Pharmacodynamics of Moxifloxacin, Meropenem, Caspofungin, and Their Combinations against *In Vitro* Polymicrobial Interkingdom Biofilms

Albert Ruiz-Sorribas,[a] Hervé Poilvache,[b,c] Françoise Van Bambeke[a]

aPharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium
bLaboratoire de Neuro Musculo Squelettique, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium
cOrthopaedic Surgery Department, Cliniques Universitaires Saint-Luc, Brussels, Belgium

ABSTRACT Biofilms colonize medical devices and are often recalcitrant to antibiotics. Interkingdom biofilms, where at least a bacterium and a fungus are present, increase the likelihood of therapeutic failures. In this work, a three-species *in vitro* biofilm model including *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* was used to study the activity of the antibiotics moxifloxacin and meropenem, the antifungal caspofungin, and combinations of them against interkingdom biofilms. The culturable cells and total biomass were evaluated to determine the pharmacodynamic parameters of the drug response for the incubation with the drugs alone. The synergic or antagonistic effects (increased/decreased effects) of the combination of drugs were analyzed with the highest-single-agent method. Biofilms were imaged in confocal microscopy after live/dead staining. The drugs had limited activity when used alone against single-, dual-, and three-species biofilms. When used in combination, additive effects against single- and dual-species biofilms and increased effects (synergy) against biomass of three-species biofilms were observed. In addition, the two antibiotics showed different patterns, moxifloxacin being more active when targeting *S. aureus* and meropenem when targeting *E. coli*. All these observations were confirmed by confocal microscopy images. Our findings highlight the interest in combining caspofungin with antibiotics against interkingdom biofilms.

KEYWORDS biofilm, moxifloxacin, meropenem, caspofungin, combination, 96-well plate, *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, antimicrobial combinations

Biofilms are consortia of microorganisms embedded in matrix produced by themselves or by the host. They can grow attached to biotic or abiotic surfaces or in aggregates (1). They are recalcitrant to antibiotics and immune defenses for several reasons. First, the matrix is a barrier that reduces the diffusion and bioavailability of drugs (2) as well as access by immune cells. Second, the scarcity of nutrients and oxygen in the biofilm triggers the appearance of dormant phenotypes. These cells with low metabolic levels are not responsive to antibiotics requiring active metabolism and replication to exert their antimicrobial effects (3). Last, dormant microorganisms surviving antibiotic exposure may regain functional metabolism and act as a reservoir, explaining relapses of the infection (4).

Medical devices are particularly prone to colonization by biofilms, i.e., vascular or urinary catheters, cardiac valves, urethral stents, endotracheal tubes, joint prostheses, etc. (1, 5). Treatment failure is frequent for such infections, leading to chronic or relapsing infections, bloodstream infection, and, in the most severe cases, death (6). A recent CDC report mentions that medical devices related infections are also associated with higher rates of antimicrobial resistance (7).

Biofilm-associated infections of medical devices are often caused by opportunistic or nosocomial pathogens like *Staphylococcus aureus*, coagulase-negative staphylococci, *Enterobacteriaceae*, *Pseudomonas* spp., *Streptococcus* spp., and *Candida* spp. (7, 8). Fungi have a higher prevalence in culture-negative cases, i.e., patients showing symptoms of infection in the absence of isolated pathogens (9, 10). In this context, polymicrobial infections, in which at least two different pathogens are identified at the same site of infection, and more particularly interkingdom biofilms, with at least a bacterium and a fungus, are more recalcitrant and require longer treatments (11–13). A retrospective clinical study (14) showed that the failure rate for treatments of polymicrobial biofilms on prosthetic joint infections was 50.2%, compared to 31.5% for monomicrobial infections and 30.2% for culture-negative infections.

Many studies have explored the relationship between dual-species interkingdom biofilms and antimicrobial therapies (see reference 15 for a review). For example, the activity of vancomycin was reduced against *S. aureus* in coculture with *Candida albicans* due to a protective effect of the secreted fungal polysaccharides against the antibiotic action (16, 17). In the same line, mixed-species biofilms of *Staphylococcus epidermidis* and *C. albicans* were less responsive to vancomycin and fluconazole than the corresponding single-species biofilms (18). This phenomenon is also observed in mixed-species biofilms involving Gram-negative bacteria, with ofloxacin proving less active against *Escherichia coli* cocultured with *C. albicans* (19).

In this study, a previously established *in vitro* three-species biofilm model (20) was used to explore the pharmacodynamics of the combinations of a broad-spectrum antibiotic and an antifungal agent. The model includes *S. aureus* as the most frequently isolated pathogen in infections developing on orthopedic devices (1), *E. coli* as a model for *Enterobacteriaceae* (21) and *C. albicans* as a model for fungal infections (22). As broad-spectrum antibiotics, we used the fluoroquinolone moxifloxacin, which is part of the gold standard treatment for bacterial infections associated with medical devices, and the carbapenem meropenem, as a potent alternative against extended-spectrum-β-lactamase (ESBL)-producing Gram-negative bacteria, which are often coresistant to fluoroquinolones (23, 24). As an antifungal agent, the echinocandin caspofungin was tested. It is a noncompetitive inhibitor of the β-1,3-glucan synthase Gsc1 in *Candida* spp. This transmembrane enzyme adds glucose residues to the β-1,3-glucan, one of the major cell wall polysaccharides (25, 26). Gsc1 is homologous to the bacterial poly-β-1,6-*N*-acetylglucosamine (PNAG) synthases IcaA in *Staphylococcus* spp. and PgaC in *Enterobacteriaceae* (see Fig. S1 in the supplemental material). These enzymes are responsible for the elongation of the chains of PNAG, a major polysaccharidic component of the matrix (27, 28). Caspofungin and another echinocandin, anidulafungin, were shown to also target the staphylococcal enzyme, reducing the amount of PNAG in the biofilm matrix and improving the activity of antibiotics, including fluoroquinolones, against *S. aureus* biofilms (29, 30). Likewise, micafungin can potentiate the effect of levofloxacin and ceftazidime against *Pseudomonas aeruginosa* biofilms, but the underlying mechanism has not been explored (31).

The interactions between drugs were assessed using the highest-single-agent model (32), which presupposes that there are an active and an inactive drug in each combination, which is expected when an antibiotic and an antifungal agent are mixed. This model establishes additivity when the effect of the combination is not different from that of the most active drug alone and synergy (increased effect) when the effect of the combination is higher than that of the most active drug alone.

In brief, we showed that the drugs had limited activity when used alone against single-, dual-, or three-species biofilms and an additive effect when they were used in combination against single- or dual-species biofilms but increased (synergistic) effects, especially when caspofungin was combined with meropenem, to reduce the biomass of three-species biofilms.

RESULTS

Mixed-species biofilm models. A three-species biofilm model in 96-wells polystyrene plates including *S. aureus*, *E. coli* and *C. albicans* was previously set up (20). The

same protocol was used to grow the corresponding bacterium-fungus dual-species biofilms and single-species biofilms. The study of the evolution of the culturable cells and biomass over time for all models showed no significant difference between 48 and 72 h (Fig. S2), i.e., the time interval during which biofilms were incubated with the antimicrobials. Thus, any difference observed during this time frame is due to the activity of the antimicrobials.

Single-drug activity against culturable cells in planktonic cultures and biofilms. The MICs of moxifloxacin and meropenem were 0.06 mg/L and 0.03 mg/L, respectively, against both bacteria but > 256 mg/L against *C. albicans*. The MIC of caspofungin was low against *C. albicans* (0.125 mg/L) but high against bacteria (64 and 32 mg/L against *S. aureus* and *E. coli*, respectively). The concentration-response curves for the activity of these antimicrobials against planktonic cultures and biofilms and the pharmacodynamic parameters maximal effect (E_{max}), C_{-1log}, and $C_{-90\%}$ are presented in Fig. 1 and Table 1. Drug activity was expressed as the change from the initial value measured at the time of drug addition (48-h precultured biofilms or initial inoculum for planktonic cultures). This reference time point was selected to compare activity in biofilms and planktonic cultures because biofilms remained stable during the incubation (Fig. S2) while planktonic cultures were growing. E_{max} corresponds to the horizontal asymptote for the reduction in culturable cells or biomass from this reference time point, extrapolated for an infinitely large concentration of the drug. C_{-1log} and $C_{-90\%}$ correspond to the concentration of the drug required to reduce 90% of culturable cells (thus 1 $\log_{10}$ CFU/well) or of biomass, respectively. Thus, they are measures of the relative potency of the drug.

Moxifloxacin (Fig. 1A to C) caused a significantly smaller reduction of culturable cells against both bacterial species in biofilms than in planktonic cultures and had no effect against *C. albicans*. It was more effective (more negative E_{max}) against *S. aureus* in the single-species biofilm than in the *S. aureus–C. albicans* or three-species biofilms and against *E. coli* in single-species or *E. coli–C. albicans* biofilms than in the three-species biofilm. C_{-1log} values were close to those measured in planktonic cultures in all biofilms and for both species, except for *S. aureus* in the three-species biofilm, against which moxifloxacin was less potent (higher C_{-1log}).

Meropenem (Fig. 1D to F) showed a reduced efficacy against *S. aureus* (though to a lesser extent against the *S. aureus–C. albicans* biofilm) and to a lower extent against *E. coli* (significant difference for single-species biofilm only) compared to the planktonic cultures. C_{-1log} values against bacteria were similar against planktonic cultures and all biofilms with the noticeable exception of the single-species biofilm of *E. coli*, against which meropenem was less potent. In other words, meropenem was more potent against *E. coli* in biofilms in which *C. albicans* was present, associated with *S. aureus* or not. No activity against *C. albicans* was observed.

Caspofungin (Fig. 1G to I) was active against planktonic *C. albicans* at low concentrations, with a C_{-1log} 10 times lower than the MIC and an E_{max} of -2.3 $\log_{10}$ CFU/well. Against bacteria, it started reducing the inoculum only at large concentrations. In biofilms, caspofungin did not show any effect against culturable *E. coli* cells and only little effect at high concentrations against *S. aureus*. Surprisingly, high concentrations were also needed to reduce *C. albicans* culturable cells in single-species and *S. aureus–C. albicans* biofilms, with C_{-1log} around 12 mg/L. The E_{max} was not reached in most biofilms at the highest concentration tested.

Single-drug activity against biomass in biofilms. The concentration-response curves for the activity of these antimicrobials against biomass in biofilms and the corresponding pharmacodynamic parameters E_{max} and $C_{-90\%}$ are presented in Fig. 2 and Table 1.

Moxifloxacin reduced biofilm biomass during the 24 h of incubation only in single-species biofilms of *S. aureus* or *E. coli*, but the E_{max} was not reached at the highest concentration tested (Fig. 2A). The biomass of dual-species and three-species biofilms slightly increased after exposure to moxifloxacin (Fig. 2B). Meropenem reduced the biomass of bacterial single-species biofilms only, to 75 and 34% of the control value in
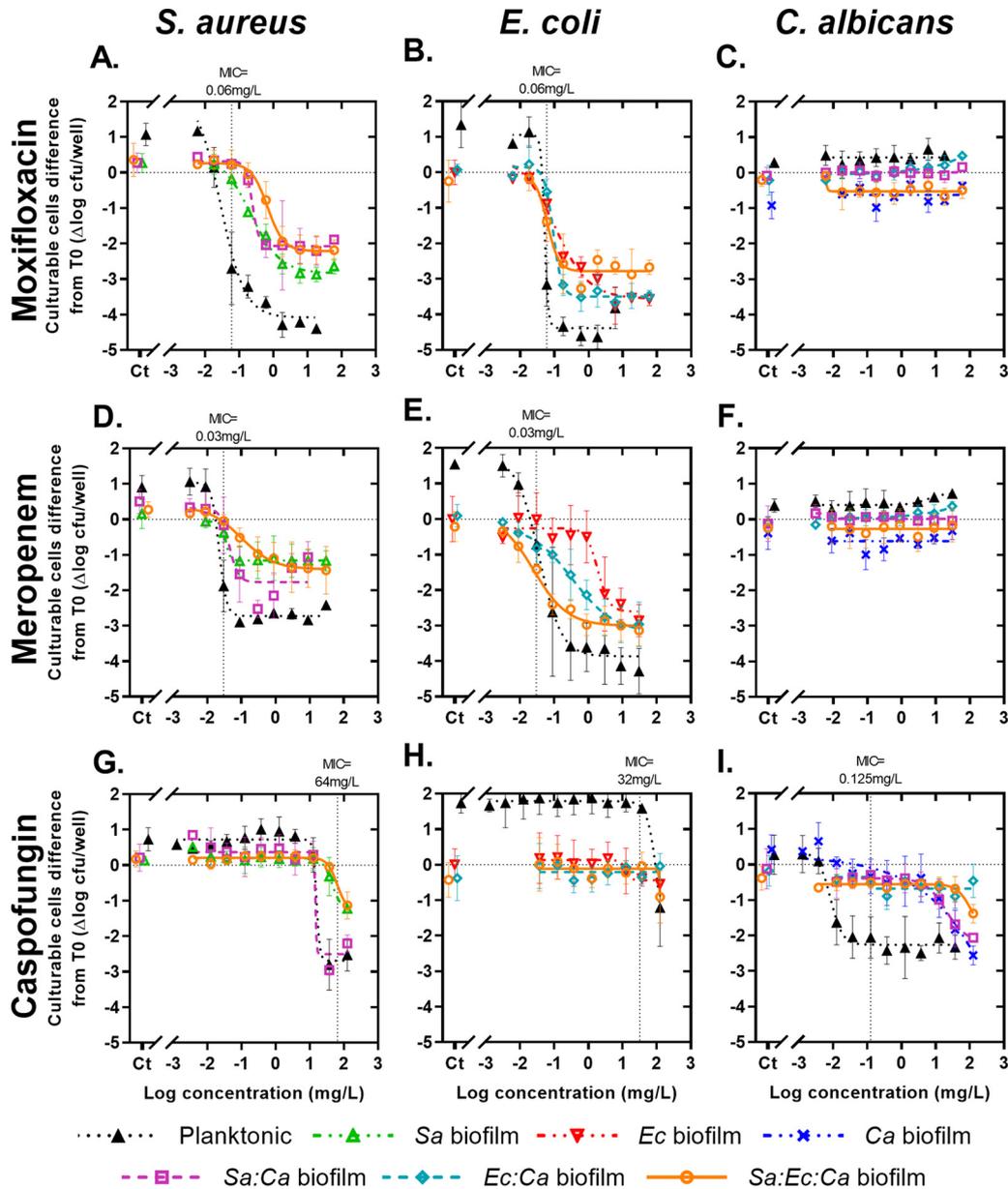
FIG 1 Activity of antimicrobials against culturable cells in planktonic cultures and biofilms after 24 h of incubation over a broad range of concentrations. Each row corresponds to one antimicrobial (moxifloxacin [A to C], meropenem [D to F], or caspofungin [G to I]); each column corresponds to one microorganism in all models, with the symbols explained at the bottom. The data are expressed as the difference between culturable cells at time zero (48-h biofilms or initial inoculum for planktonic bacteria) and 24 h treatment (72-h biofilms or 24 h of incubation for planktonic bacteria). n = 3 to 6. The horizontal dotted line shows the initial value of culturable cells; the vertical dotted line shows the MIC of each antimicrobial against the studied species. Sa, $S. aureus$; Ec, $E. coli$; Ca, $C. albicans$.

