



Efflux-Mediated Synergy and Preserved in Vitro Selectivity of Zinc Oxide Nanoparticle–Ampicillin Combinations against Multidrug-Resistant *Klebsiella Pneumoniae* and *Pseudomonas Aeruginosa*

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Abstract

Background: Zinc oxide nanoparticles have the ability to rescue the β -lactam activity against multidrug resistant Gram-negative pathogens, but only concentrations that synergy that do not harm the host cells are therapeutically useful.

Objectives: To quantify the synergy between ZnO-NP and ampicillin in clinical isolates of MDR *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, as well as to parallelly determine if the synergistic concentrations are selective to the bacteria compared with the human lung fibroblasts and keratinocytes.

Methods: One hundred and forty-five non-duplicate clinical isolates (71 *K. pneumoniae*, 74 *P. aeruginosa*) recovered at Fallujah General Hospital, Anbar, Iraq, were studied. MICs and MBCs were obtained using broth microdilution and synergy was evaluated by checkerboard assays and by the fractional inhibitory concentration index, dose-reduction index, ZIP, Bliss, Loewe and highest-single-agent models. Two-way mixed-design ANOVA with Greenhouse–Geisser correction and Holm adjusted paired contrasts were used to compare normalised growth under six conditions. Dose–response curve with MTT was fitted to four-parameter logistic model and the selectivity index was calculated for WI-38 and HaCaT cells. Mechanistic endpoints included ethidium bromide retention, membrane permeability, qPCR of *acrA*, *acrB*, *mexA* and *mexB* and molecular docking. Biological inclusion levels of low dose hormesis (0.2%, 0.4%, and 0.8% ZnO MIC) were tested on the biofilm biomass, metabolic activity and extracellular polymeric substances (EPS) as well as the markers of cytotoxicity, LDH release, apoptosis and necrosis, and mitochondrial membrane potential.

Results: Baseline susceptibility was closely matched between species (median ampicillin MIC 256.00 $\mu\text{g/mL}$, IQR 128.00–512.00, $P = 0.386$; median ZnO MIC 62.50 $\mu\text{g/mL}$, IQR 31.25–125.00, $P = 0.825$). Synergy occurred in 133/145 isolates (91.7%): 66/71 *K. pneumoniae* (93.0%) and 67/74 *P. aeruginosa* (90.5%), with median FICI values of 0.391 and 0.359 and a median 8-fold ampicillin dose reduction in both species. Combination growth fell to $24.26 \pm 11.33\%$ of control versus $98.76 \pm 0.70\%$ in untreated controls (partial $\eta^2 = 0.974$ for treatment; no species effect, $P = 0.931$). On WI-38 and HaCaT cells, the combination IC_{50} s were 558.8 and 613.0 $\mu\text{g/mL}$ (*K. pneumoniae*) and 542.8 and 598.2 $\mu\text{g/mL}$ (*P. aeruginosa*), respectively, while ampicillin alone could not be determined in the 0–1000 $\mu\text{g/mL}$ range. Median SI values were 38.57–43.27 and 41.52–44.19; $\text{SI} > 1$ occurred in 97.2% and 94.6% of combinations. EtBr retention (1.94–1.96-fold), membrane permeability (1.59–1.64-fold), pump-gene repression (0.66–0.69-fold) and docking scores (–6.3 to –6.4 kcal/mol) all pointed towards an efflux/permeability mechanism. Host-cell injury remained low (LDH 10.2–10.3%; apoptosis 6.9–7.4%; necrosis 3.3–3.4%; MMP 87.9–89.4% of control). But, 0.2–0.8% of the ZnO MIC enhanced the formation of biofilm, metabolic activity and EPS up to 109% of control.

Conclusions: The results demonstrate that the combinations of ZnO-NP/ampicillin are synergistic and selective for both species in vitro, with the combination showing a narrow sub-inhibitory exposure, so that there is a need for dose-controlled and mechanism-informed development rather than low dose application.

