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Effects of ceragenins and conventional antimicrobials on *Candida albicans* and *Staphylococcus aureus* mono and multispecies biofilms

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ABSTRACT

It is known that synergy between *Candida albicans* and *Staphylococcus aureus* results in enhanced biofilm formation and increased resistance to antimicrobials. Ceragenins (CSAs) are derivatives of cholic acid designed to mimic the antimicrobial activities of endogenous antimicrobial peptides. In this study, various CSAs were tested on *C. albicans* and methicillin-susceptible *S. aureus* or methicillin-resistant *S. aureus* mono or multispecies biofilms at 2 different concentrations (16 and 64 μg/mL) and compared with conventional antimicrobials. CSA-8 was active agent both with mono and multispecies biofilms ($P < 0.05$). Among antifungals, amphotericin B and, among antibacterials, ciprofloxacin and gentamicin were active agents against all studied microorganisms. This study suggests that CSAs, especially CSA-8, have useful antibiofilm effects against monomicrobial or fungal–bacterial multispecies biofilms.

1. Introduction

Biofilm growth is one of the leading infectious causes of morbidity and mortality, and it has been estimated that over half of infections originate from biofilms. Biofilm-embedded microorganisms exhibit resistance to both antimicrobial agents and host immune responses when compared to their planktonic forms (Tobudic et al. 2012). Multispecies biofilms, where polymicrobial species are closely associated, represent an understudied and clinically relevant health problem, with the potential to serve as an infectious reservoir (Harriott and Noverr 2009; Harriott et al. 2016). Furthermore, organisms within multispecies biofilms exhibit uniquely altered gene expression, amplified pathogenic phenotypes, and altered antimicrobial susceptibility (Li et al. 2015; Tavernier et al. 2017). Synergistic, mutualistic, and antagonistic interactions can occur between microorganisms and contribute to the development of multispecies biofilm communities (Zago et al. 2015). Previous biofilm research has been focused on single-species biofilms; nevertheless, less is known about polymicrobial interactions, particularly those in fungal–bacterial biofilms.

It has been estimated that 27% of patients with candidemia have polymicrobial blood cultures, with *S. aureus* as the third most common organism isolated with *C. albicans* (Zago et al. 2015; Harriott and Noverr 2010). *C. albicans* and *S. aureus* have also been co-isolated from various mucosal surfaces such as vaginal and oral mucosa, including biofilm forms (Peters et al. 2010). *C. albicans* forms hyphae at the base of biofilms, and *S. aureus* adheres preferentially to *Candida* hyphae within the biofilm (Harriott and Noverr 2009). Synergy between *C. albicans* and *S. aureus* in a biofilm results in enhanced biofilm formation and increased resistance to antimicrobial agents (Harriott and Noverr 2009; Harriott and Noverr 2010; Li et al. 2015). Furthermore, synergistic interactions between *C. albicans* and *S. aureus* enhance mortality in animal models (Carlson 1983). Multispecies biofilms are more complicated and difficult to eradicate than monomicrobial biofilms, in part because both antifungal and antibacterial agents would be necessary to impact multispecies biofilms, and few therapeutics are available for treating fungal (Sardi et al. 2013) and bacterial biofilms (Otto 2012; Tong et al. 2015). Multispecies biofilms of *C. albicans* and *S. aureus* increase the resistance of microorganisms to antimicrobials (Harriott and Noverr 2009; Harriott and Noverr 2010); consequently, new management options for eradication of multispecies biofilms are urgently required.

Endogenous antimicrobial peptides (AMPs) display broad-spectrum antimicrobial activities and may be considered for eradication of

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multispecies biofilms; however, many AMPs are difficult to synthesize and purify due to their complexity and size, and they can be substrates for proteases, which limit their *in vivo* half-lives (Savage et al. 2002). Ceragenins (CSAs) were designed to mimic the activities of AMPs; these compounds are not peptide based, are not salt sensitive, and are relatively simple to prepare and purify on a large scale. CSAs associate with anionic cell surfaces and cause formation of transient pores in microbial membranes, resulting in membrane depolarization and cell death. As well as their antibacterial activities against resistant strains, these molecules also display antifungal, antiviral, antiparasitic, anticancer, and antibiofilm effects. A large number of different CSAs have been synthesized and studied. The molecules between CSA-1 and CSA-50 that have been synthesized are members of the first series/generation of CSAs to be well characterized. Molecules synthesized later than CSA-50 are later generations of CSAs (Guan et al. 2000; Savage et al. 2002). Among the first generation, CSA-13 is the best studied CSA and has potent antimicrobial activities against Gram-positive bacteria, Gram-negative bacteria, fungi, virus, and parasites (Bozkurt-Guzel et al. 2018; Bozkurt-Guzel et al. 2014; Howell et al. 2009; Lara et al. 2010; Leszczynska et al. 2013). A variety of studies confirmed that other derivatives, including second-generation CSAs CSA-131 and CSA-192, possess high antimicrobial activity, including activity against antimicrobial-resistant clinical isolates such as *Candida* spp. and colistin-resistant Gram-negative bacteria (Bozkurt-Guzel et al. 2018; Hacioglu et al. 2018; Hashemi et al. 2017). Additionally, a few studies have evaluated antibiofilm activity of CSAs (CSA-13, CSA-90, CSA-131, CSA-138, CSA-142, CSA-192) against *Candida sp.* and *S. aureus* (Durnaś et al. 2016; Hacioglu et al. 2018; Olekson et al. 2017). However, there are no published studies about CSA antibiofilm effectiveness against bacteria–yeast multispecies biofilms. In this study, we aimed to determine the effects of representative conventional antimicrobial agents and representative CSAs against *C. albicans* and *S. aureus* [methicillin-susceptible *S. aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA)] mono and multispecies biofilms.