$S. aureus$ and $E. coli$ biofilms, respectively (Fig. 2C and D). Caspofungin caused significant reductions of biomass against dual-species, $E. coli$, and $C. albicans$ biofilms, with the $S. aureus$–$C. albicans$ biofilm being the most affected (Fig. 2E and F).

Activity of the moxifloxacin-caspofungin combination. A combination of moxifloxacin and caspofungin was then tested. Concentrations were adjusted to 1, 10, and 100 times their MICs against bacteria and $C. albicans$, respectively. These concentrations were selected to cover the range of concentrations over which moxifloxacin displayed a concentration-dependent effect when used alone as well as a range of clinically relevant concentrations (human maximum concentrations [C_{max}], 4 mg/L for

TABLE 1 Pharmacodynamic parameters describing the activity of moxifloxacin, meropenem and caspofungin on culturable cells and biomass after 24 h of incubation with planktonic or biofilm cultures[a]

Test	Microorganism	Model[b]	E_{max} (log CFU/well or % AU)[c]			C_{-1log} or $C_{-90\%}$ (mg/L)[d]		
			Moxifloxacin	Meropenem	Caspofungin	Moxifloxacin	Meropenem	Caspofungin
Culturable cells	S. aureus	Planktonic	−4.1 ± 0.4 a, A	−2.8 ± 0.2 a, B	−2.7 ± 0.5 a, B	0.03 ± 0.01 d, D	0.02 ± 0.01 d, D	14.6 ± 13.1 d, D
		Sa biofilm	−2.8 ± 0.2 b, A	−1.1 ± 0.2 b, B	−1.3 ± 0.9*	0.19 ± 0.05 d, D	0.04 ± 0.04 d, D	71 ± 34.1 e, E
		Sa-Ca biofilm	−2.1 ± 0.2 c, A	−1.8 ± 0.4 c, A	−2.2 ± 0.4 a, A	0.25 ± 0.14 d, D	0.05 ± 0.03 d, D	31 ± 17.2 d, E
		Sa-Ec-Ca biofilm	−2.2 ± 0.2 c, A	−1.2 ± 0.3 b, B	−1.3 ± 0.2*	0.68 ± 0.23 e, D	0.42 ± 0.85 d, D	100 ± 19.7 f, E
	E. coli	Planktonic	−4.5 ± 0.2 a, A	−3.9 ± 0.5 a, A	−1.2 ± 1.4*	0.05 ± 0.04 d, D	0.03 ± 0.01 d, D	120.6 ± ND
		Ec biofilm	−3.4 ± 0.3 b, A	−2.8 ± 0.7 b, A	0 ± 0.2*	0.05 ± 0.02 d, D	1.37 ± 0.81 e, E	>125
		Ec-Ca biofilm	−3.5 ± 0.2 b, A	−3.2 ± 0.8 ab, A	−0.4 ± 0.2*	0.07 ± 0.01 d, D	0.08 ± 0.07 d, D	>125
		Sa-Ec-Ca biofilm	−2.8 ± 0.2 c, A	−3 ± 0.2 ab, A	−0.9 ± 0.7*	0.05 ± 0.02 d, D	0.01 ± 0.01 d, D	>125
	C. albicans	Planktonic	0.5 ± 0.1*	0.5 ± 0.1*	−2.3 ± 0.2 a	>60	>30	0.01 ± 0 d
		Ca biofilm	−0.6 ± 0.1*	−0.6 ± 0.1*	−2.5 ± 0.3*	>60	>30	12.9 ± 4.6 e
		Sa-Ca biofilm	0 ± 0.1*	0 ± 0*	−2.2 ± 0.9 a	>60	>30	12.2 ± 8 e
		Ec-Ca biofilm	0.1 ± 0.1*	0.3 ± 0*	−0.6 ± 0.4*	>60	>30	>125
		Sa-Ec-Ca biofilm	−0.5 ± 0.1*	−0.5 ± 0.2*	−1.4 ± 0.3*	>60	>30	73.5 ± 20.5 f
Biomass		Sa biofilm	54.4 ± 20.6 a	77.6 ± 17.6 a	115.5 ± 12*	>60	>30	>125
		Ec biofilm	64.7 ± 8.2*	33.5 ± 6.5*	69.7 ± 32 a	>60	>30	>125
		Ca biofilm	112.3 ± 13 ab, A	112.8 ± 27.6 b, A	81.9 ± 4.8 a, B	>60	>30	>125
		Sa-Ca biofilm	127 ± 17*	91 ± 8.7 a	49 ± 5.7*	>60	>30	>125
		Ec-Ca biofilm	139 ± 12.7 a, A	133.2 ± 10.6 b, A	81 ± 1.4 a, B	>60	>30	>125
		Sa-Ec-Ca biofilm	106.7 ± 8.4 b, A	87.2 ± 7.8 a, A	85 ± 16.5 a, A	>60	>30	>125

[a]Data are based on concentration-response curves shown in Fig. 1 and 2. Statistical analysis for each microorganism and test was done with two-way ANOVA and Tukey posttest. Significant differences ($P < 0.05$) between models for the same drug are highlighted by different lowercase letters in the same column; those between different drugs for the same model are indicated by different uppercase letters in the same row. *, effect at 60, 30 or 125 mg/L of moxifloxacin, meropenem, or caspofungin, respectively (highest concentrations tested). Not considered for the statistical comparisons of E_{max}.

[b]Sa, S. aureus; Ca, C. albicans; Ec, E. coli.

[c]Maximal reduction for culturable cells (log CFU/well) or biomass (% AU), extrapolated from the concentration-response curve for an infinitely large concentration.

[d]Concentration needed to reduce of 90% the culturable cells (−1 log) or of 90% the biomass ($C_{-90\%}$), calculated from the concentration-response curve. ND, not determined.
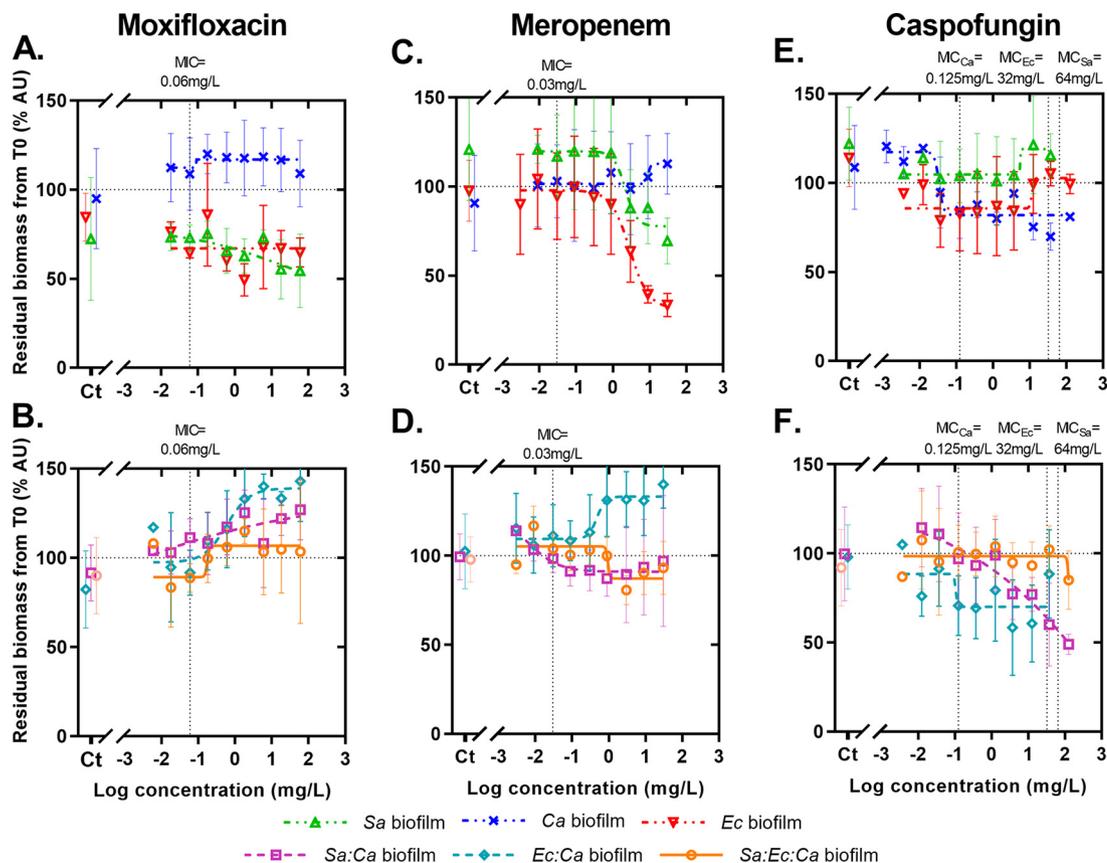
FIG 2 Activity of antimicrobials against biomass of biofilms after 24 h of incubation over a broad range of concentrations. Each column corresponds to one antimicrobial (moxifloxacin [A and B], meropenem [C and D], and caspofungin [E and F]); the top row shows results for single-species biofilms, and the bottom row shows to dual- or three-species biofilms, with the symbols defined at the bottom. The data are expressed as the difference between culturable cells at time zero (48-h biofilms) and 24 h treatment (72-h biofilms). *n* = 3 to 6. The horizontal dotted line shows the biomass value at time zero (100%); the vertical dotted line shows the MIC of each antimicrobial against the studied species. *Sa*, *S. aureus*; *Ec*, *E. coli*; *Ca*, *C. albicans*.

moxifloxacin [70 times the MIC against bacteria] [33] and 13.8 mg/L [free drug; highly protein-bound drug] for caspofungin [110 times the MIC against *C. albicans*] [34]).

Figure S3 and Fig. 3 show the results obtained for culturable cells of each microorganism in planktonic cultures and each biofilm, respectively. In planktonic cultures, an additive effect was generally observed, with reductions in culturable cells close to the effect of the active single agent in most cases. However, a decreased effect (antagonism) was noticed at high concentrations of caspofungin against *S. aureus* or at low concentrations of moxifloxacin against *E. coli* (Fig. S3A and B), or an increased effect (synergy) at a high concentration of caspofungin and low concentration of moxifloxacin against *C. albicans* (Fig. S3C).

An additive effect was observed in single-species, *S. aureus*–*C. albicans*, and three-species biofilms for all organisms (Fig. 3A to E and H to J). A decreased effect was noticed regarding *E. coli*, which reached significance in the *E. coli*–*C. albicans* biofilm but not in the *E. coli* single-species or the three-species biofilms (Fig. 3B and F). No interaction was observed regarding *C. albicans* in the same biofilm exposed to drug combinations (Fig. 3G).