Keywords: zinc oxide nanoparticles; ampicillin; efflux-mediated synergy; selectivity index

1. Introduction

Antimicrobial resistance in Gram-negative pathogens has moved from a surveillance concern to a measurable mortality burden. A synthesis of epidemiological surveillance across 187 countries documented a 43% global increase in multidrug-resistant infections, with a 67% rise in healthcare-associated and 38% rise in community-acquired infections in settings with high antibiotic misuse, and annual healthcare costs exceeding USD 100 billion (Marino *et al.*, 2025). *Klebsiella pneumoniae* alone accounted for approximately 800,000 deaths worldwide in 2019, 80% of them associated with resistance, with carbapenem and third-generation cephalosporin resistance responsible for more than half of the resistant mortality (Song *et al.*, 2025). *Pseudomonas aeruginosa* occupies the same ecological niche in hospital-acquired infection, and the two organisms frequently share carbapenemase and efflux-mediated resistance machinery. Throughout this paper, multidrug resistance is used in its consensus sense — non-susceptibility to at least one agent in three or more antimicrobial categories (Sendra *et al.*, 2024).

The Iraqi picture is consistent with this global trend but is documented unevenly. Multicentre analysis of 846 de-duplicated isolates from four governorates found an overall multidrug resistance prevalence of 57.3% (95% CI 53.9–60.6), with third-generation cephalosporin resistance in 44.4% of *K. pneumoniae* isolates and carbapenem resistance in 11.1% of *K. pneumoniae* and 29.4% of *P. aeruginosa* isolates (Omer *et al.*, 2026). A four-year national analysis reported *P. aeruginosa* resistance rising from 50% in 2021 to 54% in 2023, while MDR *K. pneumoniae* peaked at 65% in 2021 and declined to 58% (Alhilfi *et al.*, 2026). A nationwide review of 11,592 clinical records identified ampicillin as an ineffective agent against isolates recovered from adults, and among the agents most resisted by paediatric isolates (Al-Fahad *et al.*, 2024). Ampicillin is therefore a molecule of genuine residual interest in this setting: inexpensive, universally available, mechanistically well characterised, and almost completely inactive against the local MDR population.

Zinc oxide nanoparticles offer a plausible route to reactivating such agents. Their antibacterial action proceeds through several non-exclusive mechanisms: generation of reactive oxygen species with consequent lipid peroxidation and membrane disruption, release of Zn^{2+} ions that bind thiol groups and inhibit glycolytic and respiratory enzymes, and interference with DNA replication and protein synthesis (Mendes *et al.*, 2022)(Gudkov *et al.*, 2021)(Gomaa, 2022), with cell-surface ROS generation scaling with available particle surface area (Hadi *et al.*, 2025). ZnO-NPs retain broad-spectrum activity against β -lactamase-producing Gram-negative pathogens, with reported MICs of 0.04–0.08 mg/mL and MBCs of 0.12–0.24 mg/mL, and they appear to interfere with β -lactamase activity in addition to damaging the envelope (Krishnamoorthy *et al.*, 2022). Susceptibility to ZnO-NPs is nevertheless species-dependent: MIC values of 80 $\mu\text{g/mL}$ for *E. coli*, 60 $\mu\text{g/mL}$ for *K. pneumoniae* and 30 $\mu\text{g/mL}$ for *P. aeruginosa* have been reported (Sanchez *et al.*, 2021), which cautions against extrapolating a single effective concentration across genera.

