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Simultaneous Versus Sequential Caspofungin-Antibacterial Treatment of Bacterial-Candida albicans Biofilms on Titanium

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Abstract

Background

The optimal coordination of antifungal and antibacterial exposure against implant-associated mixed-species biofilms remains uncertain. We compared simultaneous and sequential caspofungin-antibacterial regimens across paired concentrations in two *Candida albicans*-bacterial biofilm models. *C. albicans-Staphylococcus epidermidis* and *C. albicans-Escherichia coli* biofilms were established on titanium discs in modified synthetic synovial fluid and exposed to two-dimensional caspofungin (CAS)-vancomycin (VAN) or CAS-meropenem (MER) concentration matrices, respectively. Regimens comprised simultaneous exposure for 24 h, CAS for 8 h followed by the antibacterial agent for 16 h, or the reverse sequence. The no-recovery endpoint required that no fungal or bacterial colonies were recovered from any of six replicate discs across three independent experiments. No-recovery boundaries were defined using drug concentrations expressed relative to the corresponding single-species minimum biofilm eradication concentrations.

Results

Across both models, simultaneous treatment produced broader regions of low or no bacterial recovery and reached the no-recovery endpoint at lower antibacterial concentrations in selected comparisons. At CAS 4,096 μg/mL, simultaneous CAS-VAN treatment required VAN 512 μg/mL, compared with 2,048 μg/mL for either sequential regimen. At CAS 2,048 μg/mL, simultaneous CAS-MER treatment required MER 512 μg/mL, compared with 1,024 μg/mL for CAS first and 2,048 μg/mL for MER first. Sequential CAS-VAN boundaries partly overlapped, with differences favoring CAS first, whereas sequential CAS-MER boundaries crossed. Neither sequence consistently yielded lower bacterial recovery across the tested concentration matrices.

Conclusions

Treatment responses depended on paired drug concentrations and the exposure schedule tested. Simultaneous treatment reached the no-recovery endpoint at lower antibacterial concentrations in selected comparisons, whereas the relative performance of sequential regimens varied with concentration and drug pairing. Because individual drug exposure durations were longer during simultaneous treatment, timing and duration effects could not be separated. Comparisons with matched individual drug exposure durations and validation under locally achievable and tolerable exposure conditions are needed before these findings can inform clinical administration strategies.

Background

Candida periprosthetic joint infection (PJI) is difficult to treat and frequently involves bacterial coinfection. In an international cohort of 269 patients, bacteria were coisolated in 51.3% of cases[1]. Antimicrobial treatment in these patients must therefore consider both fungal and bacterial pathogens. However, how antifungal and antibacterial exposures should be coordinated to improve activity against implant-associated mixed-species biofilms remains uncertain.

Local antimicrobial delivery provides a clinical setting in which this question is relevant. Intra-articular antibiotic infusion has been investigated as an adjunct to revision surgery in selected patients with PJI[2–5]. Pharmacokinetic measurements after intra-articular infusion have demonstrated high synovial concentrations of vancomycin (VAN) and meropenem (MER), with different rates of decline over time[6]. These observations suggest that drug selection and administered concentration alone may not fully describe the exposure experienced by microorganisms within the joint. They provide a rationale for examining exposure schedules but do not determine the relative activity of simultaneous and sequential antifungal-antibacterial regimens against mixed-species biofilms[7].

Fungal-bacterial interactions provide a biologic basis for comparing these regimens. In *Candida albicans* (*C. albicans*)-*Staphylococcus aureus* biofilms, fungal matrix β-1,3-glucan contributed to impaired VAN penetration and increased bacterial tolerance, whereas subinhibitory caspofungin (CAS) reduced this protection[8, 9]. These findings suggest that modifying the fungal component or its matrix may influence antibacterial activity, but they do not establish a preferred treatment order or its applicability to other organism-drug combinations. Previous studies of CAS-antibacterial combinations during concurrent exposure have also shown that activity varies with the microbial community, drug combination, and endpoint assessed[10, 11]. Whether the relative activity of simultaneous and sequential regimens changes across paired concentrations and bacterial-antibacterial drug combinations remains unclear.

Therefore, we established *C. albicans-Staphylococcus epidermidis* (*S. epidermidis*) and *C. albicans-Escherichia coli* (*E. coli*) mixed-species biofilms on titanium discs in modified synthetic synovial fluid and evaluated CAS combined with VAN or MER, respectively. Within a 24-hour treatment period, we compared simultaneous exposure with CAS-first and antibacterial-first sequential regimens across paired drug concentrations (Fig. 1). We aimed to compare simultaneous treatment with CAS-first and antibacterial-first sequential regimens across paired drug concentrations, assessing posttreatment microbial recovery and the concentration combinations at which neither organism was recovered. We further examined whether either sequential regimen consistently showed greater activity or whether its relative performance varied with concentration and bacterial-antibacterial drug pairing.

Methods

Microorganisms and inoculum preparation

C. albicans SC5314 (ATCC MYA-2876), *S. epidermidis* ATCC 35984, and *E. coli* ATCC 25922 were used throughout the study. Before each experiment, *C. albicans* was freshly subcultured on Sabouraud

dextrose agar (SDA) at 30°C for 24 hours. A single colony was inoculated into yeast extract-peptone-dextrose (YPD) broth and cultured overnight at 30°C with shaking at 180 rpm for 16 to 18 hours. *S. epidermidis* and *E. coli* were similarly freshly subcultured on tryptic soy agar (TSA) at 37°C for 18 to 24 hours. A single colony of each bacterial strain was then inoculated into tryptic soy broth (TSB) and cultured overnight at 37°C with shaking at 150 rpm for 16 to 18 hours. The overnight cultures were harvested by centrifugation and washed twice with sterile phosphate-buffered saline (PBS). *C. albicans* cells were resuspended in RPMI 1640 medium buffered with 165 mM 3-(N-morpholino) propanesulfonic acid (MOPS; pH 7.0) and adjusted to 1×10^6 CFU/mL. The bacterial suspensions were adjusted to 1×10^6 CFU/mL before use in subsequent experiments.

Preparation of modified synthetic synovial fluid

An RPMI-1640-based modified synthetic synovial fluid (mSSF) was prepared for biofilm formation and antimicrobial exposure. The concentrations of the principal macromolecular components were adapted from the SSF2 formulation[12, 13] .

RPMI-1640 medium containing D-glucose, HEPES, and L-glutamine (Solarbio, Beijing, China; Cat. No. 10491) was supplemented with bovine serum albumin (BSA; Solarbio, Beijing, China; Cat. No. A8020), hyaluronic acid (Macklin, Shanghai, China; Cat. No. H909938; molecular weight, 0.8–1.5 MDa), and fibrinogen from bovine plasma (Macklin, Shanghai, China; Cat. No. F823833; CAS No. 9001-32-5) to final concentrations of 25 g/L, 3 g/L, and 0.100 g/L, respectively.