Considering then the biomass of each type of biofilm (Fig. 4), an additive effect against single-species or dual-species biofilms was observed (Fig. 4A to E), but a strong synergy (increased effect) was noticed against the three-species biofilm when at least one of the two antimicrobials was present at the highest concentration tested, decreasing the biomass by 20% to 30% (Fig. 4F).
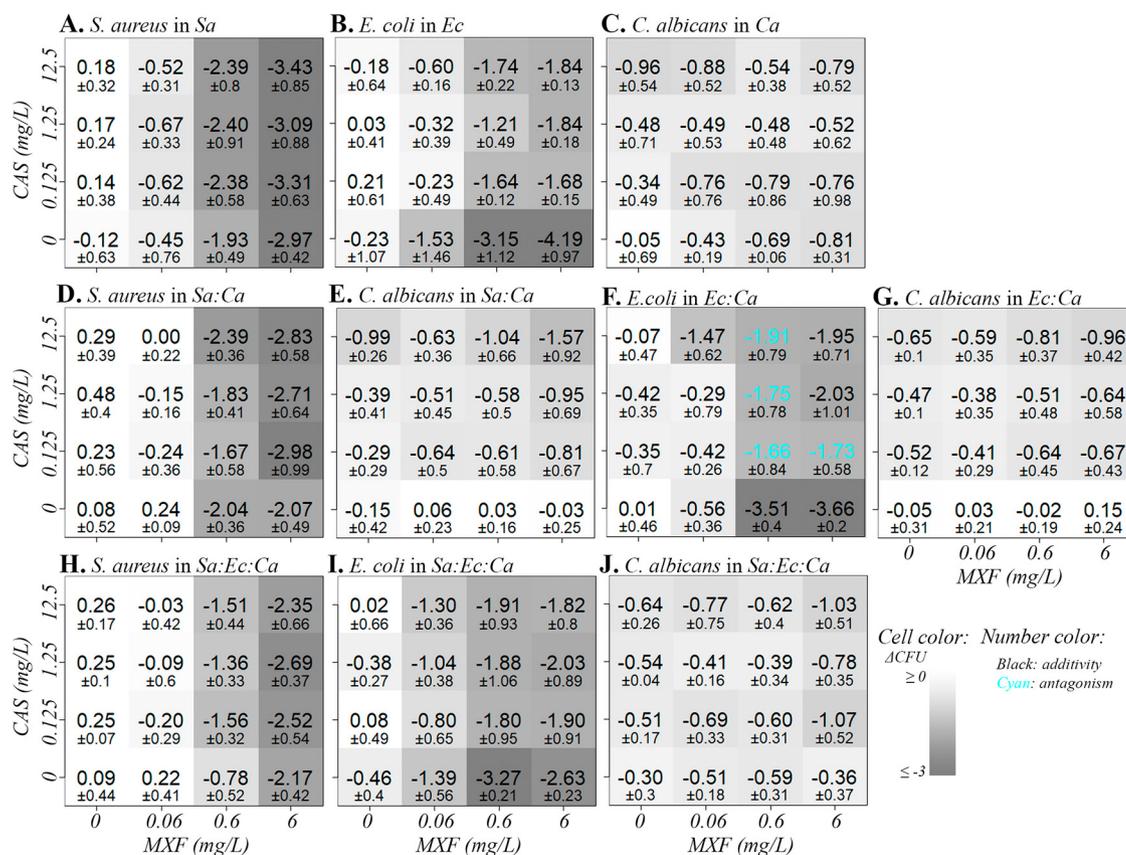
FIG 3 Effect (average and standard deviation [SD]) against the culturable cells in biofilms ($\Delta\log_{10}$ CFU/well) of the combination of moxifloxacin and caspofungin for 24 h and its synergy/antagonism (increased/decreased effect). Each drug was used at three selected concentrations; data for single drugs at the concentrations used in combination are repeated for clarity. The effect is shown in gray scale (darker shade means more effect). Black numbers show additivity (no interaction); cyan numbers show a decreased effect (antagonism; $P < 0.05$). Moxifloxacin (MXF) and caspofungin (CAS) concentrations are in milligrams per liter.

Activity of the meropenem-caspofungin combination. A combination of meropenem and caspofungin was also tested following a similar design, except that the concentrations selected for meropenem were 10 times higher than those of moxifloxacin (10, 100, and 1,000 times the MIC) to fully cover the zone of concentration dependency of its activity, to reach the plateau of maximal efficacy in all biofilms, and to better cover the range of clinically relevant concentrations for this drug (human C_{max}, 40 mg/L for a dose of 2 g [670 times the MIC against bacteria] [35]).

The combination was examined against culturable cells in planktonic cultures and biofilms (Fig. S4 and Fig. 5). In planktonic cultures, additive effects were observed in most of the cases, and thus, there was no gain or loss from the effect of the active single agent. Nevertheless, a synergy at a high concentration of caspofungin against *C. albicans* (Fig. S4C) and a decreased activity at a high concentration of caspofungin and low concentration of meropenem against *E. coli* (Fig. S4B) were observed.

In the single-species biofilms, meropenem and caspofungin showed significant interactions but with different patterns. Caspofungin increased the effect of meropenem at 0.3 mg/L against *S. aureus* (Fig. 5A) and at any concentration against *E. coli* (Fig. 5B). Conversely, meropenem increased the effect of caspofungin at 0.125 mg/L against *C. albicans* (Fig. 5C). For the dual-species biofilms, an additive effect was observed against *S. aureus* (Fig. 5D), but caspofungin increased the effect of meropenem at 0.3 mg/L against *E. coli* (Fig. 5F). Again, meropenem slightly increased the effect of caspofungin against *C. albicans* in both dual-species biofilms, but the synergy reached significance only in the *S. aureus*–*C. albicans* biofilm and for the lowest concentrations of caspofungin (Fig. 5E, G).
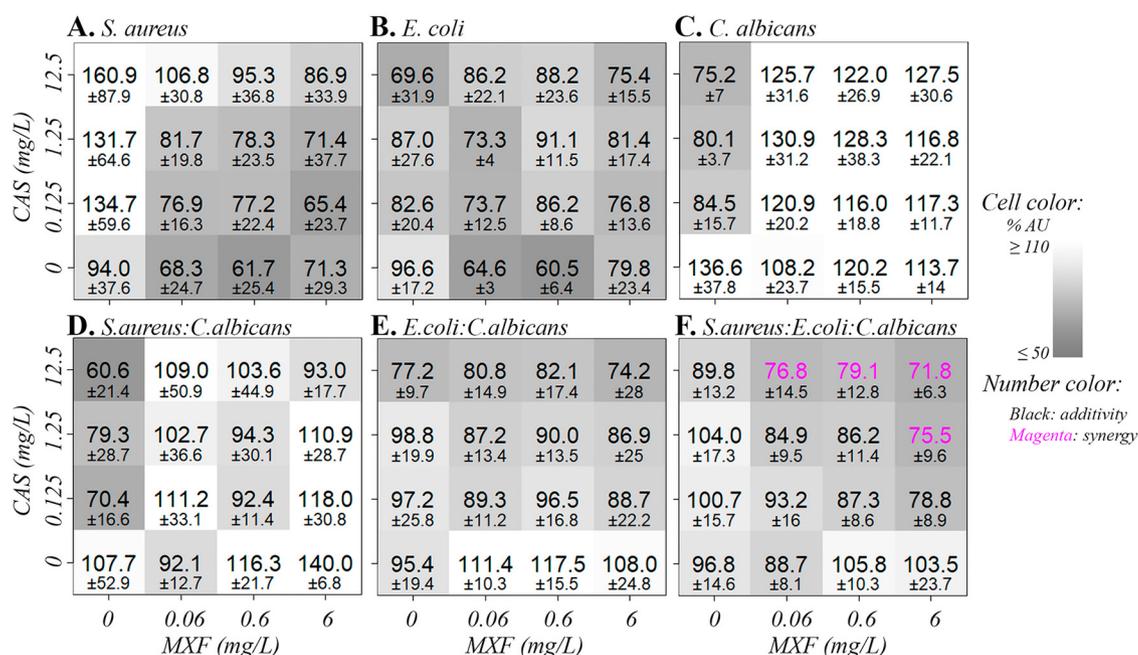
A. *S. aureus*

CAS (mg/L)	MXF 0	MXF 0.06	MXF 0.6	MXF 6
12.5	160.9 ±87.9	106.8 ±30.8	95.3 ±36.8	86.9 ±33.9
1.25	131.7 ±64.6	81.7 ±19.8	78.3 ±23.5	71.4 ±37.7
0.125	134.7 ±59.6	76.9 ±16.3	77.2 ±22.4	65.4 ±23.7
0	94.0 ±37.6	68.3 ±24.7	61.7 ±25.4	71.3 ±29.3

B. *E. coli*

CAS (mg/L)	MXF 0	MXF 0.06	MXF 0.6	MXF 6
12.5	69.6 ±31.9	86.2 ±22.1	88.2 ±23.6	75.4 ±15.5
1.25	87.0 ±27.6	73.3 ±4	91.1 ±11.5	81.4 ±17.4
0.125	82.6 ±20.4	73.7 ±12.5	86.2 ±8.6	76.8 ±13.6
0	96.6 ±17.2	64.6 ±3	60.5 ±6.4	79.8 ±23.4

C. *C. albicans*

CAS (mg/L)	MXF 0	MXF 0.06	MXF 0.6	MXF 6
12.5	75.2 ±7	125.7 ±31.6	122.0 ±26.9	127.5 ±30.6
1.25	80.1 ±3.7	130.9 ±31.2	128.3 ±38.3	116.8 ±22.1
0.125	84.5 ±15.7	120.9 ±20.2	116.0 ±18.8	117.3 ±11.7
0	136.6 ±37.8	108.2 ±23.7	120.2 ±15.5	113.7 ±14

D. *S.aureus:C.albicans*

CAS (mg/L)	MXF 0	MXF 0.06	MXF 0.6	MXF 6
12.5	60.6 ±21.4	109.0 ±50.9	103.6 ±44.9	93.0 ±17.7
1.25	79.3 ±28.7	102.7 ±36.6	94.3 ±30.1	110.9 ±28.7
0.125	70.4 ±16.6	111.2 ±33.1	92.4 ±11.4	118.0 ±30.8
0	107.7 ±52.9	92.1 ±12.7	116.3 ±21.7	140.0 ±6.8

E. *E.coli:C.albicans*

CAS (mg/L)	MXF 0	MXF 0.06	MXF 0.6	MXF 6
12.5	77.2 ±9.7	80.8 ±14.9	82.1 ±17.4	74.2 ±28
1.25	98.8 ±19.9	87.2 ±13.4	90.0 ±13.5	86.9 ±25
0.125	97.2 ±25.8	89.3 ±11.2	96.5 ±16.8	88.7 ±22.2
0	95.4 ±19.4	111.4 ±10.3	117.5 ±15.5	108.0 ±24.8

F. *S.aureus:E.coli:C.albicans*

CAS (mg/L)	MXF 0	MXF 0.06	MXF 0.6	MXF 6
12.5	89.8 ±13.2	*76.8 ±14.5*	*79.1 ±12.8*	*71.8 ±6.3*
1.25	104.0 ±17.3	84.9 ±9.5	86.2 ±11.4	*75.5 ±9.6*
0.125	100.7 ±15.7	93.2 ±16	87.3 ±8.6	78.8 ±8.9
0	96.8 ±14.6	88.7 ±8.1	105.8 ±10.3	103.5 ±23.7

MXF (mg/L): 0, 0.06, 0.6, 6

Cell color:
%AU
≥ 110
≤ 50

Number color:
Black: additivity
Magenta: synergy

FIG 4 Effect (average and SD) against the biomass of the biofilms (% absorbance units [AU]/well) of the combination of moxifloxacin and caspofungin for 24 h, and its synergy/antagonism (increased/decreased effect). Each drug was used at three selected concentrations; data for single drugs at the concentrations used in combination are repeated for clarity. The effect is shown in gray scale (darker shade means more effect). Black numbers show additivity (no interaction); magenta numbers show increased effect (synergy; $P < 0.05$). Moxifloxacin (MXF) and caspofungin (CAS) concentrations are in milligrams per liter.

In the three-species biofilm, an additive effect was observed for *S. aureus* (Fig. 5H). Interestingly, the patterns against *E. coli* and *C. albicans* were opposite to each other (Fig. 5I and J): caspofungin reduced the effect of meropenem against *E. coli*, while meropenem markedly increased the effect of caspofungin against *C. albicans*.

Against biomass (Fig. 6), an increase in activity was observed for the combination between meropenem and caspofungin against the single-species and the three-species biofilms (Fig. 6A to C and F). The reduction in biomass in the three-species biofilm reached around 40%. However, only an additive effect was observed against the dual-species biofilms (Fig. 6D and E).

Confocal microscopy with live/dead staining. Figure 7 shows images from confocal microscopy of biofilms grown on Ti coupons and exposed to a combination of moxifloxacin-caspofungin or meropenem-caspofungin at the highest concentrations tested previously (i.e., 6/12.5 and 30/12.5 mg/L, respectively), for 24 h. The pictures show qualitative trends similar to those observed in biofilms grown in microtiter plates.

For single-species biofilms, the highest proportion of dead bacteria was observed in the *S. aureus* biofilm after incubation with moxifloxacin-caspofungin. A similarly high reduction of approximately 3 $\log_{10}$ CFU was measured in microtiter plates for *E. coli* biofilms exposed to meropenem-caspofungin, but the predominant effect seen in the confocal image was the marked reduction in biomass, also described in quantitative experiments (28% of residual biomass). The images of *S. aureus* biofilm exposed to meropenem-caspofungin and *E. coli* biofilms exposed to moxifloxacin-caspofungin looked similar, in accordance with our quantitative results. A slightly higher proportion of dead cells were visible in the treated *C. albicans* biofilm than in the corresponding control.