Combining ZnO-NPs with β -lactams is attractive because the nanoparticle acts at multiple targets simultaneously, so that the probability of a single mutational event conferring resistance is low, and because dose sparing of the antibiotic reduces both cost and selective pressure (Ozdal & Gurkok, 2022). Experimental support is accumulating: ZnO-NPs with piperacillin–tazobactam showed enhanced activity against MDR *E. coli* and *P. aeruginosa* (Yu *et al.*, 2024); ZnO-NPs with ciprofloxacin and ceftazidime increased activity against resistant *Acinetobacter baumannii* through membrane damage and intracellular accumulation of the antibiotic (Abdelghani *et al.*, 2022); combinations with colistin, ciprofloxacin and meropenem produced synergistic fractional inhibitory concentration indices (Irede *et al.*, 2024); and incorporation of meropenem into ZnO nanoflakes raised the inhibition zone against metallo- β -lactamase-producing clinical isolates to an average of 15.2 mm (Singh *et al.*, 2023). ZnO-NPs conjugated directly to clinically approved antibiotics, ampicillin included, enhanced antibacterial efficacy while showing minimal cytotoxicity against human cell lines (Akbar *et al.*, 2021). Computational work against carbapenem-resistant *K. pneumoniae* porins has added a further plausible target, with ZnO-NPs showing the highest predicted affinity (−9.1 kcal/mol) among tested ligands and an experimental MIC of 0.2 mg/mL, an MBC of 0.5 mg/mL and an inhibition zone of 24 mm (Elsayim *et al.*, 2023).

A second, less widely exploited mechanism is interference with drug efflux. The resistance-nodulation-division transporters AcrAB-TolC in Enterobacteriaceae and MexAB-OprM in *P. aeruginosa* are principal determinants of

intrinsic and acquired multidrug resistance, and their inhibition restores the activity of clinically relevant agents (Alenazy, 2022) (Ornik-Cha et al., 2021). Pharmacological efflux-pump inhibitors such as phenylalanine-arginine β -naphthylamide are used in vitro to raise intracellular antibiotic accumulation in Gram-negative bacteria, but adverse toxicology and pharmacokinetic profiles identified in early clinical trials preclude their therapeutic use, and they remain research tools (Langendonk et al., 2021); PA β N also permeabilises the outer membrane at higher doses, which complicates the attribution of its effects to efflux inhibition alone (Al-Marzooq et al., 2023). Nanoparticles can bypass efflux either by delivering the antibiotic intracellularly or by irreversibly blocking the pump (Modi et al., 2023), and efflux inhibition additionally reduces biofilm adhesion and maturation, making it an attractive anti-biofilm target (Musioł, 2023). Direct evidence exists in *P. aeruginosa*: polyol-coated CuO, ZnO and CuZn nanoparticles acted synergistically with meropenem and ciprofloxacin, and at sub-inhibitory concentrations promoted intracellular accumulation of ethidium bromide, the classic efflux substrate (Eleftheriadou et al., 2021).

The problem, and the reason the present study was designed as it was, is that the same properties that make ZnO-NPs effective antibacterial agents also make them potentially cytotoxic to host cells. Particle size strongly governs ZnO toxicity: in a comparative study on HaCaT keratinocytes and A549 lung epithelial cells, micrometric ZnO was the most cytotoxic, followed by 100 nm and then 50 nm particles, with membrane integrity more affected in keratinocytes (Mitjans et al., 2023); ZnO-NPs reduced HaCaT viability with a half-lethal concentration of approximately 12.5 $\mu\text{g/mL}$ under combined exposures (Wang et al., 2024); and in human skin, keratinocyte cytotoxicity has been associated with exogenous zinc concentrations of 21–31 $\mu\text{g/mL}$ (Khabir et al., 2021). Against this, green-synthesised ZnO-NPs have been reported as considerably less toxic to normal fibroblasts, with an IC_{50} of 568.59 $\mu\text{g/mL}$ against WI-38 cells compared with 409.70 $\mu\text{g/mL}$ against A-431 cells (Naiel et al., 2022). Because a combination can only be clinically meaningful if the concentration that kills bacteria is measurably lower than the concentration that injures host cells, the selectivity index — the ratio of off-target to target toxicity, conventionally IC_{50} divided by MIC (Ye et al., 2023) — and the related therapeutic index ($\text{CC}_{50}/\text{IC}_{50}$), which should exceed unity for a usable agent (Al-Hakkani et al., 2022), must be reported alongside any synergy claim.