BSA was dissolved in RPMI-1640 and sterilized by filtration through a 0.22-μm membrane. Hyaluronic acid was sterilized separately and aseptically added to the filtered medium, followed by incubation at 37°C with shaking at 250 rpm for 30 minutes. Fibrinogen was freshly prepared at 2.5 mg/mL in sterile 0.9% saline and added aseptically as the final component to achieve a concentration of 0.100 g/L.

The pH was adjusted to approximately 7.4. The prepared mSSF was stored at 4°C protected from light and used within 48 hours. The same formulation was used for biofilm formation and subsequent antimicrobial exposure.

Biofilm formation on titanium discs

Sterile titanium discs measuring 8 mm in diameter and 2 mm thick were aseptically placed in 24-well tissue culture plates. For single-species *C. albicans* biofilms, 1 mL of fungal suspension at 1×10^6 cells/mL was added to each well and incubated statically at 37°C for 90 minutes to allow initial adherence. The discs were washed twice with sterile PBS, transferred to fresh wells containing 1 mL of mSSF, and incubated for an additional 24 hours at 37°C. For single-species *S. epidermidis* and *E. coli* biofilms, 1 mL of the corresponding bacterial suspension at 1×10^6 CFU/mL was added to each well, and

discs were incubated at 37°C for 24 hours[14]. After incubation, discs were washed twice with sterile PBS to remove nonadherent and loosely adherent cells.

For the *C. albicans-S. epidermidis* mixed-species model, fungal and bacterial suspensions were prepared at approximately 1.0×10^6 and 1.0×10^7 CFU/mL, respectively. Equal volumes (0.5 mL each) were added to each well, yielding initial concentrations of approximately 5×10^5 CFU/mL for *C. albicans* and 5×10^6 CFU/mL for *S. epidermidis*, and the discs were incubated at 37°C for 24 hours. To establish *C. albicans-E. coli* mixed-species biofilms, *C. albicans* and *E. coli* suspensions were prepared separately and combined at a 1:1 ratio immediately before inoculation[15]. The mixed suspension was adjusted in mSSF to an initial concentration of approximately 5×10^5 CFU/mL for each organism. One milliliter of the mixed inoculum was added to each well containing a titanium disc, followed by incubation at 37°C for 24 hours.

Scanning electron microscopy of single- and dual-species biofilms

Single- and mixed-species biofilms were established on sterile titanium discs. After biofilm formation, discs were gently washed twice with PBS and fixed in 2.5% (v/v) glutaraldehyde for 4 hours. Samples were subsequently washed with PBS and dehydrated through a graded ethanol series of 30%, 50%, 70%, 85%, 95%, and 100% for 15 minutes at each concentration; the 100% ethanol step was repeated twice. Samples were critical-point dried, mounted on aluminum stubs, and sputter-coated with an approximately 8-nm Au/Pd conductive layer. Biofilm morphology and architecture were examined by scanning electron microscopy (SEM; Helios 5 CX, Thermo Fisher Scientific). SEM was used to assess microbial adherence, surface colonization, and biofilm architecture and to confirm the successful establishment of the single- and dual-species biofilm models.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of CAS against *C. albicans* SC5314 was determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) M27 reference method[16]. The final *C. albicans* inoculum was adjusted to 0.5×10^3 to 2.5×10^3 CFU/mL in RPMI-1640 buffered with 165 mM MOPS to pH 7.0. CAS was tested using twofold serial dilutions. Plates were incubated at 35°C for 24 hours. The CAS MIC was defined as the lowest concentration producing a prominent reduction (≥ 50%) in growth relative to the drug-free growth control.

VAN and MER MICs against *S. epidermidis* and *E. coli*, respectively, were determined by broth microdilution according to CLSI M07[17], with interpretation based on CLSI M100 criteria[18]. Bacterial suspensions were prepared in cation-adjusted Mueller-Hinton broth to a final inoculum of approximately 5×10^5 CFU/mL. VAN and MER were prepared as twofold serial dilutions and tested against *S. epidermidis* and *E. coli*, respectively. Microdilution plates were incubated at 35°C in ambient air for 18 hours. The MIC was defined as the lowest antimicrobial concentration that completely inhibited visible

bacterial growth. MIC testing was performed in three independent experiments, with each condition tested in triplicate.

Minimum biofilm eradication concentrations

Single-species minimum biofilm eradication concentration (MBEC) was determined for CAS against *C. albicans*, VAN against *S. epidermidis*, and MER against *E. coli* single-species biofilms. After the 24-hour biofilm growth period, titanium discs were washed twice with sterile PBS and transferred to fresh wells containing 1 mL of mSSF with twofold serial dilutions of the corresponding antimicrobial agent. Biofilms maintained in drug-free mSSF under the same conditions served as growth controls. The discs were exposed to the antimicrobial for 24 hours at 37°C.

After antimicrobial exposure, the discs were washed twice with sterile PBS and transferred individually to sterile tubes containing 1 mL of PBS. Biofilm-associated microorganisms were recovered by vortexing for 30 seconds, bath sonication at 40 kHz for 5 minutes, and vortexing for an additional 30 seconds[19]. The recovered suspensions were serially diluted and quantitatively cultured on the corresponding agar medium. The MBEC was defined as the lowest antimicrobial concentration at which no viable colonies were recovered after biofilm disruption and culture.

Sequential and simultaneous antimicrobial treatment of mixed-species biofilms

C. albicans-S. epidermidis and *C. albicans-E. coli* biofilms formed on titanium discs were treated with CAS combined with VAN or MER, respectively. Twofold serial dilutions of CAS and the corresponding antibacterial agent were arranged in a two-dimensional concentration matrix. Each concentration pair was evaluated under three administration regimens: CAS followed by the antibacterial agent, the antibacterial agent followed by CAS, and simultaneous administration.

Treatments lasted 24 hours in 1 mL of mSSF at 37°C and consisted of an initial 8-hour phase followed by a 16-hour phase. At the end of the first phase, discs were transferred without washing to fresh wells containing 1 mL of drug-supplemented mSSF. The transfer and medium-renewal procedure was identical across all three regimens.

In the CAS-first regimen, biofilms were exposed to CAS for 8 hours and subsequently to VAN or MER for 16 hours. In the antibacterial-first regimen, biofilms were exposed to VAN or MER for 8 hours and subsequently to CAS for 16 hours. In the concurrent regimen, CAS and VAN or MER were present throughout both treatment phases; after 8 hours, discs were transferred to fresh medium containing the same drug combination and concentrations for the remaining 16 hours. Each concentration pair and treatment regimen were evaluated using six replicate titanium discs.

Biofilm recovery and species-specific CFU enumeration

At the end of the 24-hour treatment period, titanium discs were washed twice with sterile PBS and transferred individually to sterile tubes containing 1 mL of sterile PBS. Biofilm-associated microorganisms were recovered by vortexing for 30 seconds, bath sonication at 40 kHz for 5 minutes, and a further 30 seconds of vortexing[20, 21]. The recovered suspensions were serially diluted in sterile PBS and plated in parallel on species-selective agar media for independent enumeration of the fungal and bacterial populations.