For the dual-species biofilms, dead cells were more visible in biofilms incubated with the combination moxifloxacin-caspofungin, while the reduction in biomass was more visible after incubation with the meropenem-caspofungin combination, as in our quantitative experiments.
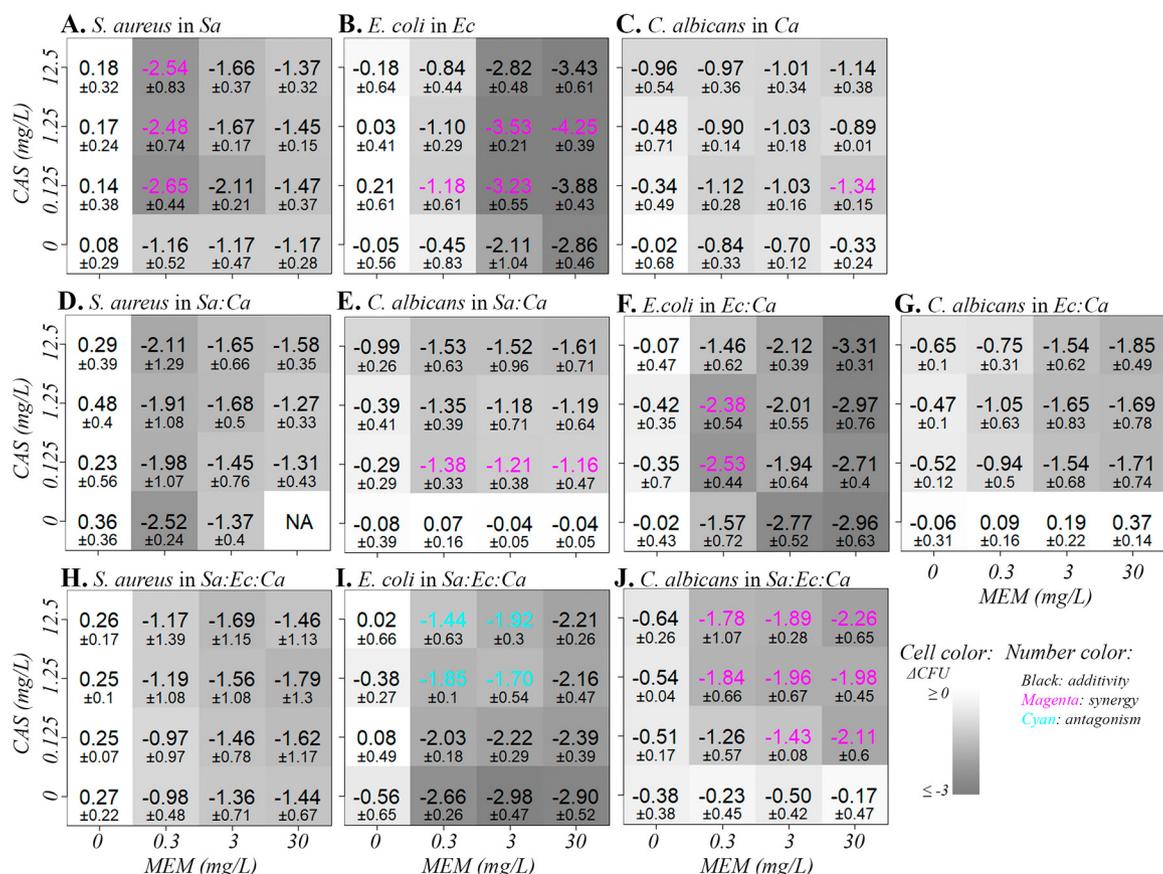
FIG 5 Effect (average and SD) against the culturable cells in biofilms ($\Delta\log_{10}$ CFU/well) after the combination of meropenem and caspofungin for 24 h and its synergy/antagonism (increased/decreased effect). Each drug was used at three selected concentrations; data for single drugs at the concentrations used in combination are repeated for clarity. The effect is shown in gray scale (darker shade means more effect). Black numbers show additivity (no interaction); magenta numbers show increased effect (synergy; $P < 0.05$); cyan numbers show decreased effect (antagonism; $P < 0.05$). Meropenem (MEM) and caspofungin (CAS) concentrations are in milligrams per liter.

For the three-species biofilm, both combinations markedly reduced the biomass, and a substantial proportion of *C. albicans* cells were dead after incubation with meropenem-caspofungin, as also observed in 96-well plates.

Relative abundance of polysaccharides. The abundance of polysaccharides was determined after incubation with moxifloxacin at 6 mg/L, meropenem at 30 mg/L, caspofungin at 12.5 mg/L and moxifloxacin-caspofungin or meropenem-caspofungin at the same concentrations. The relative abundance in each sample (i.e., the quantity of polysaccharides normalized to the total biomass of the corresponding biofilm assessed by the absorbance of crystal violet) was compared to the value measured in the corresponding control biofilm (incubation without antimicrobials) (Fig. 8; values of absolute amounts of polysaccharides and optical density [OD] of crystal violet are presented in Fig. S5).

A lower relative abundance of polysaccharides was observed in all biofilms incubated with each of the combinations of antimicrobials (moxifloxacin-caspofungin or meropenem-caspofungin) compared to control biofilms, except for the *E. coli* biofilm (Fig. 8B). For drugs used alone, caspofungin was the only one to reduce the polysaccharide relative abundance in all biofilms (except *E. coli* biofilms, for which an increase was observed), although this effect did not reach statistical significance.

DISCUSSION

While some studies have explored the activity of antibiotics and antifungals against both single- and dual-species biofilms (17, 30, 36), only a few of them have included
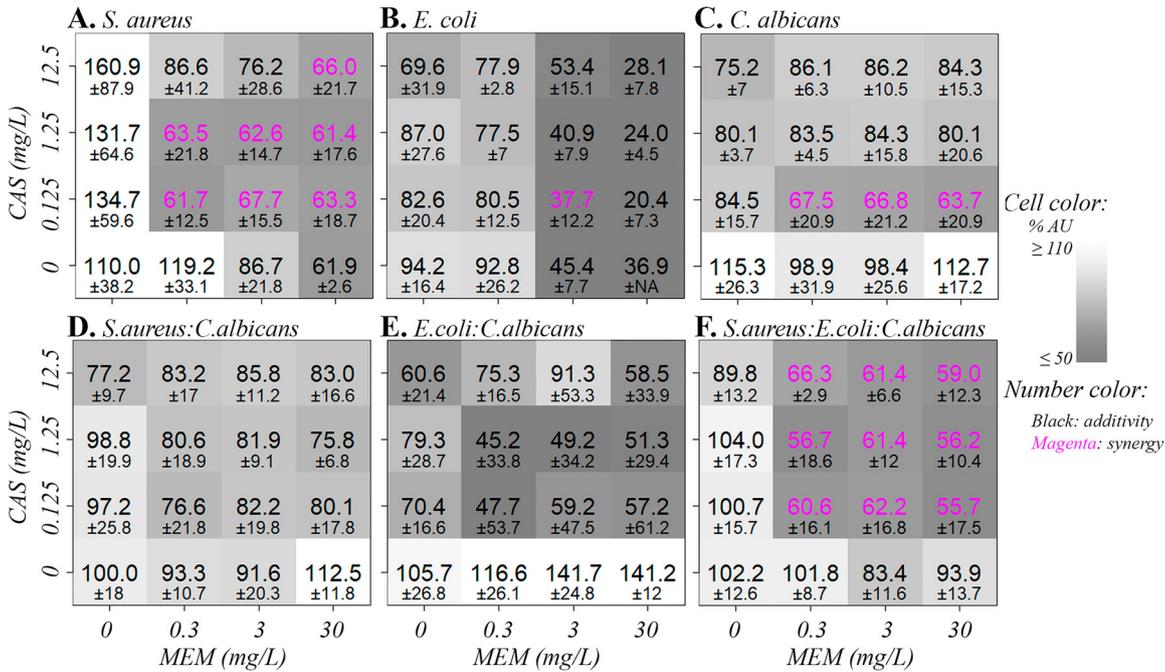
FIG 6 Effect (average and SD) against the biomass of the biofilms (% AU/well) after the combination of meropenem and caspofungin for 24 h and its synergy/antagonism (increased/decreased effect). Each drug was used at three selected concentrations; data for single drugs at the concentrations used in combination are repeated for clarity. The effect is shown in gray scale (darker shade means more effect). Black numbers show additivity (no interaction); magenta numbers show increased effect (synergy; $P < 0.05$). Meropenem (MEM) and caspofungin (CAS) concentrations are in milligrams per liter.

the selection of antimicrobials explored in this work (37, 38) or tested them against interkingdom biofilms (16, 18, 19, 39). Moreover, this study is, to the best of our knowledge, the first one to assess on a pharmacodynamic basis the nature of the drug interactions against the pathogens used in the three-species biofilm model.

Considering first the activity of single agents against single-species biofilms, we globally observed that antibiotics are less effective, but not necessarily less potent, against biofilms than planktonic bacteria, while caspofungin was less potent in *Candida* biofilms and showed only minimal efficacy except at high concentrations. Although few studies ran full concentration-response curves allowing for a direct comparison with our work, some previous publications also showed that moxifloxacin was more effective than meropenem against *S. aureus* biofilms (29, 40, 41), that the fluoroquinolone levofloxacin was more potent and effective than meropenem against *E. coli* biofilms (42), or that caspofungin potency was reduced in biofilms (43). The lack of effect of caspofungin against single-species or *E. coli*–*C. albicans* biofilms was reported previously (16, 19). Despite this global loss of efficacy, it is worth mentioning that reductions superior to 2.5 $\log_{10}$ CFU were observed against *E. coli* biofilm for both antibiotics or moxifloxacin against *S. aureus* when they were used at concentrations close to their respective human C_{max} (around 4 and 40 mg/L for doses of 400 mg and 2 g, respectively [33, 35]). Nevertheless, there was always a high proportion of bacterial persistence, ranging from 10^3 to 10^5 CFU/well in all biofilms.

Regarding the activity of single agents against mixed-species biofilms, moxifloxacin was less effective against *S. aureus* in the three- and dual-species biofilm than against the single-species biofilm. It was also less effective against *E. coli* in the three-species biofilm than in the single-species or dual-species biofilm. This could be ascribed to a protective role of the *C. albicans* polysaccharidic matrix (16, 17), but the reason why this does not apply to meropenem remains to be established. This behavior contrasts with that of meropenem, for which the major difference observed among biofilm models concerns its potency. It was markedly reduced against *E. coli* in single-species
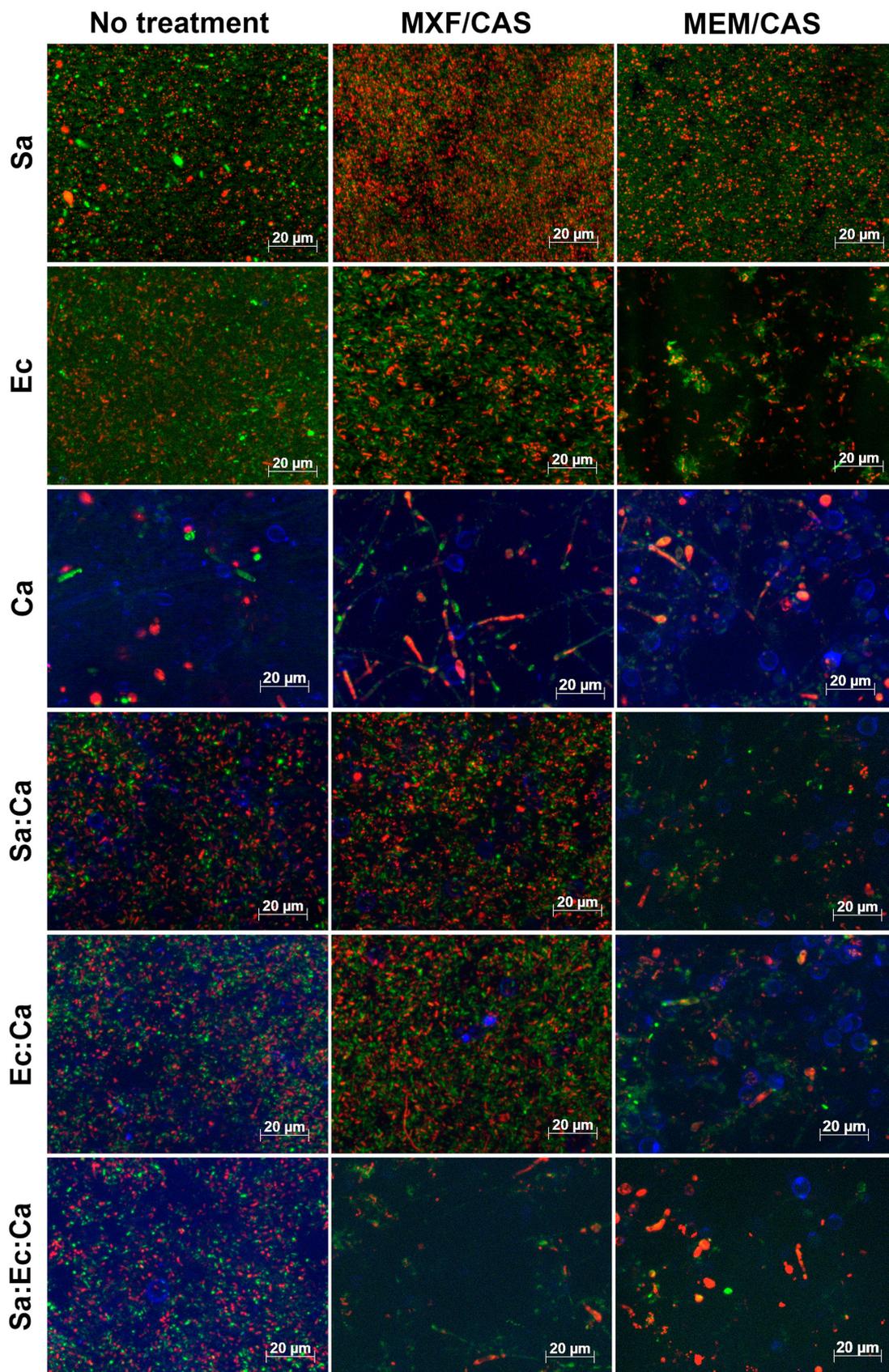
FIG 7 Confocal images of biofilms after live/dead staining. Biofilms grown on Ti alloy coupons were incubated for 24 h with combinations of moxifloxacin-caspofungin (6 and 12.5 mg/L) or meropenem-caspofungin (30 and 12.5 mg/L). Green, intact cells (live); red, disrupted cells (dead); blue, *C. albicans* yeasts and hyphae. Sa, *S. aureus*; Ec, *E. coli*; Ca, *C. albicans*.
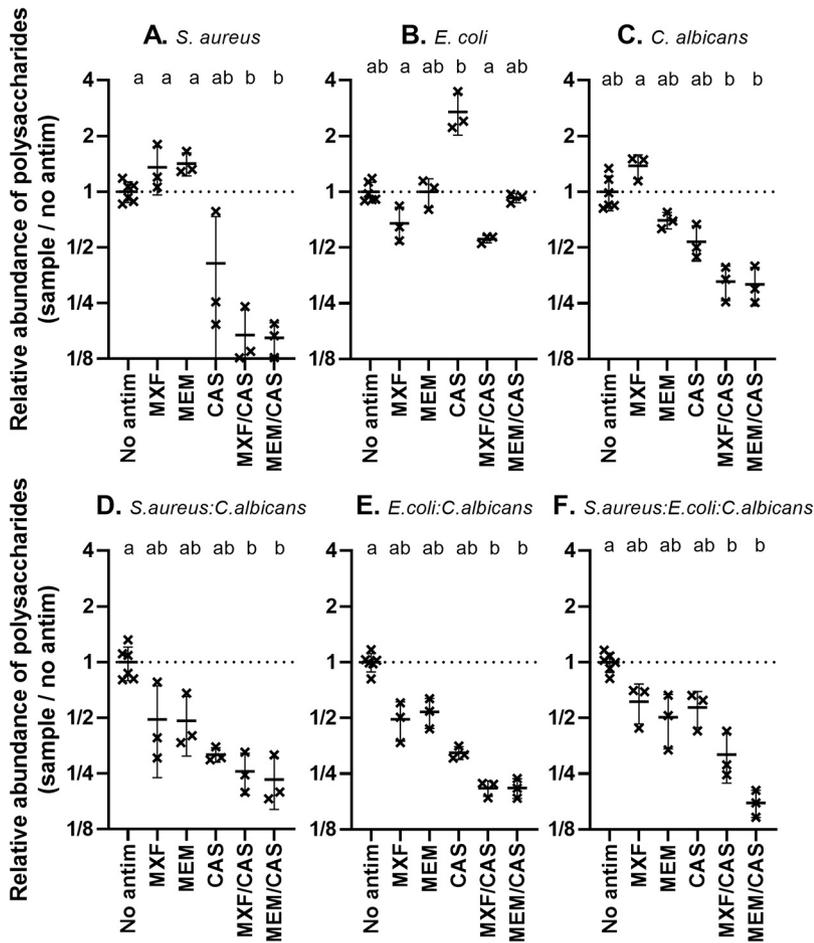
FIG 8 Relative abundance of polysaccharides after incubation with antimicrobials, expressed as the ratio to the value measured in the corresponding control (no antimicrobial added). The abundance itself was calculated as the ratio between the quantity of polysaccharides, estimated with the periodic acid-Schiff method, and the total biomass, estimated with the crystal violet assay. MXF, moxifloxacin (6 mg/L); MEM, meropenem (30 mg/L); CAS, caspofungin (12.5 mg/L). $n = 3$ to 6. Different letters denote significant differences ($P < 0.05$; one-way nonparametric analysis of variance [ANOVA], Tukey posttest).