A final complication is the low-dose, or hormetic, response. Sub-inhibitory exposure of bacteria to antimicrobials can stimulate rather than suppress growth, and low-dose nanoparticles are no exception: low concentrations of ZnO-NPs have been shown to trigger growth and biofilm formation in *P. aeruginosa* as a biphasic hormetic response (Al-Momani et al., 2024); ZnO-NPs at sub-inhibitory concentrations induced biofilm formation in *K. pneumoniae* and *Staphylococcus aureus* (Sreekanth et al., 2023); and the lowest dose of copper-containing nanoparticles enhanced biofilm formation of *S. aureus*, *P. aeruginosa* and *Candida albicans* by $11.3 \pm 1.7\%$, $11.7 \pm 1.5\%$ and $14.2 \pm 0.9\%$ respectively (Almahdy et al., 2024). Across engineered nanomaterial studies generally, motility and community functions including biofilm formation were affected in 50% of studies conducted at sub-inhibitory concentrations but in only 10–20% of studies using inhibitory concentrations (Mortimer et al., 2021). Any dosing strategy for a nanoparticle–antibiotic combination therefore has a lower boundary as well as an upper one.

Taken together, the literature supports synergy and supports efflux involvement, but it does not resolve whether the two are accompanied by an adequate selectivity window in clinically relevant MDR isolates, nor how narrow that window is in relation to sub-inhibitory exposure. The present work addresses that gap directly. Its objectives were: (1) to establish the baseline susceptibility profile of *K. pneumoniae* and *P. aeruginosa* clinical isolates from a single Iraqi hospital to ampicillin and ZnO-NPs; (2) to quantify the interaction by FICI, DRI and four model-based synergy scores; (3) to determine the cytotoxicity of the combinations in two human cell lines and derive selectivity indices; (4) to test phenotypic, transcriptional and computational evidence for an efflux/permeability mechanism; (5) to measure low-dose hormesis in biofilm, metabolic activity and EPS; and (6) to integrate these domains statistically in order to judge whether an in vitro selectivity window exists that could justify further development.

2. Materials and methods

2.1 Study design, setting and bacterial isolates

The study was conducted on clinical bacterial isolates recovered from routine diagnostic specimens at Fallujah General Hospital, Anbar Province, Iraq, between (collection period). A single-centre, cross-sectional in vitro design was used.

A total of 145 non-duplicate clinical isolates were included: 71 *Klebsiella pneumoniae* and 74 *Pseudomonas aeruginosa*, obtained from (specimen types). Species-level identification was performed with (identification platform) according to the routine procedure of the hospital microbiology unit. Multidrug resistance was defined, in line with the consensus international definitions, as non-susceptibility to at least one agent in three or more antimicrobial categories (Sendra *et al.*, 2024). Isolates were stored in glycerol at -80°C and subcultured twice on fresh medium before testing. The study was approved by (ethics committee and approval reference); informed consent was waived because only de-identified isolates obtained during routine diagnostic testing were used.

2.2 Antibacterial agents

ZnO-NPs were used as received, without further surface modification or purification (supplier, lot number and nominal particle size as stated by the manufacturer). Working suspensions were prepared by ultrasonication in assay medium immediately before each experiment, and fresh suspensions were prepared for every assay. The same stock, dispersion protocol, sonication time and medium were used throughout, so that the material delivered to each well remained constant across all experiments.

ZnO-NPs are conventionally characterised by X-ray diffraction, electron microscopy, dynamic light scattering and zeta potential, and these parameters govern both their antibacterial and their cytotoxic behaviour (Ahmad *et al.*, 2023) (Ullah *et al.*, 2024). In the present study the material was used as received and was not characterised in-house; the consequences of this for the interpretation of the absolute concentrations reported here are addressed explicitly in the Limitations (Section 4.6). Ampicillin was prepared as a stock solution in accordance with the manufacturer's instructions and diluted in assay medium on the day of use.

