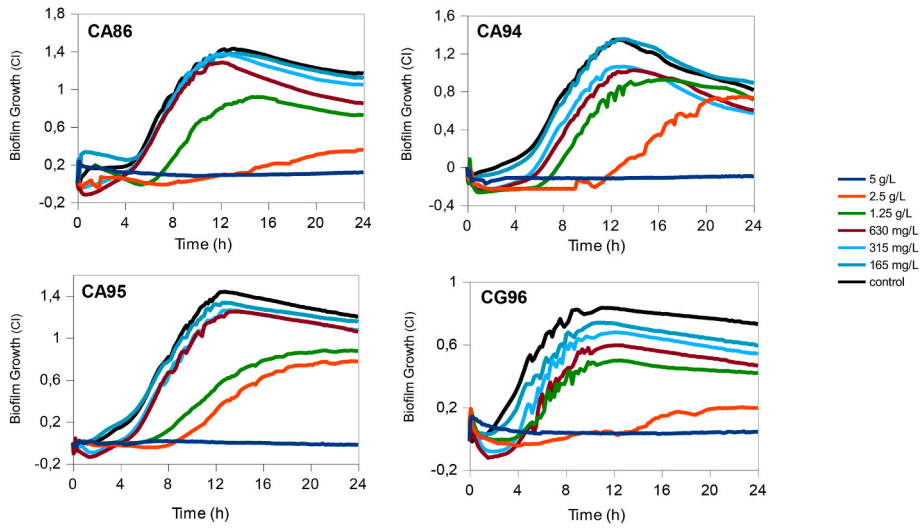

Fig. 5. Effect of andrographolide on biofilm formation in real-time of *Candida albicans* (CA) and *C. glabrata* (CA) strains. Andrographolide was added together with fungal cells at concentrations ranging from 5 g/L to 0.165 g/L. Biofilm growth was registered every 10 min for 24 h at 37 °C. Black line indicates the untreated control. Each line is the mean of two replicates. SDs are not shown for clarity.

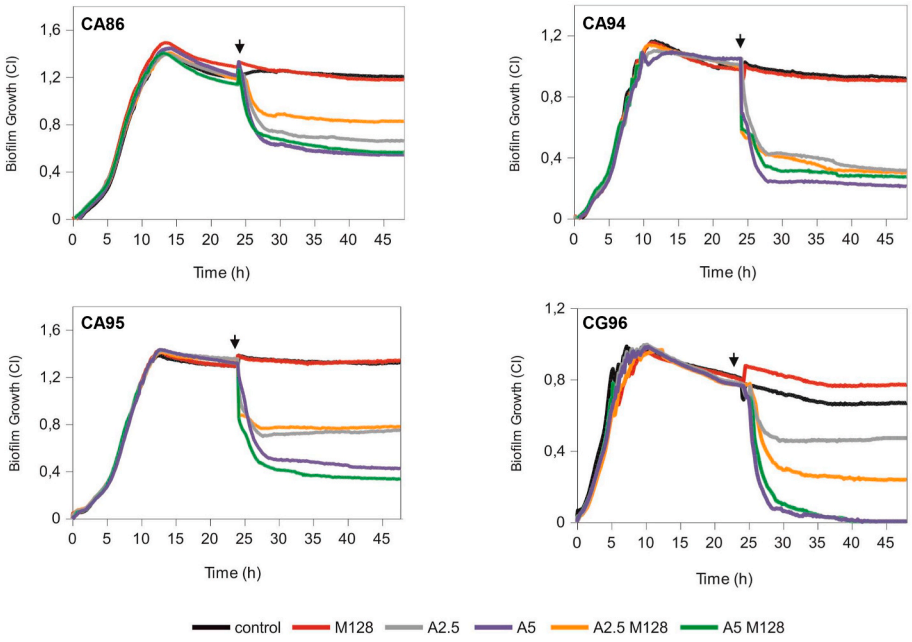

Fig. 6. *Candida albicans* (CA) and *C. glabrata* (CG) biofilm eradication using either andrographolide or micafungin alone and their combination. Both compounds were added on mature 24 h old biofilms (indicated by black arrows) and the biofilms were grown for an additional 24 h. Black lines represent untreated controls. SDs are not shown for clarity. **M128** - micafungin 128 mg/L; **A2.5** - andrographolide 2.5 g/L; **A5** - andrographolide 5 g/L; **A2.5M128** - andrographolide 2.5 g/L + micafungin 128 mg/L; A5M128 - andrographolide 5 g/L + micafungin 128 mg/L.

detachment capacity in *C. glabrata* strain 96 as measured by impedance, cell viability of this strain was decreased less when compared to *C. albicans*. These results suggest that *C. glabrata* biofilms might have different biofilm architecture and composition when compared to those of *C. albicans*. Additionally, SEM images confirmed the effect of andrographolide on mature *Candida* spp. biofilm detachment and eradication. For instance, these results indicate that andrographolide shows considerably higher efficacy on mature biofilms than both caspofungin or micafungin. On the other hand, we have also demonstrated that andrographolide and micafungin did not show a significant synergistic effect. In conclusion, obtained results strongly indicate the anti-biofilm properties (prevention and eradication) of andrographolide against *Candida* spp. (Figs. 7–8 and Fig. S4).