2. Material and methods

2.1. Strains and growth conditions

Three reference strains were used in this study: *C. albicans* wild-type strain (SC 5314) was subcultured from freezer stocks onto Sabouraud Dextrose agar (SDA, Difco, Sparks, MD) plates and incubated at 30°C overnight to generate cultures used for the experiments (Ramage et al. 2001). Two reference strains of *S. aureus*, methicillin-susceptible (MSSA-ATCC 25923) and methicillin-resistant (MRSA-ATCC 43300) strains, were selected because they are capable of biofilm formation *in vitro* (Zago et al. 2015). For each experiment, strains were subcultured from freezer stocks of *S. aureus* and *C. albicans* in mannitol salt agar (MSA, Oxoid, Hampshire, United Kingdom) and SDA plates, respectively, and incubated at 37 °C overnight.

2.2. Antimicrobial agents

Amphotericin B, fluconazole, anidulafungin, ciprofloxacin, linezolid, daptomycin, and gentamicin were kindly provided by their manufacturers. Stock solutions from dry powders were prepared according to the manufacturers' recommendations and stored frozen at −80 °C for up to 6 months. CSA-8, CSA-13, CSA-44, CSA-131, CSA-138, CSA-142, CSA-13 NDSA (1,5-naphthalenedisulfonic acid), and CSA-192 were synthesized from a cholic acid scaffold technique as previously described (Fig. 1) (Durnaś et al. 2016; Lai et al. 2008). We used CSA-8, CSA-13, CSA-13 NDSA, and CSA-44 as representative of first-generation CSAs. For second-generation CSAs, CSA-131, CSA-138, and CSA-142 are studied. Stock solutions of CSAs from dry powders were prepared in water and stored frozen at −80 °C, and frozen solutions were used within 6 months.

2.3. Biofilm formation

To prepare yeast and bacteria inocula, a loop full of the overnight cultures was transferred to 5 mL of yeast nitrogen base broth (Difco, Becton Dickinson Sparks, MD) supplemented with 100 mM glucose and tryptic soy broth (TSB, Difco, Sparks, MD), respectively, and incubated at 37 °C overnight in an orbital shaker (75 rpm). Both *Candida* and *Staphylococcus* cultures were centrifuged (about 3000 rpm, 5–10 min) and washed twice with sterile phosphate-buffered saline (PBS) and resuspended in TSB containing 0.2% glucose (TSB-g) to a cellular density equivalent to 1×10^7 colony-forming units/mL (CFU/mL). TSB-g was previously determined to be an optimal medium for supporting both candidal and staphylococcal biofilm growth (Peters et al. 2013; Ramage et al. 2001). Biofilms were formed by pipetting 100 μL of the standardized cell suspension (*C. albicans* + MSSA and *C. albicans* + MRSA) into selected wells of sterilized polystyrene flat-bottomed 96-well tissue culture microtiter plates (Greiner Bio-One, Kremsmuenster, Austria) and incubating for 24 h at 37 °C. This resulted in a final organism population in the wells of 10^6 CFU/mL for both *C. albicans* and MSSA or MRSA. Control wells contained *C. albicans* alone and MSSA or MRSA alone or medium alone.

2.4. Biofilm antimicrobial assay

Biofilms were formed as described above. After incubation, the waste medium was aspirated gently, and nonadherent cells were removed by washing the biofilms 3 times with sterile PBS. Stock solutions of antimicrobials were diluted in TSB-g. Fluconazole (10 μg/mL and 5 μg/mL), amphotericin B (4 μg/mL), anidulafungin (2 μg/mL), ciprofloxacin (75 μg/mL and 150 μg/mL), linezolid (64 μg/mL), daptomycin (8 μg/mL), and gentamicin (8 μg/mL) and CSAs (16 and 64 μg/mL) were added to the washed biofilm, according to the previous studies and closest toxic concentrations (Nagant et al. 2013; Tavernier et al. 2017). The plates were incubated for an additional 24 h, and fungal and bacterial viability was monitored by a CFU assay (Peters et al. 2013; Ramage et al. 2001).

2.5. CFU assay

After 24 h of administration of antimicrobials to the biofilms, the antibiotic solutions were removed. The wells were rinsed with PBS. Next, biofilms were detached by vortexing (900 rpm) and sonication (both 5 min) by ultrasonic cleaner. Briefly, the plates are placed to the apparatus of the sonicator. After biofilm is destroyed in 10 min by the sonication, plates are put on the minishaker (Biosan) in order to homogenize the solution, followed by collection of the content of the wells in sterile tubes. The vortexing and sonication steps were repeated after the addition of PBS to each well. Serial dilutions were made in sterile PBS and plated onto SDA supplemented with ampicillin (0.008 μg/mL) and erythromycin (0.075 μg/mL) (for *C. albicans* enumeration) or MSA (for *S. aureus* enumeration) using the drop plate method; plates were incubated at 37 °C (Harriott and Noverr 2009; Kart et al. 2014; Zago et al. 2015). Following 24 h of growth, colonies were counted and expressed as the number of CFU/mL.

2.6. Metabolic activity assay

In addition to the CFU assay, the biofilm was also assayed using a colorimetric method utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, St. Louis, MO) to measure biofilm formation inhibition. After biofilm growth in 24-well plates, antibiotics and CSAs were applied with the indicated amounts and incubated at 37 °C with 10% CO_2 for 24 h. MTT (5 mg/mL) was added onto wells and was decanted