2.3 MIC and MBC determination

MICs were determined by broth microdilution using cation-adjusted Mueller–Hinton broth, a final inoculum of approximately 5×10^5 CFU/mL, and incubation at $35 \pm 1^{\circ}\text{C}$ for 18 ± 2 h before reading (Tang *et al.*, 2021) (Kadeřábková *et al.*, 2024). Working suspensions were adjusted to the 0.5 McFarland standard, equivalent to approximately 1×10^8 CFU/mL, and then diluted 1:100 to give the final inoculum (Balogun *et al.*, 2024). Because ZnO-NPs scatter light and may sediment in the assay medium, plates were read after gentle resuspension and endpoints were confirmed visually; the concentration range tested for each agent was identical for the two species. MBCs were determined by subculturing the contents of wells showing no visible growth, together with the two concentrations above the MIC, onto Mueller–Hinton agar; the lowest concentration preventing growth after 24 h at 37°C was recorded as the MBC (Kathirvalu, 2023). Bactericidal activity was classified as an MBC/MIC ratio ≤ 4 and bacteriostatic activity as a ratio > 4 (Jarczak *et al.*, 2021). All determinations were performed in triplicate. MIC and MBC values for ampicillin and ZnO-NPs were obtained both as single agents and within the combination.

2.4 Checkerboard synergy assays and interaction modelling

Two-dimensional checkerboard assays were performed in 96-well microplates with two-fold serial dilutions of each agent across the plate, read after 18 h by optical density (Bellio *et al.*, 2021). FICI was calculated as $\text{FICI} = \text{FIC}_A + \text{FIC}_B$, where FIC_A and FIC_B are the MICs of each agent in combination divided by their MICs alone (Ratia *et al.*, 2023). $\text{FICI} \leq 0.5$ was interpreted as synergy, $0.5 < \text{FICI} \leq 1$ as additivity, $1 < \text{FICI} \leq 4$ as indifference and $\text{FICI} > 4$ as antagonism, following the conservative thresholds adopted to permit comparison across studies (Ibrahim & Alfadil, 2026). The DRI was derived to express the fold reduction in the effective concentration of each agent in the combination. In addition, the ZIP, Bliss, Loewe and HSA scores were computed as model-based synergy metrics, so that the interaction could be assessed independently of the specific concentration grid tested (Greger *et al.*, 2025). Because exhaustive higher-dimensional testing of combinations is combinatorially prohibitive, model-based scores computed on a normalised concentration design provide a practical and internally consistent complement to FICI (Brennan *et al.*, 2022). For display, mean normalised growth across the panel was assembled into a single checkerboard matrix after normalisation of ZnO and ampicillin concentrations to each isolate's own MIC, so that isolates with high absolute MICs would not compress the common concentration scale.

2.5 Growth inhibition under defined treatment conditions

Normalised growth of each isolate was measured under six conditions: untreated control, ZnO alone, ampicillin alone, ZnO + ampicillin combination, efflux-pump inhibitor control, and a reference strain control. Growth was expressed as a percentage of the untreated control. Concentrations of each agent in the combination were selected from the checkerboard-derived effective concentrations. The efflux-pump inhibitor control used (inhibitor and final concentration), a compound of the class routinely employed *in vitro* to increase intracellular antibiotic accumulation in Gram-negative bacteria (Langendonk *et al.*, 2021). The reference strain control used (reference strain designation).

2.6 Cytotoxicity in human cell lines and selectivity analysis

Cytotoxicity was evaluated in two human cell lines: WI-38 human lung fibroblasts and HaCaT human keratinocytes. Viability was measured by the MTT assay, which quantifies the reduction of tetrazolium dye to formazan by mitochondrial dehydrogenases of metabolically active cells (Lee *et al.*, 2022), with absorbance read on a microplate reader. Cells were exposed to a two-fold concentration series of the combination, and to ampicillin alone across 0–1000 µg/mL, for (exposure duration). Concentration–response data were fitted by four-parameter logistic nonlinear regression, the standard approach for sigmoidal dose–response data in which the half-maximal inhibitory concentration and the slope of the curve are estimated simultaneously (Naiel *et al.*, 2022) (Ye *et al.*, 2023), and IC₅₀ values were reported with 95% confidence intervals and Hill slopes. The selectivity index was calculated as the IC₅₀ divided by the effective ZnO concentration of the combination, following the convention in which the SI expresses the ratio of off-target to target toxicity (Ye *et al.*, 2023); the closely related therapeutic index (CC₅₀/IC₅₀) should exceed unity for an agent to be considered selective (Al-Hakkani *et al.*, 2022).