Overall, the data presented in the current manuscript underline the importance of testing new natural compounds with biocidal or detaching properties in order to obtain improved clinical outcomes from fungal biofilm-associated infections than those obtained with existing conventional therapies [5,9,44]. Our study demonstrates a potent effect of andrographolide against *C. albicans* and *C. glabrata* fungal biofilm formation and eradication. Thus, we suggest that future studies should evaluate the *in vivo* effect of andrographolide to treat fungal infections.

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CRediT authorship contribution statement

Miglė Žiemytė: conceived and designed the study, performed the experiments, and, Formal analysis, Writing – original draft. **Juan C.**

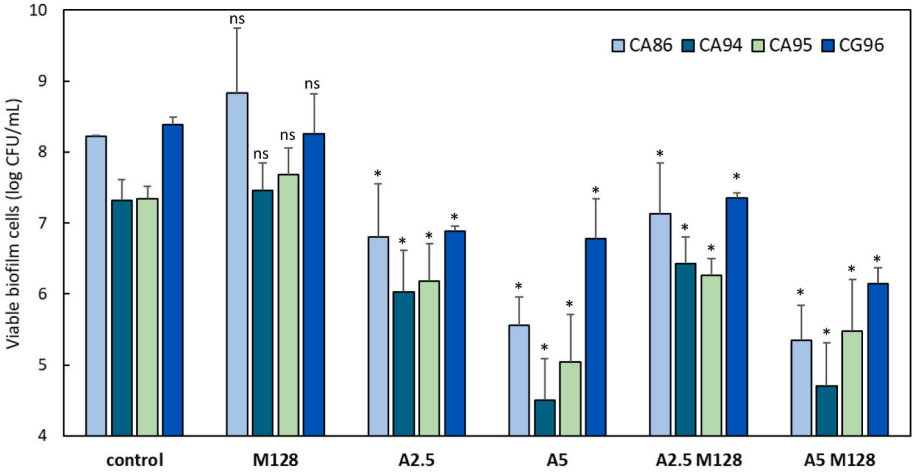

Fig. 7. Andrographolide effect alone and in combination with micafungin on mature *Candida* spp. biofilm viability. Both compounds were added at 24 h of biofilm growth and viable cell counting was performed after an additional 24 h. Data show the average of log CFUs counts from three independent biological replicates. **p-value* < 0.05, **ns** – not significant. **CA** - *C. albicans*; **CG** - *C. glabrata*; **M128** - micafungin 128 mg/L; **A2.5** - andrographolide 2.5 g/L; **A5** - andrographolide 5 g/L; **A2.5M128** - andrographolide 2.5 g/L + micafungin 128 mg/L; A5M128 - andrographolide 5 g/L + micafungin 128 mg/L.

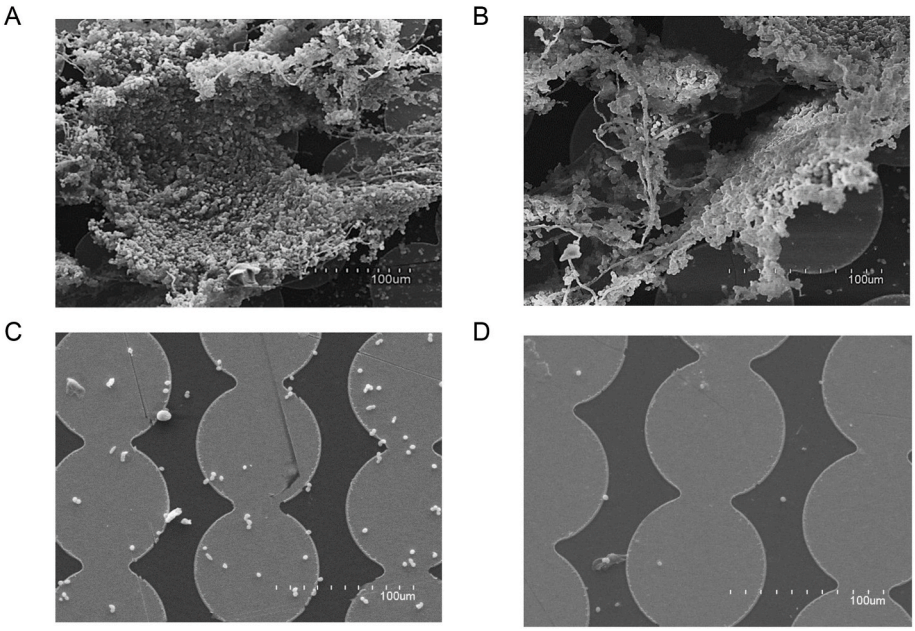

Fig. 8. Scanning electron microscopy (SEM) micrographs of 24 h *Candida albicans* strain 86 biofilms treated with andrographolide, micafungin or their combination. Both compounds were added at 24 h of biofilm growth and biofilms were grown for additional 24 h. **a** – untreated control; **b** – biofilms treated with 128 mg/L of micafungin; **c** – biofilms treated with 5 g/L of andrographolide; **d** – biofilms treated with both andrographolide (5 g/L) and micafungin (128 mg/L). Scale bar: 100 μm.

Rodríguez-Díaz: conceived and designed the study, provided bacterial strains. **María P. Ventero-Martín:** performed E-tests, performed E-tests, provided bacterial strains. **Alex Mira:** conceived and designed the study, provided reagents, Writing – original draft. **María D. Ferrer:** conceived and designed the study, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Alex Mira reports financial support was provided by Spanish Ministry of Science and Innovation. Migle Ziemyte reports financial support was provided by Spanish Ministry of Universities.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2023.100134.

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