C. albicans was selectively recovered on acidified Sabouraud glucose agar (peptone 10 g/L, D-glucose 40 g/L, and agar 15 g/L; pH 4.5). *S. epidermidis* was recovered on modified mannitol salt agar containing peptone (5 g/L), NaCl (75 g/L), D-mannitol (10 g/L), agar (15 g/L), and amphotericin B (5 mg/L)[22]. *E. coli* was recovered on selective tryptic soy agar supplemented with VAN (5 mg/L) and amphotericin B (5 mg/L). Antimicrobial supplements were added after the autoclaved media had cooled below 60°C. The selectivity of each medium was confirmed using the corresponding single-species cultures before mixed-species biofilm analysis. Plates were incubated aerobically at 37°C; *C. albicans* colonies were enumerated after 24 hours, *S. epidermidis* after 48 hours, and *E. coli* after overnight incubation. Viable counts were expressed as CFU per titanium disc.

Concentration-Response Analysis and No-Recovery Boundary Definition

For each titanium disc, total recoverable CFU was calculated by adding the *C. albicans* CFU to the CFU of the corresponding bacterial species. Fungal and bacterial counts were summed before logarithmic transformation, and the resulting total recoverable CFU values were used to construct the concentration-response matrices.

A concentration combination met the no-recovery endpoint only when total recoverable CFU was zero for all six titanium discs evaluated across the three independent experiments. Thus, neither fungal nor bacterial colonies were recovered from any of the six discs at that tested concentration combination.

Within each mixed-species biofilm model and administration regimen, a no-recovery combination was considered nondominated when no other tested combination that also met the no-recovery endpoint used concentrations of both antimicrobials that were no higher and a concentration of at least one antimicrobial that was lower. Antimicrobial concentrations were expressed relative to the corresponding single-species MBECs: CAS against *C. albicans*, VAN against *S. epidermidis*, and MER against *E. coli*. The observed nondominated concentration combinations defined the no-recovery boundary.

Use of generative artificial intelligence

ChatGPT (OpenAI) was used on a limited basis for minor language polishing and assistance with the preparation of a schematic illustration. All final content was reviewed and approved by the authors.

Results

Morphologic Characteristics of Single- and Mixed-Species Biofilms on Titanium Discs

SEM showed that *C. albicans*, *S. epidermidis* and *E. coli* formed single-species biofilms on the surfaces of titanium discs after 24 hours of incubation. Each biofilm contained multilayered cellular aggregates, with only small amounts of extracellular polymeric substance (EPS)-like material.

After 24 hours of coculture, *C. albicans* and *S. epidermidis* formed a compact, densely organized dual-species biofilm. *S. epidermidis* aggregates were closely apposed to *C. albicans* yeast cells and hyphae, indicating close spatial association between the two organisms. Cellular aggregation was more extensive than in the single-species biofilms, and the *C. albicans* hyphae were more prominent. EPS-like material was also more abundant and broadly distributed, with prominent matrix coverage at fungal-bacterial contact sites. This spatial arrangement gave the dual-species biofilm a cohesive and integrated architecture.

The *C. albicans-E. coli* dual-species biofilm exhibited similar features of close fungal-bacterial organization. Prominent *C. albicans* hyphae were interspersed with aggregates of rod-shaped *E. coli*. The bacterial cells accumulated around and partially enveloped *C. albicans* yeast cells and hyphae. EPS-like material was evident around the *E. coli* aggregates and was particularly prominent at the interfaces between the bacterial and fungal cells (Fig. 2). The two populations consequently showed close spatial integration within the biofilm.

Antimicrobial Susceptibility of Planktonic Cells and Single-Species Biofilms

The MBEC exceeded the corresponding planktonic MIC for each organism-antimicrobial pair (Fig. 3). For MER against *E. coli*, the MIC was 0.0625 µg/mL and the MBEC was 512 µg/mL, resulting in an MBEC-to-MIC ratio of 8,192. For VAN against *S. epidermidis*, the MIC was 4 µg/mL and the MBEC was 4,096 µg/mL, resulting in a ratio of 1,024. For CAS against *C. albicans*, the corresponding concentrations were 1 and 1,024 µg/mL, respectively, also resulting in a ratio of 1,024.

No-Recovery Boundary Relative to Single-Species MBECs

Using the single-species MBECs reported above as reference values, antimicrobial concentrations in the dual-species matrices were expressed as multiples of the corresponding single-species MBECs (Fig. 4). Nondominated no-recovery boundary combinations were those at which all six replicate determinations yielded 0 CFU and neither antimicrobial concentration could be reduced while maintaining this endpoint.

For the *C. albicans-S. epidermidis* biofilm, the nondominated CAS-VAN boundary differed among administration schedules. When CAS was followed by VAN, the boundary comprised CAS/VAN combinations of 2×/1×, 4×/0.5×, and 8×/0.25× their respective single-species MBECs. When VAN was

followed by CAS, the corresponding combinations were 2×/2×, 4×/0.5×, and 8×/0.25×. With simultaneous administration, the boundary combinations were 1×/2×, 2×/1×, and 4×/0.125×. At CAS 4× its single-species MBEC, simultaneous administration reached the no-recovery endpoint with VAN at 0.125× its single-species MBEC, whereas both sequential regimens required VAN at 0.5× its single-species MBEC. At CAS 2× its single-species MBEC, the corresponding VAN requirement was 1× with simultaneous administration and CAS followed by VAN, compared with 2× when VAN was followed by CAS.

For the *C. albicans-E. coli* biofilm, the nondominated CAS-MER boundary also differed among administration schedules. When CAS was followed by MER, the boundary comprised CAS/MER combinations of 1×/16×, 2×/2×, and 8×/1× their respective single-species MBECs. When MER was followed by CAS, the corresponding combinations were 1×/16×, 2×/4×, 4×/1×, and 8×/0.5×. With simultaneous administration, the boundary combinations were 0.5×/8×, 2×/1×, and 4×/0.5×. At CAS 2× its single-species MBEC, simultaneous administration reached the no-recovery endpoint with MER at 1× its single-species MBEC, compared with 2× when CAS was followed by MER and 4× when MER was followed by CAS. When MER was fixed at 1× its single-species MBEC, the corresponding CAS concentrations at the no-recovery boundary were 8× for CAS followed by MER, 4× for MER followed by CAS, and 2× for simultaneous administration.

Across both dual-species models, all nondominated no-recovery combinations contained at least one antimicrobial at or above its corresponding single-species MBEC. The concentration required for one antimicrobial varied with the concentration of the companion antimicrobial and the administration schedule. With simultaneous administration, portions of the no-recovery boundary occurred at lower concentrations of one antimicrobial at matched concentrations of the other, including at CAS 4× its MBEC in the CAS-VAN matrix and at CAS 2× or MER 1× its MBEC in the CAS-MER matrix.