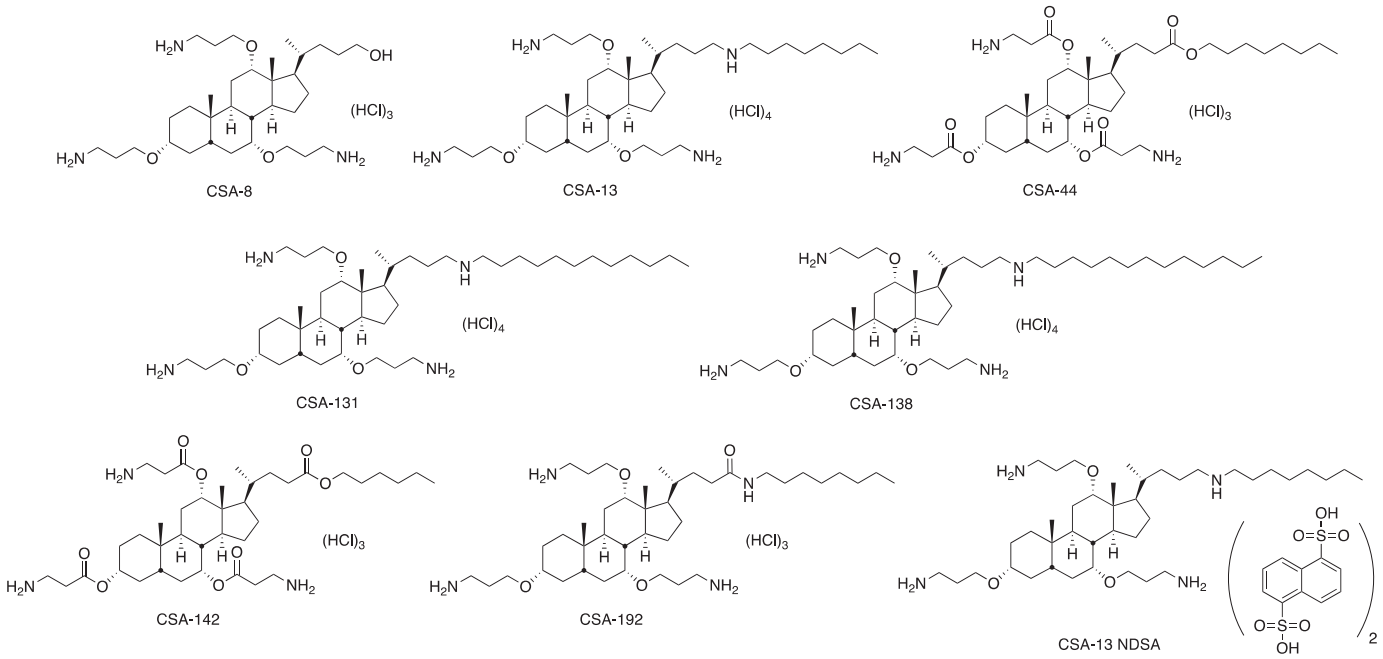

Fig. 1. Chemical structures of CSAs used in this study.

carefully after 4 h. Dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO) was added to solubilize the produced product, and absorbance was measured at a wavelength of 570 nm (Chandra et al. 2008).

2.7. Fluorescence microscopy

Following antibiotic and CSA treatments, biofilms were stained with the acridine orange (AO, Invitrogen, Carlsbad, CA) and propidium iodide (PI, Invitrogen, Carlsbad, CA) to investigate live/dead presence in biofilms. AO is a cell-permeant cationic fluorescent dye that emits green fluorescence when bound to dsDNA. PI is a fluorescent dye impermeable to the cellular membrane and generally excluded from viable cells; thus, live cells fluoresce green, while dead cells with damaged membranes are red (Li et al. 2007). After biofilm formation on coverslips (13 mm) in 24-well plates and antimicrobial treatment, the dye solution [1 μL AO (5 mg/mL) and 1 μL PI (3 μg/mL) in 1 mL PBS] was added. Biofilms were visualized using the Olympus BX51 fluorescence microscope and imaging with Olympus DP72 camera and DP2-TWAI software (Bektaş et al. 2011).

2.8. Statistical analysis

All experiments were performed in triplicate in 2 separate sets of experiments. All data are expressed as mean values with corresponding standard deviations. One-way analysis of variance and Bonferroni's multiple comparison tests were used to compare the differences between mono and multispecies biofilms, and a P value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of antimicrobials on C. albicans cells in mono and multispecies biofilms

The antimicrobial activities of antimicrobial agents were evaluated against *C. albicans* mono and multispecies (*C. albicans* + MSSA and *C. albicans* + MRSA) biofilms. Among the studied antimicrobials, the most effective CSAs were CSA-142 at 16 μg/mL and CSA-8 at 64 μg/mL (Fig. 2a and d), and among the antifungals, amphotericin B was the most effective with *C. albicans* monospecies biofilms (Fig. 3a). Amphotericin B, CSA-8, CSA-13, and CSA-192 were found to be the most effective agents against *C. albicans* + MSSA and *C. albicans* + MRSA multispecies biofilms ($P < 0.05$). Anidulafungin, CSA-142, and CSA-13 NDSA were found to be the least effective agents on *C. albicans* in multipecies biofilms. Notably, *C. albicans* became more resistant to CSAs and antifungals in multispecies biofilms, according to CFU assays (Figs. 2a and 3a) (both with MSSA or MRSA).

3.2. Effects of antimicrobials on MRSA cells at mono and multispecies biofilms

The activities of antimicrobial agents were also investigated and compared against MRSA mono and multispecies (*C. albicans* + MRSA) biofilms. CSA-8 (Fig. 2b and e) and ciprofloxacin (Fig. 3b) were the most effective agents (16 μg/mL, 64 μg/mL CSA-8 and ciprofloxacin decreased 1 log, 4 log and 2 log, respectively) against MRSA in both mono and multispecies biofilms ($P < 0.05$). CSA 192, daptomycin, and gentamicin also showed antibacterial activity against MRSA in multispecies biofilms, whereas CSA-138 and linezolid showed the least activity against MRSA in mono and multispecies biofilms.

3.3. Effects of antimicrobials on MSSA cells at mono and multispecies biofilms

The susceptibility of MSSA to antimicrobial agents in mono and multispecies (*C. albicans* + MSSA) biofilms was measured, and CSA-8 was found to be the most active agent against MSSA cells in both mono and multispecies biofilms (up to 7 log and 3 log reduction of cell counts in mono and multispecies biofilms, respectively) at 64 μg/mL (Fig. 2c and f) ($P < 0.05$). Ciprofloxacin, daptomycin, and gentamicin displayed 1–4 log reduction against MSSA cells in both mono and multispecies biofilms (Fig. 3c). CSA-138 and linezolid showed the least antimicrobial activity against MSSA in multispecies biofilms.