biofilms versus planktonic cultures or mixed species biofilms, in spite of the fact that *E. coli* activates stress responses in interkingdom biofilms (20).

Both moxifloxacin and meropenem reduced biomass in the bacterial single-species biofilms but not in *C. albicans* biofilm, which was expected. The fact that moxifloxacin increased the biomass in both dual-species biofilms and meropenem in the *E. coli–C. albicans* biofilm might be explained by the killing of bacterial cells by the antibiotics, which reduces competition for *C. albicans* to produce matrix. The poor effect of meropenem against *S. aureus* may explain why a similar trend was not observed in the *S. aureus–C. albicans* biofilm incubated with this antibiotic. Caspofungin effects on biomass were essentially observed against *S. aureus–C. albicans* biofilms, probably related to its capacity to also reduce not only the production of polysaccharides but also the number of culturable cells of *S. aureus* and of *C. albicans* (at high concentrations). The latter contribute to biomass due to their large volume. Reactive oxygen species (ROS) production, bacterial killing, and decreased biofilm formation have been documented in methicillin-resistant *S. aureus* (MRSA) exposed to caspofungin, but only when very high concentrations were used in low-ionic-content solutions and not in RPMI medium (44).

Comparing the activity of single drugs and their combinations against planktonic cultures showed that the activity of caspofungin at high concentrations was potentiated by

moxifloxacin or meropenem, which is in agreement with previous works combining caspofungin with β-lactams *in vitro* or *in vivo* (45, 46). On the other hand, the decreased activity observed for specific combinations against *S. aureus* and, more often, against *E. coli* planktonic cultures have never been described and remained unexplained. Unexpected effects of caspofungin on bacterial metabolism have been described but only for *Enterococcus faecium*, in which caspofungin instead facilitates the activity of vancomycin (47).

In biofilms, additive effects were observed in most cases, ensuring a reduction of all microorganisms in the biofilms as well as of the global biomass. However, salient increases in activity were observed in the biomasses of three-species biofilms exposed to both types of combinations. Additional synergies were noticed for specific concentration ratios of combinations with meropenem; they concern bacterial counts in single- or dual-species biofilms and fungal counts in three-species biofilms, as well as biomass of single-species biofilms. A reason why synergy is more frequently seen when the combined antibiotic is meropenem could reside in its own lower activity compared to moxifloxacin. Previous studies demonstrated the important activity of antibiotic-echinocandin combinations in *S. aureus*–*C. albicans* biofilms *in vitro* or *in vivo*, but the analyses were not performed in such a way as to be able to establish whether the interaction was pharmacologically synergistic (39). It is also remarkable that increases in activity are primarily seen with biomass and related to a reduction in the relative abundance of polysaccharides. This may at least partially result from the previously described effects of echinocandins on bacterial biofilms, i.e., impairment of PNAG synthesis in *S. aureus* biofilms (29) or a reduction in biofilm production for another Gram-negative species, *Pseudomonas aeruginosa* (31). Indeed, PNAG plays multiple other roles in both fungi and bacteria, which could be perturbed as well by the echinocandin, including hypha formation, production of fimbriae and curli fibers in *E. coli*, regulation of metabolic pathways, and building of peptidoglycan (48). This mechanism does not seem to apply to *E. coli* in our case, since PNAG relative abundance in *E. coli* biofilm was not reduced by caspofungin. Although present in *E. coli* biofilms (49), PNAG is considered a minor constituent of the matrix, which is mainly made up of proteinaceous curli fibers and flagella and the polysaccharide cellulose (50). Incidentally, a significant reduction in activity regarding culturable *E. coli* cells was noticed with moxifloxacin in single- and dual-species biofilms and with meropenem in three-species biofilms at specific concentrations, in the line with our observations in planktonic cultures.

The model used in this study has several limitations. First, it is an endpoint model under optimal conditions that does not consider the dynamics of the biofilm over time. Second, the polystyrene surface or geometry of the wells used for growing biofilms is not clinically relevant and may influence the architecture of the biofilm or the nature of the produced matrix (51). The implementation of surfaces like Ti, steel, or silicone, not only for microscopy, might be useful, although it would be impractical and expensive for relatively high-throughput experiments. Third, we used reference strains, which may differ from clinical isolates in their capacity to form biofilms or in the nature of the matrix they produce (52–54). This was a necessary step to set up a new model, but expanding the experiments to clinical strains would allow us to confirm our findings in a broader context.

Nevertheless, our *in vitro* findings highlight the interest of combining caspofungin with antibiotics for the treatment of interkingdom biofilms, with moxifloxacin being preferable when targeting *S. aureus* and meropenem when targeting *E. coli*, at least for isolates demonstrating susceptibility to these drugs.

MATERIALS AND METHODS

Strains, growth conditions, and materials. We used the reference strains *S. aureus* ATCC 25923, *E. coli* ATCC 47076, and *C. albicans* ATCC 24433, commonly used as references in experiments dealing with the evaluation of antimicrobial agents against biofilms (for a few examples, see references 55–59). Their ability to form biofilms was controlled in comparison to clinical isolates in Fig. S6. They were maintained in Mueller-Hinton broth with 10% glycerol at −80°C. For all experiments, precultures were prepared from a frozen aliquot on tryptone soy agar (TSA; Becton Dickinson [BD], Franklin Lakes, NJ) and Sabouraud glucose agar (SGA; peptone [10 g/L], D-glucose [40 g/L], agar [15 g/L]), for the bacteria and *C. albicans*, respectively, and incubated overnight at 37°C. Aliquots were discarded after thawing.

Two media were used to culture the biofilms, both based on RPMI 1640 supplemented with L-glutamine (Sigma-Aldrich, St. Louis, MO) and filter sterilized: RGP is RPMI with phosphate buffer (KH$_2$PO$_4$ [50 mM], Na$_2$HPO$_4$ [74.1 mM]; pH 7.4) and 10 g/L of glucose, and RH is RPMI with 25 mM HEPES. Inocula were prepared in phosphate-buffered saline (PBS; NaCl [137 mM], KCl [2.7 mM], Na$_2$HPO$_4$ [8 mM], KH$_2$PO$_4$ [1.5 mM]). The following selective agar media were used for CFU counting: modified mannitol salt agar (MSA; peptone [5 g/L], NaCl [75 g/L], D-mannitol [10 g/L], agar [15 g/L], amphotericin B [5 mg/L]) for *S. aureus*; selective TSA (TVA; TSA with 5 mg/L vancomycin and 5 mg/L amphotericin B) for *E. coli*; selective SGA (S4; SGA with 15 g/L agar [pH 4.5]) for *C. albicans*. Antimicrobials were added when the temperature was below 60°C after autoclaving the media.

Moxifloxacin HCl (microbiological standard; potency 97%) was kindly provided by Bayer, Leverkusen, Germany. The other drugs were obtained as the corresponding branded products registered for human parenteral use in Belgium or as microbiological standards with the following potencies: meropenem, 100% (Hospira, Brussels, Belgium); vancomycin, 100% (Mylan, Hoeilaart, Belgium); caspofungin, 50% (as Cancidas; MSD, Kenilworth, NJ); cyclosporine, 100%; and soluble amphotericin B, 45% (Sigma-Aldrich). The concentrations of excipients in the formulations of caspofungin and amphotericin B, notably acetic acid and deoxycholate acid, respectively, are below those described as harmful for microbial cultures (60, 61) under our experimental conditions. Stock solutions were prepared at 1 mg/ml in sterile Milli-Q water for moxifloxacin and meropenem and 2 mg/ml in dimethyl sulfoxide (DMSO) for caspofungin and cyclosporine, stored at −20°C, and used within 3 weeks. Amphotericin B and vancomycin were prepared in sterile Milli-Q water and used immediately.

MIC. The microdilution broth assay was performed as described in CLSI and EUCAST guidelines for bacteria and *C. albicans*, respectively, using RGP instead of standard medium (62, 63). For *C. albicans*, and to avoid a paradoxical growth (Fig. S7), 1 mg/L of cyclosporine was added to all concentrations of caspofungin (MIC of cyclosporine versus *S. aureus*, *E. coli*, or *C. albicans*, >32 mg/L).

Biofilm culture. Biofilms were cultured as described by Ruiz-Sorribas et al. (20). Briefly, biofilms were grown in polystyrene tissue culture plates (96 wells, F surface treated; VWR, Radnor, PA). The inoculum was adjusted to 1.5×10^7 CFU/ml *S. aureus* in RGP, 6×10^6 CFU/ml *E. coli* in RGP, or 2.5×10^6 CFU/ml *C. albicans* in RH, in order to obtain a stable biofilm (20). The plates were incubated 24 h at 37°C, and the medium was discarded by inversion to a beaker, for bacteria that were firmly attached to the bottom of the plates, or aspiration with a pipette, for *C. albicans*. In order to avoid any risk of cross-contamination among the different biofilm models, each plate contained a single type of biofilm. The absence of gross contamination from well to well or from the environment was also checked by including noninoculated control wells at the edges of each plate. For single-species biofilms, fresh RGP was added very gently to the wells. For dual- or three-species biofilms, RGP was inoculated with *S. aureus* and/or *E. coli* at the inocula listed above to precultured *C. albicans* 24-h biofilms. Plates were incubated for another 24 h at 37°C.

Alternatively, the same protocol was used to culture the biofilms on Ti alloy disc coupons (Ti-6Al-4V ELI; diameter, 12.7 mm; thickness, 3.8 mm; BioSurface Technologies, Bozeman, MT). Coupons were incubated with gentle agitation at 50 rpm. Coupons were reconditioned according to an adapted protocol from BioSurface Technologies (64). Briefly, used coupons were immersed in 0.1% (vol/vol) RBS soap and sonicated for at least 10 min, rinsed in running water, and sonicated repeatedly in water until no foam was produced, after which they were immersed in 2 M HCl for 2 h, rinsed with Milli-Q water, let dry at 60°C, and autoclaved.

Incubation of biofilms and planktonic cultures with antimicrobials. Precultured 48-h biofilms in 96-well plates were exposed to antimicrobials alone or in combination. Biofilms were washed once with PBS. Fresh RGP containing moxifloxacin, meropenem, caspofungin, or combinations of each of the two antibiotics with caspofungin was added. Cyclosporine (1 mg/L) was added when caspofungin was present (we checked that cyclosporine did not prevent the growth of the controls). Plates were incubated for 24 h at 37°C.

In parallel, the same drugs or combinations were tested against planktonic cells. In a 15-ml tube, 2 ml of RGP with moxifloxacin, meropenem, caspofungin, or combinations of each of the antibiotics with caspofungin was inoculated with either 7.5×10^6 CFU/ml *S. aureus*, 3×10^6 CFU/ml *E. coli*, or 1.25×10^6 CFU/ml *C. albicans*. Caspofungin solutions were always supplemented with 1 mg/liter of cyclosporine. Tubes were incubated 24 h at 37°C under gentle agitation at 130 rpm. Cells were washed with PBS by two successive centrifugations at $3,000 \times g$ (a force low enough to preserve cell viability [65]) well and resuspended in the initial volume in PBS. An appropriate dilution was performed, and 50 μL was transferred to TSA or SGA. Colonies were counted after overnight incubation.