Recoverable Bacterial Burden Varied With Antimicrobial Concentration and Administration Schedule

Across both dual-species biofilm models, recoverable bacterial CFU generally decreased as the concentration of either antimicrobial increased (Fig. 5A-C, E-G). Higher bacterial recovery was concentrated primarily in the lower-concentration regions of the matrices, whereas increasing the concentration of one or both antimicrobials was associated with broader regions of low or no recoverable CFU. The distribution of residual bacterial recovery differed among the three administration schedules. Simultaneous administration showed a smaller high-CFU region and a broader low- or no-recovery region than either sequential schedule, particularly across low-to-intermediate concentration combinations. At higher combined antimicrobial concentrations, the concentration-response surfaces for the three schedules converged toward low or no recovery. The relative bacterial recovery of the two sequential schedules varied across the concentration matrices; neither sequence yielded lower CFU at every tested combination.

For the *C. albicans-S. epidermidis* biofilm, bacterial recovery decreased as CAS, VAN, or both increased under all three administration schedules (Fig. 5A-C). With simultaneous administration, the high-CFU region occupied a smaller portion of the tested matrix, whereas residual bacterial recovery extended across a broader range of low-to-intermediate concentration combinations with both sequential schedules. The two sequential CAS-VAN response surfaces showed substantial overlap, and their relative bacterial recovery varied with the specific concentration combination; neither sequence yielded lower CFU across all tested combinations.

Differences among the CAS-VAN schedules were also evident near the transition to no recovery (Fig. 5D). At CAS 4096 μg/mL, simultaneous administration reached the no-recovery endpoint with VAN 512 μg/mL, whereas both sequential schedules required VAN 2048 μg/mL. At CAS 2048 μg/mL, simultaneous administration and CAS followed by VAN reached the endpoint with VAN 4096 μg/mL, whereas VAN followed by CAS required VAN 8192 μg/mL. Within the tested concentration range, simultaneous administration was also the only schedule to reach no recovery at CAS 1024 μg/mL, in combination with VAN 8192 μg/mL. Where the two sequential CAS-VAN boundaries separated, CAS followed by VAN required a lower VAN concentration than VAN followed by CAS.

For the *C. albicans-E. coli* biofilm, bacterial recovery likewise decreased as CAS, MER, or both increased under all three administration schedules (Fig. 5E-G). With simultaneous administration, the high-CFU region occupied a smaller portion of the tested matrix, whereas residual bacterial recovery extended across a broader range of low-to-intermediate concentration combinations with both sequential schedules. The relative bacterial recovery of the two sequential CAS-MER schedules varied across the concentration matrix, and neither sequence yielded lower CFU throughout the tested range.

Differences among the CAS-MER schedules were similarly evident near the transition to no recovery (Fig. 5H). At CAS 2048 μg/mL, simultaneous administration reached the endpoint with MER 512 μg/mL, compared with MER 1024 μg/mL when CAS was followed by MER and MER 2048 μg/mL when MER was followed by CAS. When MER was fixed at 512 μg/mL, the CAS concentration at the no-recovery boundary was 2048 μg/mL with simultaneous administration, 4096 μg/mL when MER was followed by CAS, and 8192 μg/mL when CAS was followed by MER. Simultaneous administration also reached the endpoint at CAS 512 μg/mL combined with MER 4096 μg/mL, whereas the lowest CAS concentration on either sequential boundary was 1024 μg/mL, combined with MER 8192 μg/mL. The two sequential CAS-MER boundaries intersected within the tested concentration range: at some fixed MER concentrations, MER followed by CAS reached the boundary at a lower CAS concentration, whereas at some fixed CAS concentrations, CAS followed by MER reached the boundary at a lower MER concentration. Thus, neither sequential CAS-MER schedule showed a consistently lower concentration requirement across the observed boundary.

Discussion

In this study, we compared simultaneous administration and two sequential dosing orders of CAS-antibacterial combinations against *C. albicans-S. epidermidis* and *C. albicans-E. coli* biofilms on titanium surfaces. Across both models, simultaneous treatment showed broader regions of low or no recovery across the tested concentration matrices and, in selected comparisons at matched CAS concentrations, reached the no-recovery endpoint at lower antibacterial concentrations than either sequential regimen. The two sequential regimens showed partly overlapping no-recovery boundaries in the CAS-VAN model, with differences favoring CAS first, whereas their relative concentration requirements varied across the boundary in the CAS-MER model. These findings suggest that administration schedule warrants consideration alongside drug concentration when evaluating antimicrobial combinations against *C. albicans*-containing mixed-species biofilms.

The structural and susceptibility findings provide context for these treatment responses. SEM supported the establishment of mixed-species biofilms on titanium surfaces, with bacterial aggregates closely associated with *C. albicans* yeast cells and hyphae. Compared with the corresponding single-species biofilms, coculture showed more extensive cellular aggregation and more prominent EPS-like material, particularly at bacterial-fungal interfaces. This spatial organization is consistent with imaging studies demonstrating a structural role for fungal hyphae in *S. aureus-C. albicans* biofilms[23, 24]. Such extracellular organization may be relevant to antimicrobial activity: Kong et al.[8] demonstrated that *C. albicans*-derived β-1,3-glucan impaired antimicrobial penetration and increased bacterial tolerance in *C. albicans-S. aureus* biofilms, while De Brucker et al.[25] reported increased ofloxacin tolerance mediated by fungal β-1,3-glucan in *C. albicans-E. coli* biofilms. Although SEM alone cannot establish whether similar protective mechanisms operated in our models, these observations provide structural context for interpreting the antimicrobial treatment responses.

The differences between planktonic MICs and single-species MBECs highlight the importance of evaluating antimicrobial activity against established biofilms. Across the three organism-drug pairs, MBECs were 1024 to 8192 times the corresponding MICs. This disparity is consistent with reduced antimicrobial susceptibility during biofilm growth. Ruiz-Sorribas et al.[10] likewise reported limited single-agent activity against established bacterial-fungal biofilms despite activity against planktonic organisms. The mixed-species experiments further showed that single-species MBECs did not directly define effective combination concentrations. Combining both agents at their respective single-species MBECs did not achieve the bacterial no-recovery endpoint under any tested schedule in either model. However, some combinations reached this endpoint with one agent below its single-species MBEC when the companion agent was present at a higher concentration. Thus, single-species MBECs provided a basis for normalizing concentrations rather than fixed efficacy thresholds for combination treatment. By applying the same bacterial endpoint across the tested concentration pairs, the boundary analysis showed how the concentration required for one agent varied with the concentration of the other and the administration schedule.