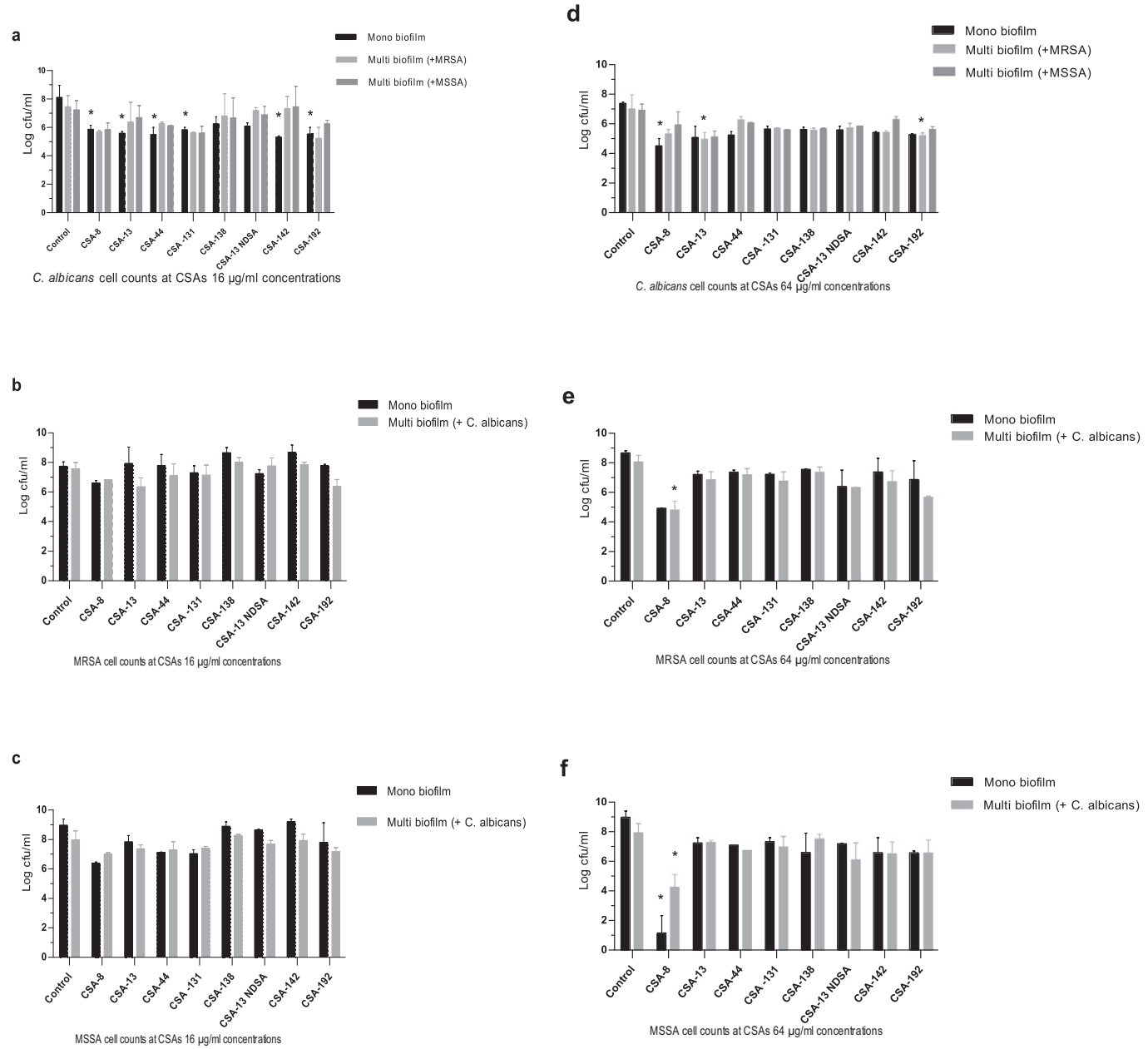

Fig. 2. Effects of CSAs on mono and multispecies biofilms. (a–c) Concentrations of 16 μg/mL; (d–f) concentrations of 64 μg/mL. Average number of CFU of the microorganisms recovered from single and multispecies biofilms is shown. Multispecies biofilms contain (a and d) *C. albicans* (SC 5314) + *S. aureus* (MRSA-ATCC 43300) or *albicans* (SC 5314) + *S. aureus* (MSSA-ATCC 25923), (b and e) *C. albicans* (SC 5314) + *S. aureus* (MRSA-ATCC 43300), or (c and f) *C. albicans* (SC 5314) + *S. aureus* (MSSA-ATCC 25923). (a) Effects of CSAs on *C. albicans* cells at 16 μg/mL. (b) Effects of CSAs on MRSA cells at 16 μg/mL. (c) Effects of CSAs on MSSA cells at 16 μg/mL. (d0 Effects of CSAs on *C. albicans* cells at 64 μg/mL. (e) Effects of CSAs on MRSA cells at 64 μg/mL. (f) Effects of CSAs on MSSA cells at 64 μg/mL. *It was found to be statistically significantly (*$P \leq 0.05$).