Culturable cells from biofilms. After removal of the medium, biofilms were detached by mechanically scratching the surface with an inoculation loop and resuspending in 200 μL of PBS with vigorous pipetting. Quality controls were routinely performed to ensure appropriate detachment of the biofilms (Fig. S8). The resuspended biofilms were disaggregated by sonication (Q700 sonicator; QSonica, Newton, CT) at 60% amplitude for 30 s directly in the well. The suspension was recovered, and two wells were pooled and diluted appropriately. In the case of three- and dual-species biofilms, 50 μL of the same dilution series was transferred to the appropriate selective agar. In the case of bacterial or *C. albicans* single-species biofilms, the same volume was transferred to TSA or SGA, respectively. Agar plates were incubated at 37°C. Colonies on TSA or TVA were counted after overnight incubation. Colonies on SGA or S4 plates were counted after 24 h, and colonies on MSA were counted after 48 h. Data were expressed as the change in CFU from the initial inoculum (time zero, corresponding to 48 h of biofilm preculture or to 0 h for planktonic cultures).

Biomass assay. Total biomass was estimated with the protocol detailed by Ruiz-Sorribas et al. (20). Briefly, after removal of the medium, biofilms were dried at 60°C. A volume of 200 μL of crystal violet (Sigma-Aldrich) at 0.5% (vol/vol) (final concentration, 115 mg/L) in water was used to stain the dried biofilms for 10 min at room temperature. Nonbound crystal violet was rinsed in running water. Bound

crystal violet was resuspended in 200 μL of acetic acid 66% (vol/vol) (Merck, Darmstadt, Germany) in water for at least 1 h in darkness. Resuspended crystal violet was quantified by absorbance at 570 nm using a microplate reader (SpectraMax Gemini XS microplate spectrophotometer; Molecular Devices LLC, San José, CA). Data were expressed as the change in absorbance, using as a reference (100%) the value measured at time zero, corresponding to 48 h of biofilm preculture.

Relative abundance of polysaccharides. The abundance of polysaccharides was estimated as the ratio between the quantity of polysaccharides and total biomass. Polysaccharides were quantified using the periodic acid-Schiff assay with a protocol adapted from those of Randrianjatovo-Gbalou et al. and Kilcoyne et al. (66, 67). Briefly, biofilms were incubated with 25 μL of PBS and 175 μL 10 mM of *ortho*-periodic acid (Fisher Scientific, Waltham, MA) in acetic acid 5% (vol/vol) for 30 min at room temperature in darkness. One hundred microliters of Schiff reagent (Sigma-Aldrich) was added and incubated for another 60 min at room temperature in darkness. The fluorescence at an excitation wavelength of 544 nm and an emission wavelength of 620 nm was read in the SpectraMax microplate reader. The values were transformed to microgram equivalents of dextran based on a calibration curve using 25 μL of serial dilutions of dextran MW 70000 from *Leuconostoc mesenteroides* (Sigma-Aldrich). The quantity of polysaccharides was normalized to the total biomass in the same sample and expressed as the ratio between the sample and the control (no antimicrobial added).

Pharmacodynamic curves and combination analysis. GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA) was used to draw concentration-response curves, calculate pharmacodynamic parameters, and run statistical analyses of data obtained with single drugs.

Data were used to fit a 4-parameter logistic or Hill curve (equation 1):

$$y = E_{\max} + \frac{E_{\min} - E_{\max}}{1 + 10^{(\log EC_{50} - x) \times h}} \tag{1}$$

where $E_{\max}$ is the maximal effect of the drug and thus the horizontal asymptote at the lowest y value, $E_{\min}$ is the minimal effect of the drug, EC_{50} is the concentration needed to reach an effect halfway between $E_{\max}$ and $E_{\min}$ (inflection point of the curve), and h is the Hill factor. The pharmacodynamic parameters $E_{\max}$ and $C_{-1\log}$ or $C_{-90\%}$ (defined as the concentration required to observe a 90% reduction in total culturable cells [1 $\log_{10}$ reduction] or biomass) were calculated from the equation of the curve.

Synergy (increased effect) or antagonism (decreased effect) for drug combinations was determined using the highest-single-agent model of the BIGL 1.4.3 package for R 3.6.2 (68, 69). Contour graphs were plotted using the plotrix 3.7-7 package (70). Briefly, the highest-single-agent model determines if the effect of a drug combination is greater than the effects produced by its single components (32). A schematic overview of the workflow and the script used in R can be found in Fig. S9 and S10, respectively. An expected additive effect ($\hat{E}_{AB}$) is calculated as the largest effect, and thus the largest reduction and lower value, of the drugs used alone at a given pair of concentrations (E_A and E_B) as detailed in equation 2. The effects of both drugs alone were extrapolated from the fitted pharmacodynamic curve.

$$\hat{E}_{AB} = \min(E_A, E_B) \tag{2}$$

As detailed by Van der Borght et al., two statistical tests based on the analysis of variances are run to compare $\hat{E}_{AB}$ with the experimental combinatory effect (E_{AB}) (68). The first test determines if the overall E_{AB} fits the $\hat{E}_{AB}$ additive model. If there is at least a significant difference, the second test identifies which E_{AB} deviates significantly from $\hat{E}_{AB}$ and if it corresponds to synergy (increased effect) or antagonism (decreased effect).

Confocal microscopy (live/dead staining). Biofilms grown on coupons were imaged after incubation with antimicrobials alone or in combination. Two hundred microliters of a mixture of FilmTracer Live/Dead (Syto9 0.01 mM, propidium iodide 0.06 mM; Invitrogen, Carlsbad, CA) and 50 mg/L calcofluor white (Sigma-Aldrich) in water was added very gently on top of the biofilms and incubated 30 min in darkness. Excess dye was rinsed off with water, and excess liquid was removed with absorbent paper. Coupons were mounted with Dako mounting oil (Agilent, Santa Clara, CA) and a glass coverslip. Z-stack pictures of the stained biofilms were taken with an AxioImager.z1-ApoTome microscope (Carl Zeiss, Oberkochen, Germany) through a multiacquisition from the top to the bottom of the biofilm. The means of filter sets (excitation/emission) were as follows: blue, 365/450 nm; green, 460/550 nm; and red, 535/590 nm. Pictures were analyzed and converted to maximal-intensity projections with AxioVision release 4.8.2.0 (Carl Zeiss).

Data availability. All raw data will be made available from the corresponding author upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

ACKNOWLEDGMENTS

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1   **Pharmacodynamics of Moxifloxacin, Meropenem, Caspofungin and Combinations**

2   **Against In Vitro Polymicrobial Inter-kingdom Biofilms**

3   Albert Ruiz-Sorribas [a], Hervé Poilvache [bc], Françoise Van Bambeke [a]

4   [a] Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université

5   catholique de Louvain, Brussels, Belgium.

6   [b] Laboratoire de neuro musculo squelettique, Institut de Recherche Expérimentale et

7   Clinique, Université catholique de Louvain, Brussels, Belgium.

8   [c] Orthopaedic surgery department, Cliniques Universitaires Saint-Luc, Brussels, Belgium.

9   Corresponding author: Françoise Van Bambeke (francoise.vanbambeke@uclouvain.be)

10

11

12   **Supplementary material**

13 **Figure S1.** A: Multiple protein sequence alignment and dendrogram of *C. albicans* Gsc1,

14 *S. aureus* IcaA, *S. epidermidis* IcaA, *Acinetobacter baumannii* PgaC and *E. coli* PgaC.

15 The highly conserved Asp132-x-Asp134 and Asp227 (in *S. aureus*) are part of the

16 predicted catalytic site. The sequence alignment highlights (red boxes) the fact that

17 residues making part of the predicted catalytic site Asp132-x-Asp134 and Asp227 (in

18 *S. aureus*) are highly conserved. B: Percentage identity matrix.

19 **A.**
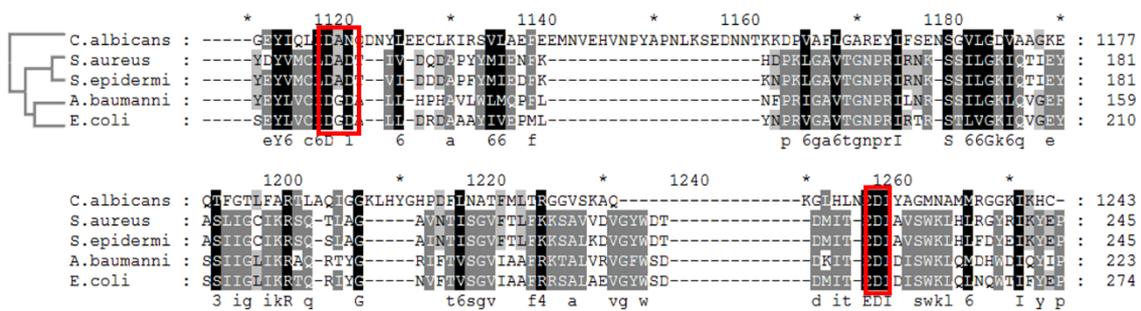
21 **B.**

	Gsc1 (*C. albicans*)	IcaA (*S. aureus*)	IcaA (*S. epidermidis*)	PgaC (*E. coli*)
IcaA (*S. aureus*)	16.71			
IcaA (S. epidermidis)	18.33	79.13		
PgaC (*E. coli*)	15.5	38.69	37.96	
PgaC (*A. baumannii*)	16.39	39.28	39.79	54.64

22

23 **Figure S2.** Evolution over time of cultivable cells and biomass in control biofilms (run

24 in parallel with biofilms were exposed to single or combined drugs). A-C: single-species

25 biofilms of *S. aureus, E. coli,* and *C. albicans,* respectively; D-E: dual-species biofilms

26 of *S.aureus:C.albicans* and *E.coli:C.albicans*; F: three-species biofilm. Each point

27 corresponds to an independent biological replicate. N=7-22. Statistical analysis: different

28 letters: significant difference (p-value<0.05; one-way ANOVA, post-Tukey test).


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50   **Figure S5.** Relative quantification of polysaccharides (A) and biomass (B) after

51   incubation with antimicrobials, expressed as the ratio with the value measured in the

52   corresponding control (no antimicrobial added). The data is complementary to Figure 8.

53   N = 3-6. Statistical analysis: different letters of same colour denote significant difference

54   (p< 0.05; one-way non-parametric ANOVA, Tukey post-test). Sa: *S. aureus*. Ec: *E. coli*.

55   Ca: *C. albicans*. Ec:Ca, Sa:Ca, Sa:Ec:Ca biofilm: dual- or three-species biofilms. MXF:

56   moxifloxacin 6 mg/L; MEM: meropenem 30 mg/L; CAS: caspofungin 12.5 mg/L.
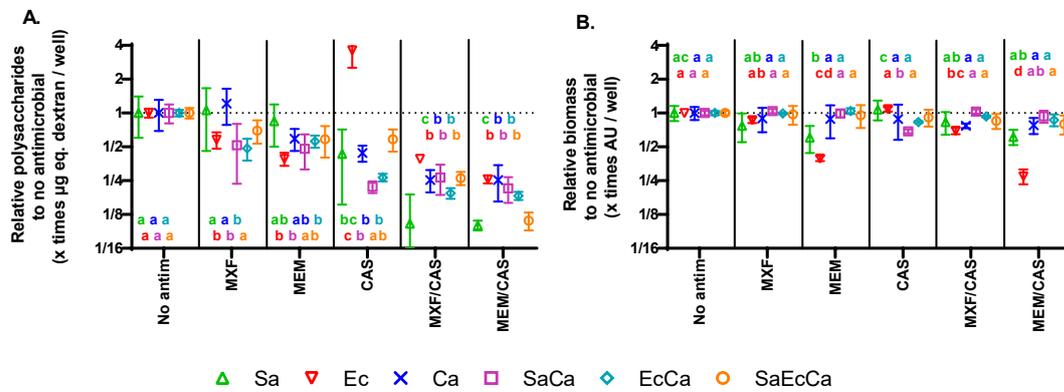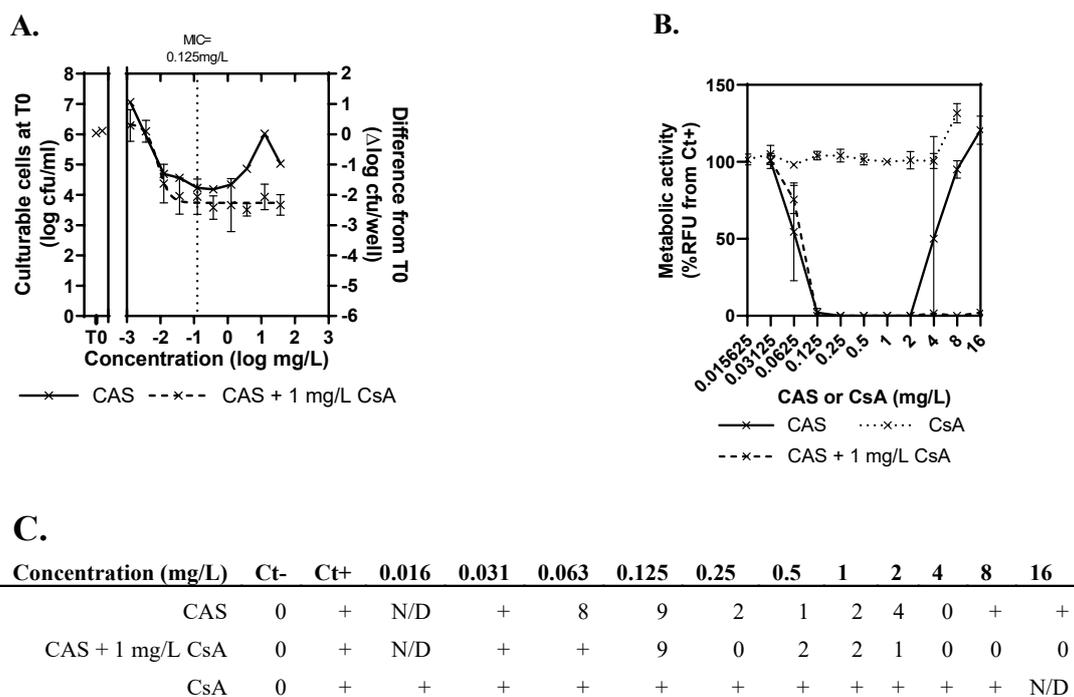

57

58 **Figure S6.** Comparison of biofilm formation on Ti coupons by reference and clinical

59 strains, as evaluated by quantification of the biomass using crystal violet staining. The

60 clinical isolates were kindly provided by Prof Hector Rodriguez-Villalobos (Clinical

61 Microbiology Department, *cliniques universitaires Saint Luc,* Brussels, Belgium). Each

62 point represents a coupon. Different letters represent a significant difference (p-value <

63 0.05; non-parametric One-way ANOVA, post-Tukey test).