The simultaneous and sequential regimens differed most clearly in selected regions of these concentration matrices. Simultaneous treatment showed broader regions of low or no recovery in both

models and lower antibacterial concentration requirements in selected no-recovery boundary comparisons. For example, at CAS 4,096 μg/mL, the VAN concentration required to reach the no-recovery endpoint was fourfold lower with simultaneous treatment than with either sequential regimen. This difference illustrates that a concentration pair sufficient to reach the endpoint under one schedule may be insufficient under another. Previous pharmacodynamic work by Ruiz-Sorribas et al.[10] characterized concentration-dependent activity and drug interactions during concurrent exposure to CAS-antibacterial combinations, including CAS-MER. Our study extends this work by comparing simultaneous treatment with both sequential orders across paired concentrations. However, individual drug exposures were longer during simultaneous treatment, which may have contributed to its favorable performance. The findings therefore apply to the regimens as tested and do not isolate the effect of concurrent exposure from that of exposure duration. Likewise, lower concentration requirements relative to sequential treatment do not establish pharmacologic synergy. At higher combined concentrations, bacterial recovery approached low or undetectable levels under all three schedules, limiting their differentiation by this outcome.

The comparison between sequential regimens revealed a concentration-dependent relationship that differed between the two bacterial-antibacterial drug pairings. In the CAS-VAN model, the no-recovery boundaries partly overlapped, with differences favoring CAS first. This advantage was confined to portions of the boundary: neither sequence consistently yielded lower bacterial counts throughout the concentration matrix. In the CAS-MER model, the relative concentration requirements reversed. At CAS 2× its single-species MBEC, CAS first required less MER to reach the endpoint, whereas at CAS 8× its MBEC, MER first required less MER. Thus, even within the same biofilm model and drug pairing, a sequence favored at one concentration could not be assumed to remain favorable at another. These findings show the value of evaluating multiple concentration pairs when comparing administration orders. The different boundary patterns should be interpreted at the level of the bacterial-antibacterial drug pairing, because both the bacterial species and the antibacterial agent differed between models. Overall, the sequential comparisons support evaluating administration order in relation to drug concentration rather than assigning a generally preferred sequence. Changes in the fungal population or its extracellular products offer a possible biological basis for schedule-dependent responses. Eshima et al.[26] found that *C. albicans* culture supernatant increased *E. coli* survival during biofilm formation in the presence of MER, with tolerance-inducing activity retained in the fraction below 10 kDa. This finding supports a contribution from soluble fungal products without requiring direct bacterial-fungal contact. Kurakado et al.[27] further showed that amphotericin B combined with MER reduced viable counts of both organisms in established *C. albicans-E. coli* biofilms, whereas micafungin combined with MER reduced fungal counts without a corresponding reduction in bacterial counts. The fungal reduction was also smaller with micafungin, indicating that the extent of the antifungal response may be relevant to the bacterial outcome. Together, these studies suggest that reducing fungal burden does not necessarily improve bacterial killing and provide a rationale for examining how the first agent alters the community encountered by the second.

For the development of local antimicrobial strategies for PJI, our findings support evaluating treatment schedule alongside drug concentration. Pharmacokinetic measurements following intra-articular infusion after revision surgery have documented high synovial concentrations of VAN and MER that change over time[6]. These observations provide a clinical context for investigating how the timing, duration, and overlap of antifungal and antibacterial exposures influence activity against mixed-species biofilms. However, the present in vitro regimens do not reproduce these dynamic concentration–time profiles, and the available antibacterial pharmacokinetic data do not establish whether the CAS concentrations tested here can be achieved and maintained locally with acceptable tissue compatibility. Likewise, a lower concentration requirement for one agent at a fixed concentration of the other does not by itself establish the clinical feasibility or safety of the combination. Further translational studies should therefore define locally achievable and tolerable exposure ranges and evaluate fungal and bacterial survival under corresponding dynamic concentration–time conditions, together with effects on local tissues.

Several limitations warrant consideration. The sequential regimens used eight- and 16-hour exposure phases, whereas both agents were present throughout the 24-hour simultaneous treatment. Differences among the tested schedules therefore reflect both administration timing and individual drug exposure duration, rather than an isolated administration-order effect. The static titanium-disc model did not reproduce host immunity, joint fluid turnover, or dynamic antimicrobial concentrations. Only one reference strain per species was tested, limiting generalisability to clinical isolates and other microbial combinations. In addition, bacterial species and antibacterial agent changed together between the CAS-VAN and CAS-MER models, preventing separation of their individual contributions. Clinical translation will require confirmation that the tested concentration ranges are locally achievable and tolerable.

Conclusion

In conclusion, the activity of caspofungin-antibacterial combinations against bacterial-*Candida albicans* mixed-species biofilms on titanium surfaces was influenced by both antimicrobial concentration and administration schedule. Simultaneous treatment demonstrated broader low- or no-recovery regions and required lower antibacterial concentrations in selected concentration comparisons, whereas the relative activity of the two sequential regimens varied according to antimicrobial pairing and concentration. These findings indicate that antimicrobial scheduling should be evaluated together with concentration rather than assuming a universally superior sequence. Further studies using matched drug exposure durations and clinically relevant dynamic exposure models are required to clarify the translational implications of these findings.

Abbreviations

BSA
Bovine serum albumin
CAS

Caspofungin
CFU
Colony-forming units
CLSI
Clinical and Laboratory Standards Institute
EPS
Extracellular polymeric substance
MBEC
Minimum biofilm eradication concentration
MER
Meropenem
MIC
Minimum inhibitory concentration
MOPS
3-(N-morpholino)propanesulfonic acid
mSSF
Modified synthetic synovial fluid
PBS
Phosphate-buffered saline
PJI
Periprosthetic joint infection
SDA
Sabouraud dextrose agar
SEM
Scanning electron microscopy
TSA
Tryptic soy agar
TSB
Tryptic soy broth
VAN
Vancomycin
YPD
Yeast extract-peptone-dextrose

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Clinical trial number

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RL: Conceptualization, methodology, investigation, formal analysis, data curation, visualization, and writing - original draft.

LY: data curation, and writing - original draft.

HX: Investigation ,and validation.

QH:Data acquisition, and validation.

AA: Methodology.

YG: Investigation, data curation, and formal analysis.

XG: Formal analysis, data interpretation, and visualization.

XZ: Methodology, supervision, and writing – review and editing.

LC: Conceptualization, supervision, project administration, and writing – review and editing.

BX: Conceptualization, supervision, project administration, methodology, and writing - review and editing.

WM: Conceptualization, supervision, project administration, methodology, and writing -review and editing.