3.4. Measurement of biofilm growth

The most and the least effective agents from the initial antimicrobial assays were screened with MTT viability assay. Antimicrobial effects of the CSAs against *C. albicans* mono and multispecies (*C. albicans* + MRSA and *C. albicans* + MSSA) biofilm were initially explored using the MTT viability assay, which measures cell viability as a function of metabolic activity. The metabolic activity of *C. albicans* during biofilm formation was reduced by ~25% with CSA-8 (64 μg/mL) (Fig. 4a). In this study, *C. albicans* mono biofilm formation was reduced by ~45% with amphotericin B treatment, and it proved to be a more effective antifungal than anidulafungin (Fig. 4a). A similar effect of CSA-8 (64 μg/mL) was observed on MRSA (~45% decrease on viability). Moreover, it was found that CSA-8 treatment inhibited MRSA biofilm growth more than ciprofloxacin and linezolid (Fig. 4b). Ciprofloxacin treatment decreased mono biofilm formation of MSSA by 42%, and CSA-8 (64 μg/mL) decreased metabolic activity by approximately 35% (Fig. 4c). These data indicate that CSA-8 is as effective as ciprofloxacin on MSSA cells. Compared to monospecies biofilms of *C. albicans* or MSSA, treatment of multispecies biofilms of *C. albicans* with MSSA resulted in a reduction of around 40% in metabolic activity with CSA-8 and CSA-192 (64 μg/mL). In addition, there was 38% inhibition of *C. albicans* and MSSA multispecies biofilm formation with amphotericin B (Fig. 4e). *C. albicans* and MRSA multispecies biofilm formation was reduced with CSA-8, CSA-13, and CSA-192 (64 μg/mL) treatment by ~47%, ~40%, and ~48%, respectively. Inhibition of metabolic activity

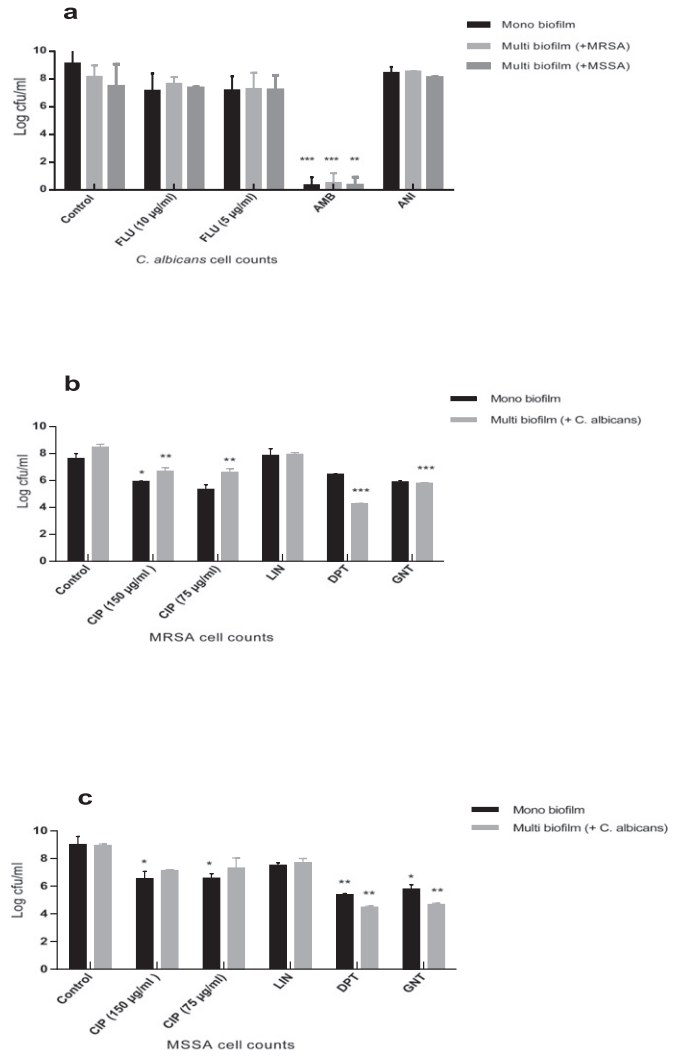

Fig. 3. Effects of antimicrobial agents on mono and multispecies biofilms (a–c). Multispecies biofilms contain (a) *C. albicans* (SC 5314) + *Staphylococcus aureus* (MRSA-ATCC 43300) or *C. albicans* (SC 5314) + *S. aureus* (MSSA-ATCC 25923), (b) *C. albicans* (SC 5314) + *S. aureus* (MRSA-ATCC 43300), or (c) *C. albicans* (SC 5314) + *S. aureus* (MSSA-ATCC 25923). (a) Effects of antifungals on *C. albicans* cells. (b) Effects of antibiotics on MRSA cells. (c) Effects of antibiotics on MSSA cells. *, **, ***It was found to be statistically significantly (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

with amphotericin B, gentamicin, and ciprofloxacin was, 47%, 70%, and 62% respectively (Fig. 4d).

3.5. Viability analysis with fluorescence microscopy

The impact of CSA-8 (64 μg/mL) on established biofilms was also observed *via* fluorescence scanning microscopy. After incubation of mono and multi species biofilms for 24 h with or without CSA-8, growth suppression of biofilm by CSA-8 was clearly seen by fluorescence microscopy with the live/dead stain using AO/PI, confirming the CFU and MTT data. The images obtained by fluorescence microscopy demonstrated the presence of a high number of round, viable cells (green) in the control group with *C. albicans* (Fig. 5a, e, and g). The images of the CSA-8 treatment group of only *C. albicans* (Fig. 5b) showed nonviable fungal cells (red). CSA-8 inhibited both mono and multispecies biofilm formation of MRSA (Fig. 5d and f), and MSSA (Fig. 5h). According to AO and PI staining, CSA-8 reduced both *C. albicans* and *S. aureus* mono and multispecies biofilm formation (Fig. 5e, f, g, and h).

4. Discussion

In this study, we demonstrated that CSA-8 was the most effective agent against both mono and multispecies biofilms, and among the second-generation CSAs, CSA-192 was the most active agent, particularly against MRSA cells in multispecies biofilms. All CSAs showed better activity against mono species biofilms as compared to multispecies bioiflms. Several recent *in vitro* studies with *C. albicans* biofilms have demonstrated efficient antifungal activities of amphotericin B similar to our results (Kuhn et al. 2002; Shuford et al. 2007; Tobudic et al. 2010). However, in our study, there was no appreciable antifungal effect for fluconazole and anidulafungin against biofilms.