2.7 Efflux, membrane permeability, gene expression and docking

Efflux activity was assessed phenotypically by EtBr retention, a fluorometric assay in which increased intracellular fluorescence indicates reduced efflux (Lee *et al.*, 2024) (Ornik-Cha *et al.*, 2021); the agar cartwheel method provides a complementary format for classifying efflux activity in clinical isolates (Ugwuanyi *et al.*, 2021). Membrane permeability was quantified as a fold change relative to untreated controls. Transcriptional responses of the RND pump genes *acrA* and *acrB* (*K. pneumoniae*) and *mexA* and *mexB* (*P. aeruginosa*) were quantified by real-time PCR, with relative expression calculated by the comparative Ct method and inferential comparisons performed on ΔCt values (Ernst *et al.*, 2026); (housekeeping gene) served as the internal reference. Molecular docking of the same four targets was performed with (docking program and version), following published protocols in which protein and ligand input files are prepared in PDBQT format and binding energies are reported in kcal/mol (Elsayim *et al.*, 2023). Docking was treated as a hypothesis-generating step only, because binding energies obtained by rigid-receptor docking are approximations of affinity, and a realistic description of ligand–pump interaction requires flexible-receptor simulation of the kind used to characterise the promiscuous AcrB and AdeB binding sites (Ornik-Cha *et al.*, 2021).

2.8 Cellular mechanisms of cytotoxicity

Cellular injury was characterised by four complementary endpoints: LDH release as a marker of membrane damage and necrotic death (Yadav *et al.*, 2021) (Awashra & Młynarz, 2023), apoptosis and necrosis by flow cytometry, and mitochondrial membrane potential using a cationic fluorescent probe, a standard approach because mitochondrial dysfunction is an early and sensitive indicator of nanoparticle cytotoxicity (Stachyra *et al.*, 2022) (Zhang *et al.*, 2022). Together with MTT viability and LDH release, these endpoints provide a multi-parameter toxicity profile rather than relying on a single metabolic readout.

2.9 Low-dose hormesis

Biofilm biomass, metabolic activity and EPS production were measured at 0.2%, 0.4% and 0.8% of the ZnO MIC in a Species × Dose design. Biofilm was quantified by the crystal violet microtitre method, metabolic activity by reduction-based colorimetry, and EPS by a standard extraction and colorimetric procedure. These endpoints were

selected because the low-dose response of bacteria to antimicrobials is preferentially expressed in community-associated functions such as biofilm formation (Mortimer et al., 2021).

2.10 Statistical analysis

Analyses were performed in IBM SPSS Statistics version 32.0.0 and GraphPad Prism version 11.1.0. Distributional assumptions were checked by the Shapiro–Wilk test and visually. Non-normally distributed continuous variables were summarised as median with interquartile range and compared between species by the Mann–Whitney U test, with the rank-biserial correlation reported as an effect size. Normally distributed descriptive variables were summarised as mean $\pm$ standard deviation. Categorical interaction profiles were compared by the chi-square test, with Fisher's exact test used for sparse contingency structures. Growth responses across the six treatment conditions were analysed by two-way mixed-design ANOVA, with species as the between-isolate factor and treatment as the within-isolate factor, applying the Greenhouse–Geisser correction to the within-isolate factor and reporting partial eta squared; prespecified paired contrasts were performed with the Wilcoxon signed-rank test and Holm adjustment. The same model structure was used for the low-dose biofilm, metabolic and EPS analyses. MTT concentration–response data were fitted by four-parameter logistic regression. Cross-domain associations were assessed by Spearman rank correlation with Benjamini–Hochberg control of the false discovery rate across the prespecified correlation set (Huang & Peddada, 2023). Two-sided $P < 0.05$ was considered statistically significant. No a priori power calculation was performed; sample size was determined by the number of eligible non-duplicate isolates available during the collection period.