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Figures

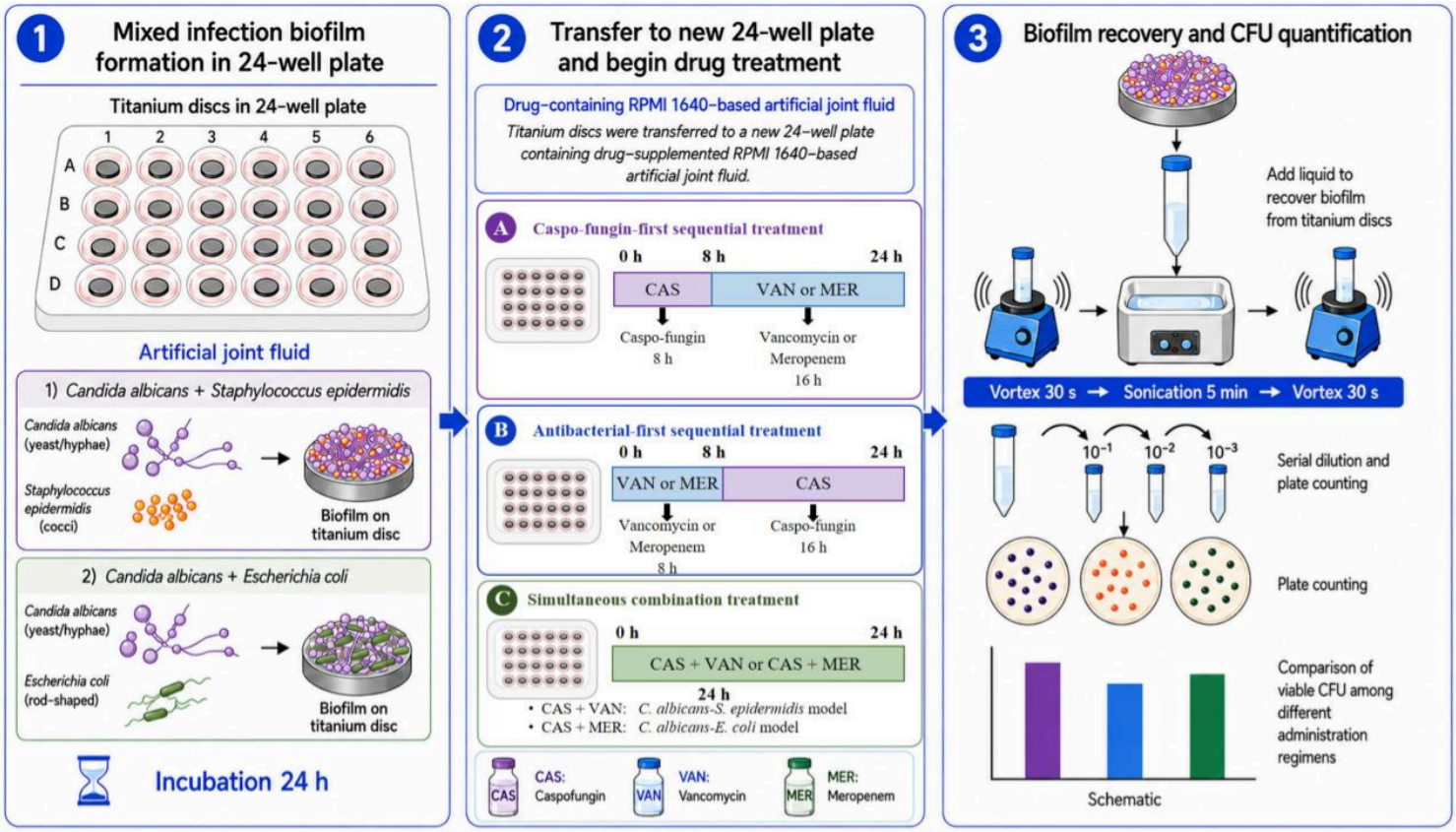


Figure 1

Experimental workflow for comparing simultaneous and sequential caspofungin-antibacterial treatment of mixed-species biofilms on titanium. *Candida albicans-Staphylococcus epidermidis* and *C. albicans-Escherichia coli* biofilms were established on titanium discs in modified synthetic synovial fluid for 24 h. For the *C. albicans-S. epidermidis* and *C. albicans-E. coli* models, caspofungin (CAS) was

combined with vancomycin (VAN) and meropenem (MER), respectively, under three treatment regimens: A) CAS for 8 h followed by the antibacterial agent for 16 h; B) the antibacterial agent for 8 h followed by CAS for 16 h; and C) simultaneous exposure to both agents for 24 h. At 8 h, discs in all regimens were transferred without washing to fresh medium containing the drug(s) assigned to the second phase. After treatment, discs were washed, and biofilm-associated microorganisms were recovered by vortexing for 30 s, bath sonication for 5 min, and further vortexing for 30 s. Recovered suspensions were serially diluted and plated on species-selective agar for separate fungal and bacterial enumeration, expressed as colony-forming units (CFU) per disc.

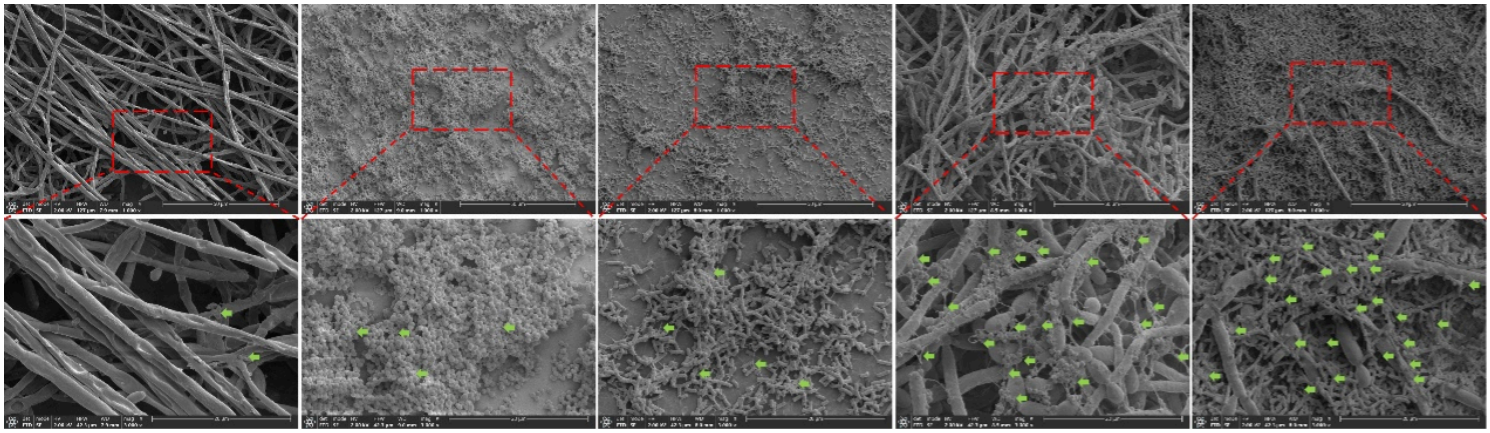


Figure 2

Scanning electron micrographs of single- and dual-species biofilms formed on titanium discs. Images were obtained at 1,000× and 3,000× magnification; scale bars = 50 μm and 20 μm, respectively. Green arrows indicate extracellular polymeric substance (EPS)-like material.