Among common antibacterial agents, ciprofloxacin displayed 1–2 log reduction against *S. aureus* in both mono and multispecies biofilms. Additionally, daptomycin and gentamicin were also effective against *S. aureus*, especially in multispecies biofilms. According to Figs. 2d and 3a, against *C. albicans* biofilms, most of the studied CSAs (at 64 μg/mL) displayed more reduction than fluconazole and anidulafungin. CSA-8 (at 64 μg/mL) was the most effective antimicrobial agent (8 log reduction) among CSAs and antibacterial agents against *S. aureus*.

The interaction of *S. aureus* and *C. albicans* is known to increase antimicrobial resistance due to the matrix that is formed that may limit penetration of the drug into the biofilm (Zago et al. 2015). We found that *C. albicans* and *S. aureus* (both MRSA and MSSA cells) become resistant to antimicrobial agents in multispecies biofilms. We did not see major differences in sensitivity to CSAs by both mono and multispecies biofilms containing MSSA. This could be explained by the fact that MRSA is intrinsically resistant to beta-lactams (presence of mecA gene) which inhibit bacterial growth by interfering with cell wall assembly; nevertheless, CSAs target cell membranes (Gordon and Lowy 2008; Savage et al. 2002).

There are few published papers describing the *in vitro* activity of CSAs against *Candida* spp. Bozkurt Guzel et al. (2018) demonstrated that the MICs of CSA-8, CSA-13, CSA-44, CSA-131, and CSA-138 ranged between 0.25 and 128 μg/mL against *Candida* spp. The antifungal activity of CSAs has also been described by Durnaś et al. (2016). They showed that CSA-13, CSA-131, and CSA-192 have stronger candidacidal activity (MFC: 0.5–32 μg/mL) than LL-37 (an AMP) (MFC: 128->256 μg/mL) and omiganan (an AMP) (MFC: 128 μg/mL) against all tested *Candida* strains. Bozkurt Guzel et al. (2017) also showed antibacterial and antifungal activities of CSA-13, CSA-142, and CSA-192 against *S. aureus* and *C. albicans*: MIC_{90} (μg/mL) values of CSA-13, CSA-142, and CSA-192 were 1, 4, and 2 against *S. aureus* and 4, 8, and 8 against *C. albicans*, respectively.

In comparison to antimicrobial effects against bacteria and fungi, the antibiofilm activity of CSAs is not as well defined. Few studies showed that CSAs, CSA-13 (100 mg/L) in particular, have antibiofilm effects on *P. aeruginosa* (Gu et al. 2013; Nagant et al. 2013). Durnas et al. (2016) also showed that 5-μg/mL concentrations of CSA-13 and CSA-131 significantly inhibited the formation of both young and mature biofilms of *C. albicans* cells. Similar to these results, Leszczynska et al. (2013) showed that CSA-13 (5 μM) exhibits activity against biofilms formed by different bacteria including *S. aureus*. In one of our studies, not only CSA-13 but also CSA-8, CSA-44, CSA-131, and CSA-138 were evaluated with adhesion and biofilm formation of *C. albicans* (Bozkurt-Guzel et al. 2018). We found that CSA-13, CSA-44, CSA-131, and CSA-138 showed similar pattern of MIC ranges as AMP-B, where the MIC values varied in a 3–4 dilution range. Recently, Olekson et al. (2017) also showed antibiofilm activities of various CSAs including CSA-13, CSA-44, CSA-131, CSA-138, CSA-142, and CSA-192 against preformed mixed-species biofilms of *P. aeruginosa* and *S. aureus*: CSA-13 decreased the amount of biofilm matrix substances detected by SYPRO Ruby stain.

The trend among the compounds argues that longer hydrophobic chains on the compounds (the lipid chain lengths are 8, 12, and 13 carbons for CSA-13, CSA-131, and CSA-138, respectively) increase activity against *Candida* sp. (Lai et al. 2008). Indeed, we observed that CSA-13,