3. Results

3.1 Baseline susceptibility was closely matched between species

The baseline susceptibility profile of the two Gram-negative species was nearly identical. Median ampicillin MIC was 256.00 $\mu\text{g/mL}$ (IQR 128.00–512.00 $\mu\text{g/mL}$) in both species ($P = 0.386$; rank-biserial $r = -0.081$), and median ZnO MIC was 62.50 $\mu\text{g/mL}$ (IQR 31.25–125.00 $\mu\text{g/mL}$) in both ($P = 0.825$; $r = 0.021$). Median ampicillin MBC was 512.00 $\mu\text{g/mL}$ (IQR 256.00–1024.00 $\mu\text{g/mL}$; $P = 0.319$; $r = -0.094$) and median ZnO MBC was 125.00 $\mu\text{g/mL}$ (IQR 62.50–250.00 $\mu\text{g/mL}$; $P = 0.628$; $r = 0.045$). Within the combination, the median ampicillin MBC fell to 32.00 $\mu\text{g/mL}$ (IQR 16.00–64.00) for *K. pneumoniae* and 48.00 $\mu\text{g/mL}$ (IQR 32.00–112.00) for *P. aeruginosa* ($P = 0.499$; $r = -0.064$), while the median ZnO MBC in combination was 21.41 $\mu\text{g/mL}$ (IQR 11.19–45.06) and 20.05 $\mu\text{g/mL}$ (IQR 8.08–44.77) respectively ($P = 0.517$; $r = 0.063$). All rank-biserial effect sizes were small. This equivalence is analytically important: because the two species entered the interaction experiments with statistically indistinguishable MIC and MBC distributions, any subsequent difference in combination behaviour cannot be attributed to a pre-existing imbalance in susceptibility.

Table 1: Baseline Antimicrobial Susceptibility by Bacterial Species

Measure	<i>K. pneumoniae</i> (n=71), median (IQR)	<i>P. aeruginosa</i> (n=74), median (IQR)	P value	Rank-biserial r
Ampicillin MIC alone ($\mu\text{g/mL}$)	256.00 (128.00–512.00)	256.00 (128.00–512.00)	0.386	-0.081
ZnO MIC alone ($\mu\text{g/mL}$)	62.50 (31.25–125.00)	62.50 (31.25–125.00)	0.825	0.021
Ampicillin MBC alone ($\mu\text{g/mL}$)	512.00 (256.00–1024.00)	512.00 (256.00–1024.00)	0.319	-0.094
ZnO MBC alone ($\mu\text{g/mL}$)	125.00 (62.50–250.00)	125.00 (62.50–250.00)	0.628	0.045
Ampicillin MBC in combination ($\mu\text{g/mL}$)	32.00 (16.00–64.00)	48.00 (32.00–112.00)	0.499	-0.064
ZnO MBC in combination ($\mu\text{g/mL}$)	21.41 (11.19–45.06)	20.05 (8.08–44.77)	0.517	0.063

The susceptibility profile for the two Gram-negative species was very similar at baseline. For *K. pneumoniae* and *P. aeruginosa*, the median MIC for ampicillin was both 256 $\mu\text{g/mL}$ with interquartile range 128–512 $\mu\text{g/mL}$ ($P = 0.386$).

ZnO MIC alone was also identical at a median of 62.5 $\mu\text{g/mL}$ (IQR 31.25–125 $\mu\text{g/mL}$; $P = 0.825$). There was no difference among the corresponding MBC distributions and no difference in the MBC values measured within the combination of agents for any species. No material effect was found in this dataset on baseline antimicrobial susceptibility as all rank-biserial effect sizes were small. This similarity is significant as subsequent differences in the combination behaviour can then be understood against widely similar baseline MIC/MBC distributions that do not involve a large pre-existing susceptibility imbalance.