64
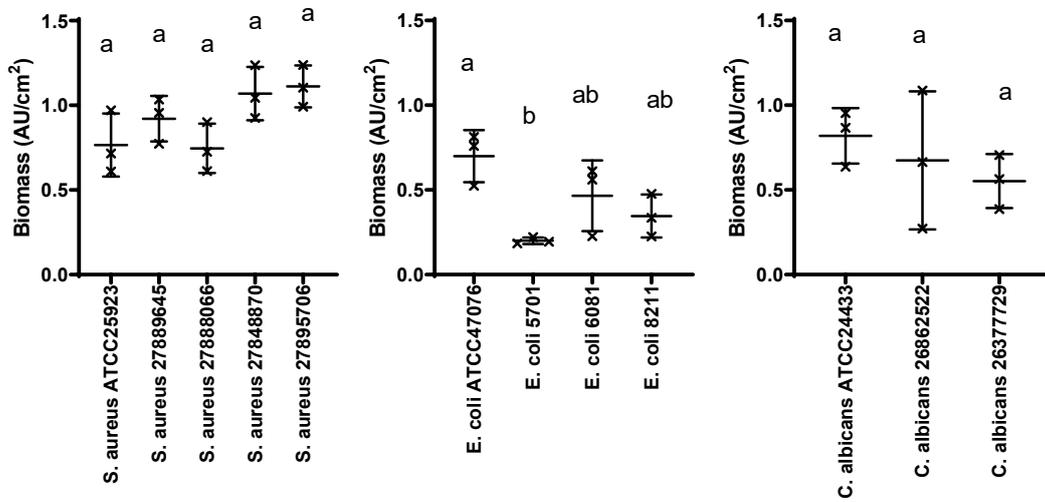
65   **Figure S7.** Paradoxical growth of *C. albicans* exposed to caspofungin in RPMI + 20mM

66   Hepes. A: planktonic growth in tubes. B: metabolic activity in 96w microplates, assessed

67   by the fluorescence of resorufin (Ex560 nm/Em590nm) after 30 min incubation with

68   0.01g/L resazurin. C: cultivability in 96w microplates, assessed by the number of colonies

69   on a dot spot with 10µL from each well on SGA. +: more colonies than able to distinguish.

70   Cyclosporin A was used to avoid the paradoxical growth at intermediate concentrations

71   of caspofungin. Some authors have reported that intermediate concentrations of

72   echinocandins, roughly between 4 to 32-64 mg/L, induce a change in the cell wall

73   polysaccharides from β-glucan to chitin, which allows the fungus to persist the

74   echinocandins. This is known as the paradoxical effect or growth and, to the best of our

75   knowledge, it has only been observed in vitro and it is limited to some strains (1).

76   Although it is not fully understood, calcineurin-pathway inhibitor molecules, like

77   cyclosporin A, avoid this transition (2, 3). Cyclosporin A has no inhibitory effect on its

78   own against *C. albicans*, *S. aureus* or *E. coli* (MIC >32 mg/L).

C.

Concentration (mg/L)	Ct-	Ct+	0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8	16
CAS	0	+	N/D	+	8	9	2	1	2	4	0	+	+
CAS + 1 mg/L CsA	0	+	N/D	+	+	9	0	2	2	1	0	0	0
CsA	0	+	+	+	+	+	+	+	+	+	+	+	N/D

79

80   **Figure S8.** Quality controls of residual biomass after detaching the biofilm from the

81   microtiter plates. Each point represents the average of one experiment. Different letters

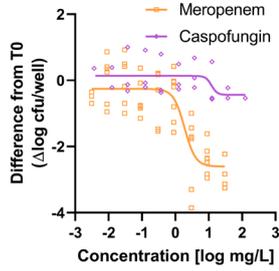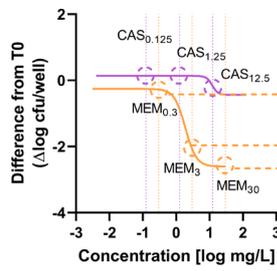82   represent a significant difference (p-value < 0.05; One-way ANOVA, post-Tukey test).

83

84
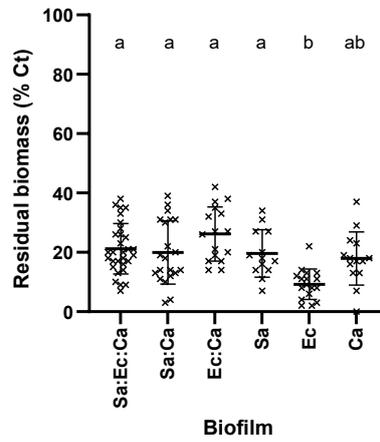
Figure S9. Schematic overview of the Highest Single-Agent model workflow.

1) Fitting concentration-response curves to single drug experiments.

**2)** Estimating the effect of single drug E_{MEM} and E_{CAS} at given concentrations.

3) Estimating the expected additive effect for the combination at each pair of concentrations according to the highest single agent model $\hat{E}_{MEM/CAS}=min(E_{MEM},E_{CAS})$.

4) Obtaining the experimental effect ($E_{MEM/CAS}$) of the combination at each pair of concentrations and of each single agent at the same concentrations in the same experiment.

5) Comparing $E_{MEM/CAS}$ **(4)** vs $\hat{E}_{MEM/CAS}$ **(3)** to determine if there is a synergy ($E_{MEM/CAS} < \hat{E}_{MEM/CAS}$), antagonism ($E_{MEM/CAS} > \hat{E}_{MEM/CAS}$) or no-interaction ($E_{MEM/CAS} \sim \hat{E}_{MEM/CAS}$).
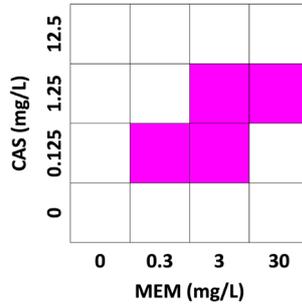
6) Plotting graphically the effects of each combination of concentrations together with that of each single drug at the same concentration, and report the significant synergy/antagonism by a color code.