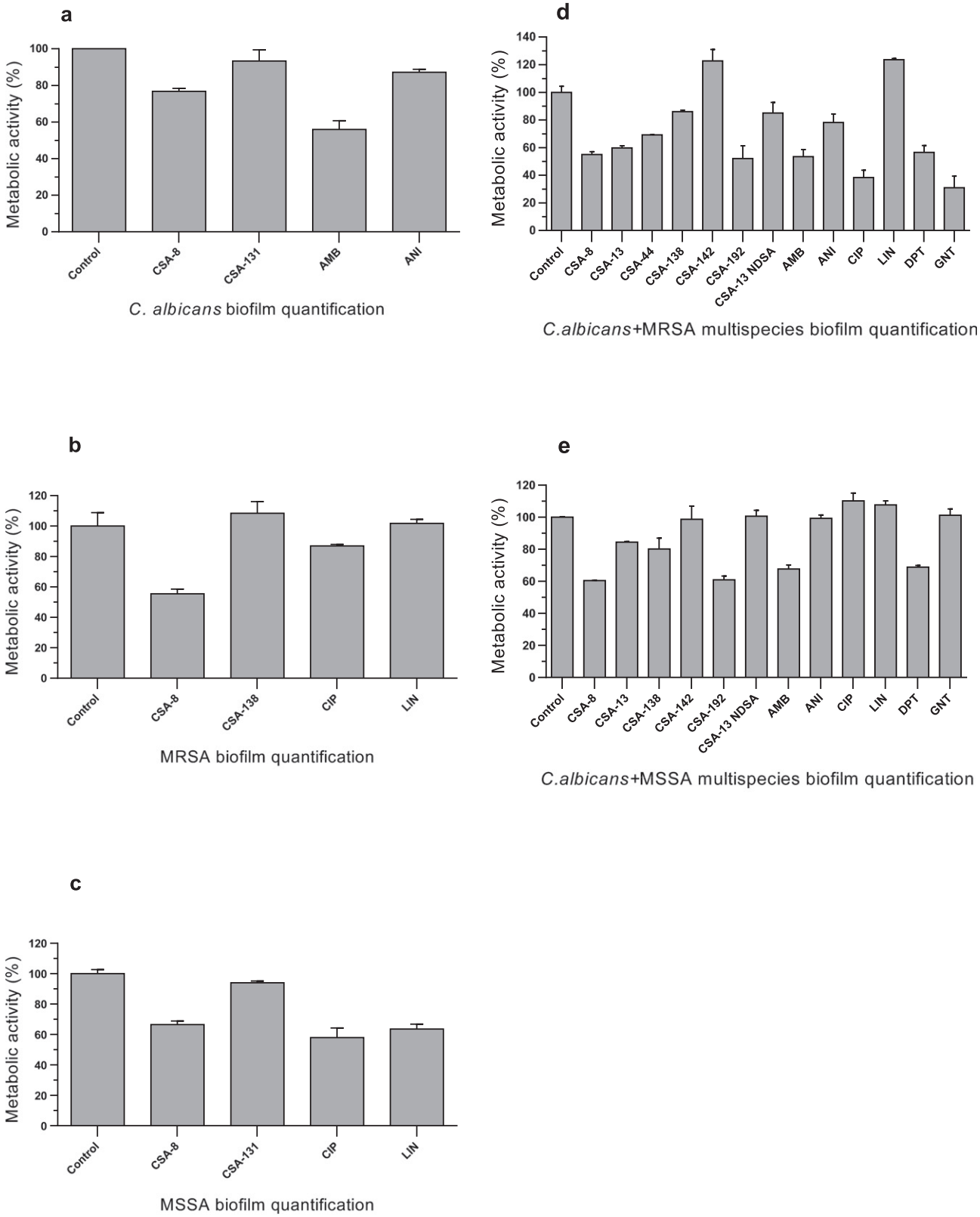

Fig. 4. The effect of antimicrobials on mono and multispecies biofilm formation. Biofilm formation, quantified using the MTT assay, was measured after 24 h of treatment. MTT formazan reduction by biofilms is expressed as a percentage of control biofilms incubated in the absence of the antimicrobials. Data are means and SEM of 3 independent experiments done in triplicate. (a) *C. albicans* mono biofilm quantification. (b) MRSA mono biofilm quantification. (c) MSSA mono biofilm quantification. (d) *C. albicans* + MRSA biofilm quantification. (e) *C. albicans* + MSSA biofilm quantification.

CSA-131, and CSA-138 showed antibiofilm activity against *C. albicans* cells in both mono and multispecies biofilms (up to 2 log reduction of cell counts). Although previous studies have shown that CSA-13 strongly inhibited the adhesion and biofilm formation of various microorganisms, in our study, we found it less effective against both mono and multispecies biofilms (Durnaś et al. 2016; Leszczynska et al.