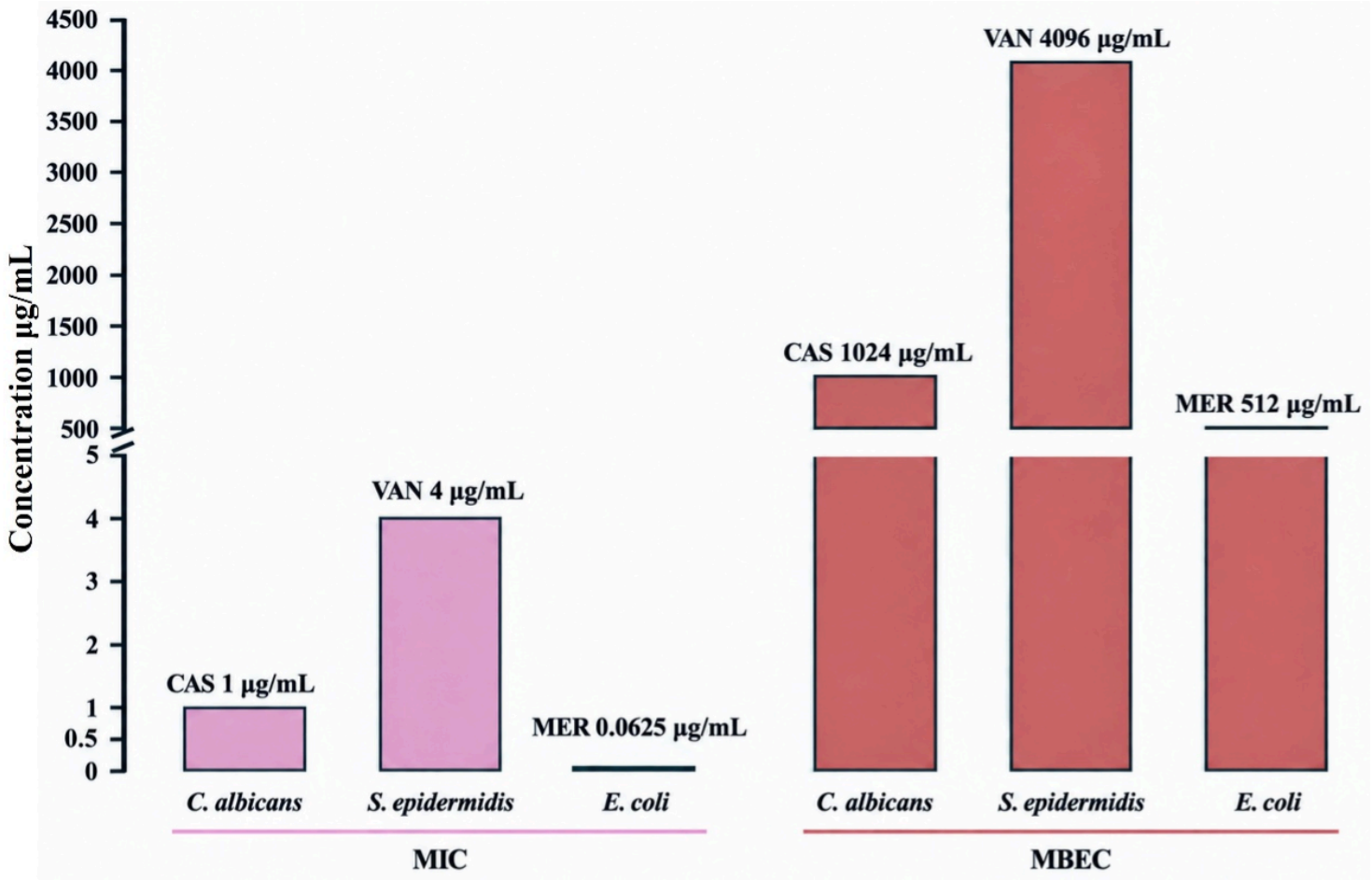


Figure 3

Minimum inhibitory concentrations and minimum biofilm eradication concentrations of the three organism-antimicrobial pairs. MIC and MBEC values are shown for CAS against *C. albicans*, VAN against *S. epidermidis*, and MER against *E. coli*.