86

87

88 **Figure S10.** Script used for the highest single-agent analysis for R.

```
#### Synergy analysis ###########################################

## Based on:
## https://cran.r-project.org/web/packages/BIGL/vignettes/analysis.html

### Packages and setting working directory #######################

install.packages("BIGL", lib = "E:/Packages directory")
install.packages("ggplot2", lib = "E:/Packages directory")
install.packages("openxlsx", lib = "E:/Packages directory")
install.packages("dplyr", lib = "E:/Packages directory")
install.packages("plotrix", lib = "E:/Packages directory")

library(BIGL, lib.loc = "E:/Packages directory")
library(ggplot2, lib.loc = "E:/Packages directory")
library(openxlsx, lib.loc = "E:/Packages directory")
library(dplyr, lib.loc = "E:/Packages directory")
library(plotrix, lib.loc = "E:/Packages directory")

setwd("E:/Working directory")

### Data input ###################################################

## The data for the analysis must come in a table with required
## columns "d1", "d2" and "effect" for doses of two compounds and
## observed cell counts respectively. Those titles cannot be changed.
## It is also required a numerical value to identify each
## subexperiment to analyze independently. In this script is called
## "experiment". Other information used in this script is a label
## for the experiment type and for the object of study: "Test",
## "Microorganism" and "Biofilm".

a1 <- "MXF"
a2 <- "CAS"
am <- c(a1,a2)

results.comb <- read.xlsx("R_data.xlsx")

subset.data <- function(data, i) {
  subset(data, experiment == i)[c("effect", "d1", "d2")]
}

nExp <- max(results.comb$experiment)

id.results <- subset(results.comb)[c("Test","Microorganism",
                                     "Biofilm","experiment")]
id.results <- unique(id.results)
paste.noNA <- function(id, sep=", ") {
  gsub(", ", sep, toString(id[!is.na(id) & id!="" & id!="NA"]))
}
sep <- ", "
id.results$id <- apply(id.results[, c(1:3)], 1,
                       paste.noNA, sep=", ")

sCFU <- seq_len(length(which(id.results$Test == "CFU")))
sBiomass <- id.results$experiment[-sCFU]

### Highest Single Agent #########################################

data.list <- list()
fit.data.list <- list()
title.list <- list()
HSA.list <- list()

for (i in seq_len(nExp)) {
  data <- subset.data(data = results.comb, i)
  fit.data <- tryCatch({
    fitMarginals(data, transforms = NULL,
                 method = "nlslm", names = am)
  }, warning = function(w) w, error = function(e) e)

  if (inherits(fit.data, c("warning", "error")))
    fit.data <- tryCatch({
```

```
162        fitMarginals(data, transforms = NULL,
163                     method = "optim", names = am)
164      })
165
166    title <- paste(id.results[i,5])
167
168    HSA <- fitSurface(data, fit.data, method = "model",
169                      null_model = "hsa",
170                      statistic = "both", B.CP = 20,
171                      parallel = FALSE)
172
173    data.list[[i]] <- data
174    fit.data.list[[i]] <- fit.data
175    title.list[[i]] <- title
176    HSA.list[[i]] <- HSA
177  }
178
179  ### Marginal fits graphs #########################################
180
181  dir.create("Marginal_fits")
182
183  plot.fit.data.list <- list()
184
185  for (i in seq_len(nExp)){
186    plot.fit.data <- plot(fit.data.list[[i]]
187                          )+ ggtitle(title.list[[i]])
188
189    plot.fit.data.list[[i]] <- plot.fit.data
190
191    tiff (filename=paste0("Marginal_fits/Marg_fit_",
192                          a1,a2,"_",i,".tiff"))
193    print(plot.fit.data.list[[i]])
194    dev.off()
195  }
196
197  ### Contour graphs #################################################
198
199  dir.create("Contour_graphs_HSA")
200
201  tiff(filename = paste0("Contour_graphs_HSA/Contour_",
202                         a1,a2,"_HSA_%02d.tiff"))
203  for (i in seq_len(nExp)) {
204    contour(HSA.list[[i]],
205            colorPalette = c("blue", "white", "red"),
206            main = title.list[[i]],
207            digits = 3, cutoff = 1,
208            xlab = paste(a1,"(mg/L)"), ylab = paste(a2,"(mg/L)"),
209            plevels = c(0.95,0.99,0.999))
210  }
211  dev.off()
212
213  pdf(paste0("Contour_",a1,a2,"_HSA.pdf"))
214  for (i in seq_len(nExp)) {
215    contour(HSA.list[[i]],
216            colorPalette = c("blue", "white", "red"),
217            main = title.list[[i]],
218            digits = 3, cutoff = 1,
219            xlab = paste(a1,"(mg/L)"), ylab = paste(a2,"(mg/L)"),
220            plevels = c(0.95,0.99,0.999))
221  }
222  dev.off()
223
224  ### Output table with results ######################################
225
226  desc.eff.list <- list()
227  desc.sd.list <- list()
228  mHSA.eff.sd.syn.list <- list()
229
230  subset.name <- function(data) {
231    subset(data, d1 != 0 & d2 != 0)[c("d1", "d2")]
232  }
233  subset.m <- function(data) {
234    subset(data, data[2] != "NA")[col.name]
235  }
236  check.syn <- function (data, j) {
237    if(data$R[j] < 0) return ("*") else("ª")
238  }
```

```r
239    define.add <- function(data, j) {
240      if(data$call[j] == "None") return(NA) else(data$check[j])
241    }
242
243    HSA.syn.list <- list()
244
245    for (i in seq_len(nExp)) {
246      desc.eff <- aggregate(effect ~ d1 + d2,
247                            data = data.list[[i]], mean)
248      desc.sd <- aggregate(effect ~ d1 + d2, data = data.list[[i]], sd)
249      desc.eff$effect <- round(desc.eff$effect, digits = 2)
250      desc.sd$effect <- round(desc.sd$effect, digits = 2)
251      desc.eff.sd <- cbind.data.frame(desc.eff$d1,desc.eff$d2,
252                                      paste(desc.eff$effect,
253                                            desc.sd$effect,sep= " ±"))
254      colnames(desc.eff.sd) <- c("d1","d2","effect.sd")
255
256      name <- subset.name(data = desc.eff)
257      col.name <- unique(name$d1)
258      col.name <- c("0", unique(name$d1))
259
260      HSA.syn <- subset(HSA.list[[i]][["maxR"]][["Ymean"]])[
261        c("d1","d2","call","R")]
262      for(j in seq_len(nrow(HSA.syn))){
263        HSA.syn$check[j] <- check.syn (data = HSA.syn, j)
264        HSA.syn$check[j] <- define.add (data = HSA.syn, j)
265      }
266      HSA.syn$R <- NULL
267      HSA.syn$call <- NULL
268      HSA.syn$call <- HSA.syn$check
269      HSA.syn$check <- NULL
270      HSA.syn$call <- as.factor(HSA.syn$call)
271
272      HSA.eff.sd.syn <- full_join (desc.eff.sd, HSA.syn,
273                                   by = c("d1", "d2"))
274      HSA.eff.sd.syn$eff.sd.syn <- apply(HSA.eff.sd.syn[,c(3:4)], 1,
275                                         paste.noNA, sep=" ")
276      HSA.eff.sd.syn$effect.sd <- NULL
277      HSA.eff.sd.syn$call <- NULL
278
279      mHSA.eff.sd.syn <- reshape(HSA.eff.sd.syn, direction = "wide",
280                                 idvar = "d2", timevar = "d1")
281      rownames(mHSA.eff.sd.syn) <- mHSA.eff.sd.syn$d2
282      mHSA.eff.sd.syn$d2 <- NULL
283      colnames(mHSA.eff.sd.syn) <- unique(desc.eff.sd$d1)
284
285      mHSA.eff.sd.syn <- as.data.frame(mHSA.eff.sd.syn)
286      mHSA.eff.sd.syn <- subset(mHSA.eff.sd.syn)[col.name]
287      mHSA.eff.sd.syn <- subset.m (data = mHSA.eff.sd.syn)
288
289      desc.eff.list[[i]] <- desc.eff
290      desc.sd.list[[i]] <- desc.sd
291      HSA.syn.list[[i]] <- HSA.syn
292      mHSA.eff.sd.syn.list[[i]] <- mHSA.eff.sd.syn
293    }
294
295    wb <- createWorkbook()
296    addWorksheet(wb, sheetName = paste0(a1,a2,"_HSA"))
297
298    for (i in seq_len(nExp)) {
299      writeData(wb, sheet = paste0(a1,a2,"_HSA"), x = title.list[[i]],
300                xy = c(1,paste(2+7*(i-1))))
301      writeData(wb, sheet = paste0(a1,a2,"_HSA"),
302                x = mHSA.eff.sd.syn.list[[i]],
303                xy = c(2,paste(3+7*(i-1))), rowNames = TRUE,
304                keepNA = TRUE, na.string = "ND")
305    }
306
307    saveWorkbook(wb, file = paste0(a1,"_",a2,".xlsx"),
308                 overwrite = FALSE)
309
310    ### Published graphs #########################################
311
312    # Need to run previous section before.
313
314    subset.mono <- function(data) {
315      subset(data, d1 == 0 | d2 == 0)[c("d1", "d2")]
```

```
316      }
317
318      mdesc.eff.list <- list()
319      mdesc.sd.list <- list()
320
321      cor.syn <- "magenta"
322      cor.ant <- "cyan"
323      cor.add <- "black"
324      cor.mono <- "black"
325
326      for (i in sCFU){
327        desc.sd.list[[i]]$sd <- paste0("±",desc.sd.list[[i]]$effect)
328        desc.sd.list[[i]]$effect <- NULL
329      }
330
331      for (i in sBiomass){
332        desc.sd.list[[i]]$sd <- paste0("±",round(desc.sd.list[[i]]$effect,
333                                               digits = 1))
334        desc.sd.list[[i]]$effect <- NULL
335      }
336
337      for (i in seq_len(nExp)){
338        mdesc.eff <- reshape(desc.eff.list[[i]], direction = "wide",
339                             idvar = "d2", timevar = "d1")
340        rownames(mdesc.eff) <- mdesc.eff$d2
341        mdesc.eff$d2 <- NULL
342        colnames(mdesc.eff) <- unique(desc.eff.list[[i]]$d1)
343        mdesc.eff <- as.data.frame(mdesc.eff)
344        mdesc.eff <- subset(mdesc.eff)[col.name]
345        mdesc.eff <- subset.m (data = mdesc.eff)
346        mdesc.eff <- as.matrix (mdesc.eff)
347
348        mdesc.sd <- reshape(desc.sd.list[[i]], direction = "wide",
349                            idvar = "d2", timevar = "d1")
350        rownames(mdesc.sd) <- mdesc.sd$d2
351        mdesc.sd$d2 <- NULL
352        colnames(mdesc.sd) <- unique(desc.sd.list[[i]]$d1)
353        mdesc.sd <- as.data.frame(mdesc.sd)
354        mdesc.sd <- subset(mdesc.sd)[col.name]
355        mdesc.sd <- subset.m (data = mdesc.sd)
356        mdesc.sd <- as.matrix (mdesc.sd)
357
358        mdesc.eff.list[[i]] <- mdesc.eff
359        mdesc.sd.list[[i]] <- mdesc.sd
360      }
361
362      mcolor.eff.list <- list()
363
364      maxCFU <- 0
365      minCFU <- -3
366      maxBiomass <- 110
367      minBiomass <- 50
368
369      for (i in sCFU){
370        desc.eff.list[[i]]$effect[
371            desc.eff.list[[i]]$effect > maxCFU] <- maxCFU
372        desc.eff.list[[i]]$effect[
373            desc.eff.list[[i]]$effect < minCFU] <- minCFU
374        color.eff <- rbind(desc.eff.list[[i]], c(10,10,maxCFU),
375                           c(10,10,minCFU))
376        color.eff$effect <- color.scale(color.eff$effect, alpha = 0.5,
377                                        extremes = c("black","white"))
378        color.eff <- color.eff[1:nrow(desc.eff.list[[i]]),]
379
380        mcolor.eff <- reshape(color.eff, direction = "wide",
381                              idvar = "d2", timevar = "d1")
382        rownames(mcolor.eff) <- mcolor.eff$d2
383        mcolor.eff$d2 <- NULL
384        colnames(mcolor.eff) <- unique(color.eff$d1)
385        mcolor.eff <- as.data.frame(mcolor.eff)
386        mcolor.eff <- subset(mcolor.eff)[col.name]
387        mcolor.eff <- subset.m (data = mcolor.eff)
388        mcolor.eff <- as.matrix (mcolor.eff)
389
390        mcolor.eff.list[[i]] <- mcolor.eff
391      }
392      for (i in sBiomass){
```

```r
    desc.eff.list[[i]]$effect[
         desc.eff.list[[i]]$effect > maxBiomass] <- maxBiomass
    desc.eff.list[[i]]$effect[
         desc.eff.list[[i]]$effect < minBiomass] <- minBiomass
    color.eff <- rbind(desc.eff.list[[i]], c(10,10,maxBiomass),
                       c(10,10,minBiomass))
    color.eff$effect <- color.scale(color.eff$effect, alpha = 0.5,
                                    extremes = c("black","white"))
    color.eff <- color.eff[1:nrow(desc.eff.list[[i]]),]

    mcolor.eff <- reshape(color.eff, direction = "wide",
                          idvar = "d2", timevar = "d1")
    rownames(mcolor.eff) <- mcolor.eff$d2
    mcolor.eff$d2 <- NULL
    colnames(mcolor.eff) <- unique(color.eff$d1)
    mcolor.eff <- as.data.frame(mcolor.eff)
    mcolor.eff <- subset(mcolor.eff)[col.name]
    mcolor.eff <- subset.m (data = mcolor.eff)
    mcolor.eff <- as.matrix (mcolor.eff)

    mcolor.eff.list[[i]] <- mcolor.eff
}

mHSA.syn.list <- list()

for (i in seq_len(nExp)){
  HSA.syn.list[[i]]$call <- as.character(HSA.syn.list[[i]]$call)
  HSA.syn.list[[i]]$call[HSA.syn.list[[i]]$call == "*"] <- cor.syn
  HSA.syn.list[[i]]$call[HSA.syn.list[[i]]$call == "ª"] <- cor.ant
  HSA.syn.list[[i]]$call[is.na(HSA.syn.list[[i]]$call)] <- cor.add
  temp <- subset.mono(data = desc.eff.list[[i]])
  temp$call <- cor.mono
  HSA.syn.list[[i]] <- rbind(temp, HSA.syn.list[[i]])
  mHSA.syn <- reshape(HSA.syn.list[[i]], direction = "wide",
                      idvar = "d2", timevar = "d1")
  rownames(mHSA.syn) <- mHSA.syn$d2
  mHSA.syn$d2 <- NULL
  colnames(mHSA.syn) <- unique(desc.eff.list[[i]]$d1)
  mHSA.syn <- as.data.frame(mHSA.syn)
  mHSA.syn <- subset(mHSA.syn)[col.name]
  mHSA.syn <- subset.m (data = mHSA.syn)
  mHSA.syn <- as.matrix (mHSA.syn)

  mHSA.syn.list[[i]] <- mHSA.syn
}

dir.create ("Summary_graphs_HSA")

tiff(filename = paste0("Summary_graphs_HSA/Summary_",
                       a1,a2,"_HSA_%02d.tiff"))
for (i in sCFU){
  color2D.matplot(mdesc.eff.list[[i]],
                  cellcolors = mcolor.eff.list[[i]],
                  show.values = 2, vcol = mHSA.syn.list[[i]],
                  vcex = 2.5,
                  xlab = paste(a1,"(mg/L)"),
                  ylab = paste(a2,"(mg/L)"), yrev = FALSE,
                  #main = title.list[[i]],
                  axes = FALSE, border = NA)
  axis(1,at=0.5:3.5,labels = c(colnames(mdesc.eff.list[[i]])),
       font = 2)
  axis(2,at=0.5:3.5,labels = c(rownames(mdesc.eff.list[[i]])),
       font = 2)

  addtable2plot(0,-0.3,table = mdesc.sd.list[[i]][nrow(
    mdesc.sd.list[[i]]):1,],
    bg = "transparent", cex = 1.9,
    display.colnames = FALSE, ypad = 3.8, xpad = 0.68,
    xjust = 0, yjust = 1)
}
for (i in sBiomass){
  color2D.matplot(mdesc.eff.list[[i]],
                  cellcolors = mcolor.eff.list[[i]],
                  show.values = TRUE, vcol = mHSA.syn.list[[i]],
                  vcex = 2.5,
                  xlab = paste(a1,"(mg/L)"),
                  ylab = paste(a2,"(mg/L)"), yrev = FALSE,
```

```
                         #main = title.list[[i]],
                         axes = FALSE, border = NA)
      axis(1,at=0.5:3.5,labels = c(colnames(mdesc.eff.list[[i]])),
           font = 2)
      axis(2,at=0.5:3.5,labels = c(rownames(mdesc.eff.list[[i]])),
           font = 2)

      addtable2plot(0,-0.3,table = mdesc.sd.list[[i]][nrow(
        mdesc.sd.list[[i]]):1,],
        bg = "transparent", cex = 1.9,
        display.colnames = FALSE, ypad = 3.8, xpad = 0.68,
        xjust = 0, yjust = 1)
}
dev.off()

### Relationship of elements ###############################

## Data (relevant)

## data.list: list of dataframes with results per experiment.
## desc.eff.list: list of dataframes with average results per experiment.
## desc.sd.list:  list of dataframes with sd results per experiment.
## fit.data.list: list of output of fitMarginals() per experiment.
## HSA.list: list of output of fitSurface() for hsa per experiment.
## HSA.syn.list:  list of dataframes with synergy/antagonism coded by
##                colours for hsa per experiment.
## id.results: dataframe with summary of experiments and acronyms.
## mcolor.eff.list: list of matrix with gradient colors depending on
##                  average results per experiment.
## mdesc.eff.list: list of matrix with average results per experiment.
## mdesc.sd.list: list of matrix with sd results per experiment.
## mHSA.eff.sd.syn.list:  list of dataframes with pasted average, sd, and
##                        synergy/antagonism for HSA per experiment.
## mHSA.syn.list: list of matrix with synergy/antagonism coded by
##                colours for hsa per experiment.
## plot.fit.data.list: list of graphs with marginal fit per experiment.
## results.comb: dataframe with input of results.
## title.list: list of characters with acronyms for titles.


## Values that need to be introduced

## a1: compound 1.
## a2: compound 2.
## cor.add: color for additivity.
## cor.ant: color for antagonism.
## cor.mono: color for monotherapy.
## cor.syn: color for synergy.
## maxBiomass and minBiomass: maximal and minimal for the range of the
##                            color for biomass graphs.
## maxCFU and minCFU: maximal and minimal for the range of the
##                    color for cfu graphs.


## Functions

## check.syn: prints a code for synergies or antagonisms for a single
##            combination from the output of a fitSurface() converted
##            to dataframe.
## define.add:  prints NA in the place of "None" or prints the existing
##              value when it is different.
## paste.noNA: merge two columns without adding NA data.
## subset.data: select the columns "d1", "d2" and "effect" from a single
##              "experiment" in a dataframe.
## subset.m: selects rows that do not have NA values.
## subset.mono: selects combinations of "d1" an "d2" that include at least
##              a 0 (zero) value.
## subset.name: selects combinations of "d1" an "d2" that do not include
##              0 (zero) values.
```

References:

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2. Shields RK, Nguyen MH, Du C, Press E, Cheng S, Clancy CJ. 2011. Paradoxical effect of caspofungin against Candida bloodstream isolates is mediated by multiple pathways but eliminated in human serum. Antimicrob Agents Chemother 55:2641–7.

3. Wiederhold NP, Kontoyiannis DP, Prince RA, Lewis RE. 2005. Attenuation of the Activity of Caspofungin at High Concentrations against Candida albicans: Possible Role of Cell Wall Integrity and Calcineurin Pathways. Antimicrob Agents Chemother 49:5146–5148.