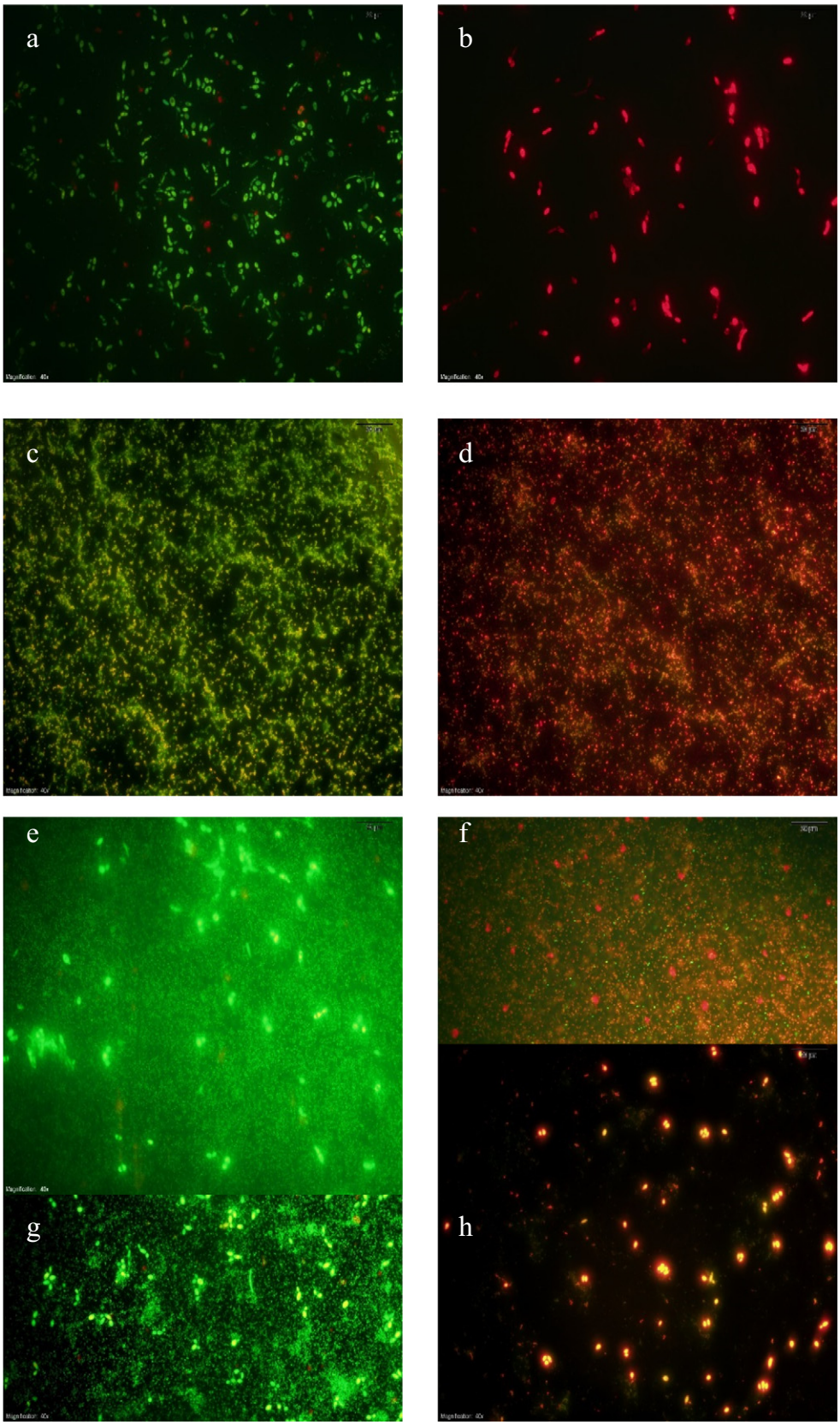

Fig. 5. Fluorescence images of mono and multispecies biofilms with or without CSA-8 treatment. (a, b) Images of *C. albicans* (a) control, (b) treated with CSA-8 (64 mg/mL). (c, d) Images of MRSA (c) control, (d) treated with CSA-8 (64 mg/mL). (e, f) Images of *C. albicans* and MRSA (e) control, (f) treated with CSA-8 (64 mg/mL). (g, h) Images of *C. albicans* and MSSA (g) control, (h) treated with CSA-8 (64 mg/mL). Live cells are stained in green and dead cells in red with using AO/PI (magnification 40×, bar = 20 μm).

2013). On the other hand, CSA-8, which is known to be more effective against Gram-positive bacteria, showed the highest antimicrobial activity against both *C. albicans* and *S. aureus* mono and multispecies biofilms, which suggests that this explains how CSA affects cell walls of both yeast and bacteria (Chin et al. 2007). Our results were similar to a previous study which was done by our study group (Bozkurt-Guzel et al. 2018).

CSA-44 and CSA-142 are not stable for long periods in water, and they are also temperature sensitive (Guan et al. 2000). Indeed, we could not find any activity against either mono or multispecies biofilms.

Pollard al. (2012) have shown that resistance can be generated by some bacteria to CSA-13. CSA-13 retained potent antibacterial activity against *S. aureus* over the course of 30 serial passages. Resistance generated in Gram-negative bacteria correlates with modifications to the outer membranes of these organisms and was not stable outside of the presence of the antimicrobial. Even if this resistance was modest in comparison with the resistance to antimicrobials such as ciprofloxacin, it could pose challenges in the future. Thus, it may be predicted that the second-generation CSAs will gain importance against biofilm and planktonic cells.

In our study, CSA-192 showed antifungal activities against *C. albicans* in both mono and multispecies biofilms. According to our results, CSA-142 showed the least antimicrobial activity against either *C. albicans* or *S. aureus* in mono and multispecies biofilms; this could be explained by decomposition of the CSA-142 (because of its chemical structure) (Guan et al. 2000). Our results are corroborated by a recent study which reported that CSA-142 showed the least antimicrobial activity against *C. albicans* and *S. aureus* planktonic cells (Bozkurt-Guzel et al. 2018). Although we observed that CSA-138 showed antibiofilm activity against *C. albicans* cells in both mono and multispecies biofilms, it showed no activity against MRSA or MSSA cells either in mono or multispecies biofilms.

The different activities of antimicrobials against yeast and bacteria can be explained by significant differences in the cell surface of the *C. albicans* and bacteria. The fungal membranes are less negatively charged compared to bacteria as they contain neutral sterols. Additionally, yeast cell walls are thicker and composed of different polysaccharides such as chitin and β glucan (Durnaś et al. 2016; Yeaman and Yount 2003).

Previous studies have demonstrated that in fungal–bacterial biofilms with *C. albicans*, the number of *S. aureus* cells increased comparatively to its single-species biofilms due to the presence of a higher quantity of glucose. The major component of the *C. albicans* matrix is glucose, and it can be used by *S. aureus* as a carbon source for biofilm formation (Zago et al. 2015). Among CSAs, CSA-192 and CSA-8 were found effective against *C. albicans* in multispecies biofilms. This can explain the increase of cell counts in multispecies biofilms of *S. aureus* due to the lack of *C. albicans* cells.

The principal aim of this study was to evaluate the quantitative effects of CSAs and antibiotics on *in vitro* mono and multispecies biofilm development by *C. albicans and S. aureus*. Our results indicated that CSAs and antibiotics modulate biofilm development to varying degrees with both *C. albicans* and *S. aureus*. It was observed that CSA-8 has significant inhibitory effects on both mono and multispecies biofilm formation, whereas CSA-192 only affects multispecies biofilm according to mono species biofilm development. Decrease of *C. albicans* mono biofilm formation observed in the CFU assay and multispecies biofilm formation by MTT assay with treatment of CSA-192 suggest that CSA-192 shows an inhibitory effect on growth of *C. albicans.* Inhibition of both mono and multispecies biofilm development with CSA-8 was confirmed through fluorescence microscopy images. It was shown that CSA-8 inhibits biofilm formation with effect on both *C. albicans and S. aureus*.

5. Conclusions

This is the first report evaluating the antibiofilm activities of the first-generation CSAs CSA-8 and CSA-44 and the second-generation of CSAs CSA-138, CSA-142, and CSA-13 NDSA against mature biofilms compared to conventional antimicrobial agents. Additionally, this is the first study investigating various CSAs against fungal–bacterial multispecies biofilms which are an understudied and a clinically relevant problem. The antibiofilm activity of CSAs, especially against fungal–bacterial multispecies biofilms, represents a promising option for the future development of antimicrobial agents. Doses necessary to reach local concentrations of the CSAs to eliminate biofilms will depend upon route of administration, and reaching these concentrations should be possible in topical and gastrointestinal applications. Further safety, efficacy, and pharmacokinetic studies, especially involving different models, should be performed to correlate the potential use of CSAs as antibiofilm agents.

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