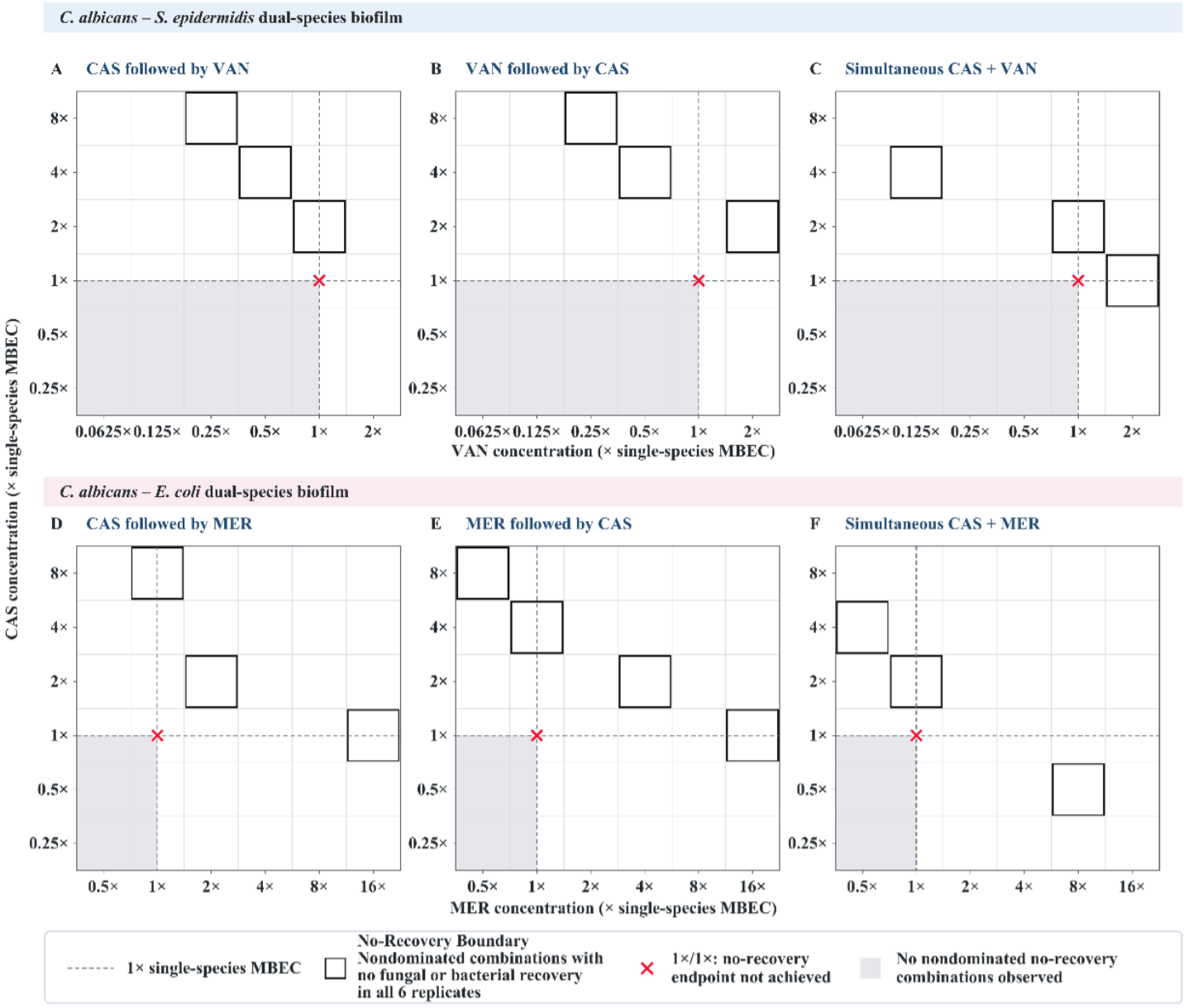


Figure 4

Nondominated no-recovery boundaries of mixed-species biofilms relative to the corresponding single-species minimum biofilm eradication concentrations (MBECs). For the *C. albicans-S. epidermidis* biofilm, administration regimens were **A)** CAS followed by VAN, **B)** VAN followed by CAS, and **C)**simultaneous CAS plus VAN. For the *C. albicans-E. coli* biofilm, administration regimens were **D)** CAS followed by MER, **E)** MER followed by CAS, and **F)** simultaneous CAS plus MER. Antimicrobial concentrations are expressed as multiples of the corresponding single-species MBECs: CAS against *C. albicans*, 1024 µg/mL; VAN against *S. epidermidis*, 4096 µg/mL; and MER against *E. coli*, 512 µg/mL. Horizontal and vertical dashed lines indicate 1× the corresponding single-species MBEC. Black outlines identify nondominated concentration combinations at which both fungal and bacterial CFU were zero for all six titanium discs across three independent experiments. The gray-shaded region denotes combinations in which both antimicrobials were below their corresponding single-species MBECs; no nondominated no-

recovery combinations were observed in this region. The red × marks the 1×/1× combination, at which recoverable CFU remained above zero.

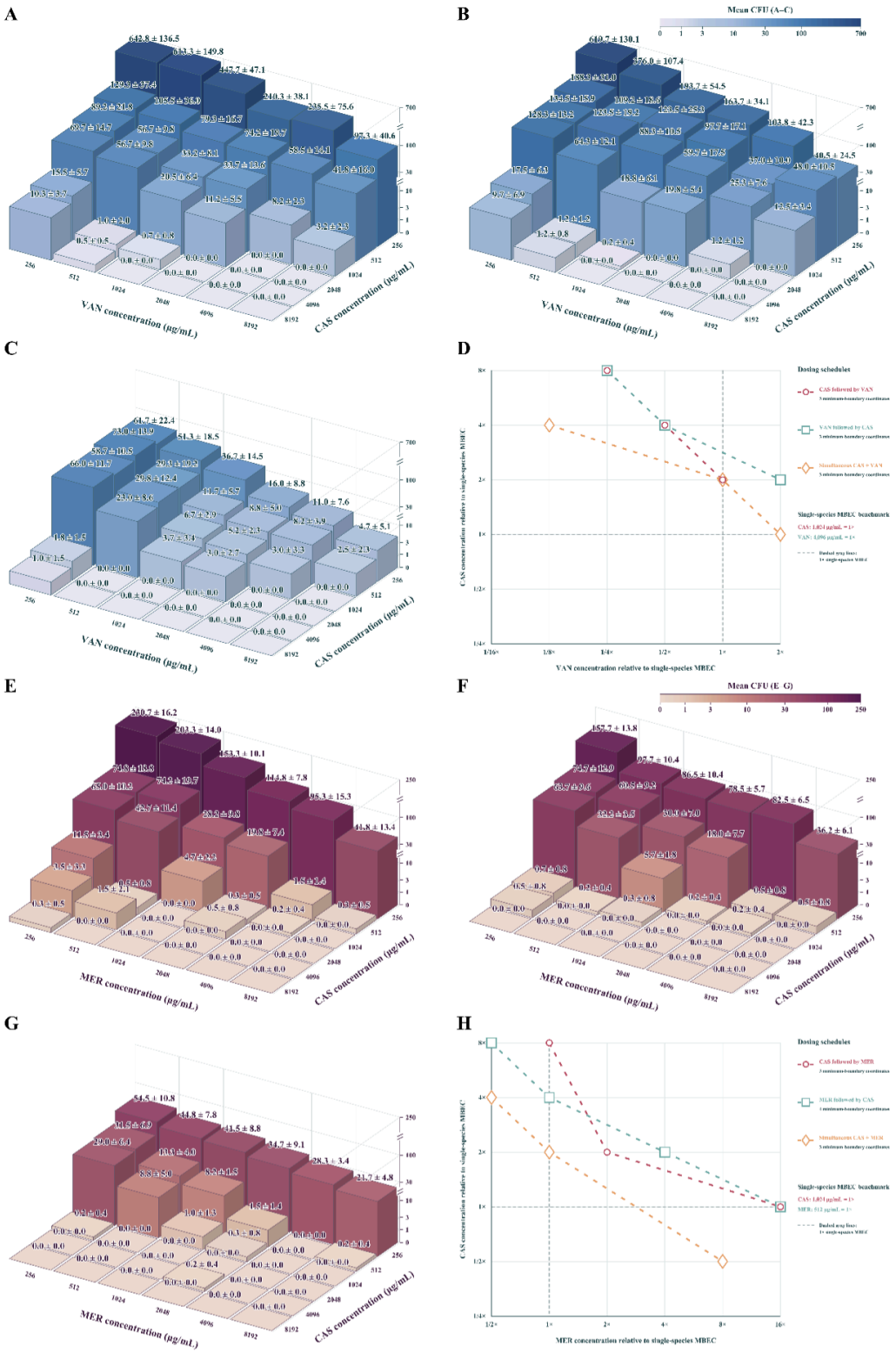


Figure 5

Concentration-response patterns and paired concentration requirements for no recovery under different antimicrobial administration regimens. For the *C. albicans-S. epidermidis* biofilm, panels **A-C**

show the concentration-response matrices for **A)** CAS followed by VAN, **B)** VAN followed by CAS, and **C)**simultaneous CAS plus VAN. Panel **D** compares the paired CAS-VAN concentrations associated with the no-recovery endpoint under the three administration regimens, expressed relative to the corresponding single-species MBECs. For the *C. albicans-E. coli* biofilm, panels **E-G** show the concentration-response matrices for **E)** CAS followed by MER, **F)**MER followed by CAS, and **G)** simultaneous CAS plus MER. Panel **H**compares the paired CAS-MER concentrations associated with the no-recovery endpoint under the three administration regimens, expressed relative to the corresponding single-species MBECs.