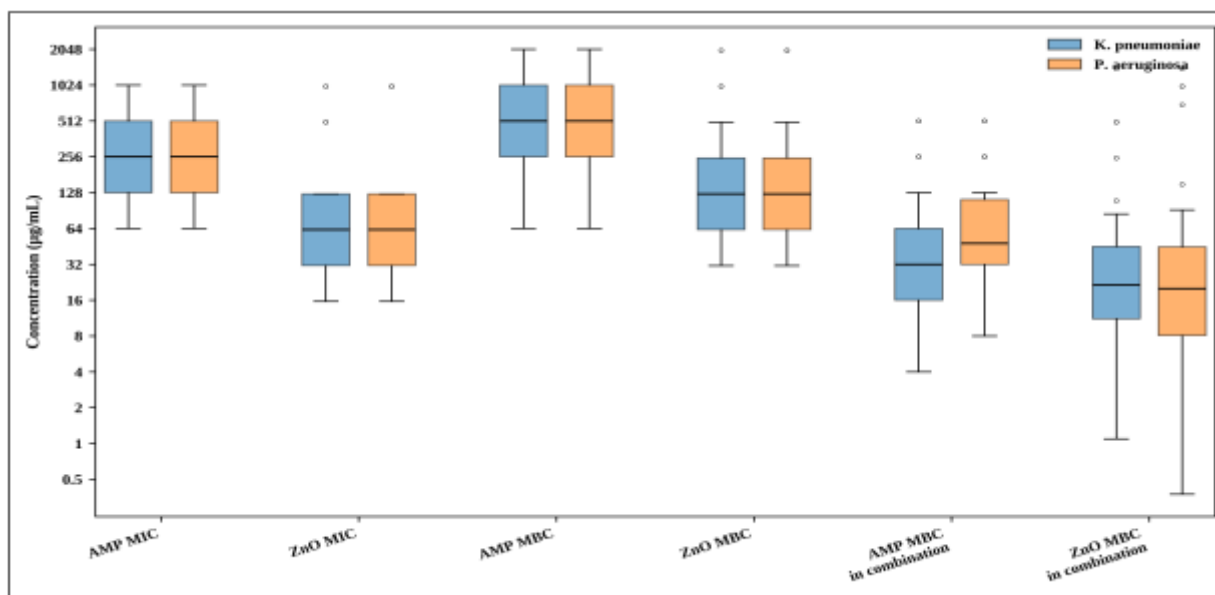


Figure 1: MIC and MBC Distribution by Bacterial Species

The initial susceptibility profile was very similar for the two Gram-negative species. The median of Ampicillin MIC was also the same 256 $\mu\text{g/mL}$ for both *K. pneumoniae* and *P. aeruginosa*, and the interquartile range was 128–512 $\mu\text{g/mL}$ ($P = 0.386$). ZnO MIC alone was also identical at a median of 62.5 $\mu\text{g/mL}$ (IQR 31.25–125 $\mu\text{g/mL}$; $P = 0.825$). The distribution of the corresponding MBCs was not significantly different among the two agents and no species difference was found in MBCs taken in the combination. All rank-biserial effect sizes were small indicating that in this dataset, baseline antimicrobial susceptibility was not materially influenced by species. This similarity is significant as the subsequent differences in combination behaviour may be interpreted in the context of broadly similar MIC/MBC distributions and not against a major pre-existing susceptibility imbalance.

3.2 The interaction was synergistic and reproducible in both species

Combination testing produced predominantly synergistic interactions. Synergy was recorded in 133 of 145 isolates (91.7%): 66/71 *K. pneumoniae* (93.0%) and 67/74 *P. aeruginosa* (90.5%). Median FICI was 0.391 in *K. pneumoniae* and 0.359 in *P. aeruginosa* ($P = 0.184$; FDR $q = 0.369$; $r = 0.128$), both well below the 0.5 synergy threshold. The median ampicillin DRI was 8-fold in both species ($P = 0.549$; $q = 0.549$; $r = 0.055$). Model-based scores were comparable between species: ZIP 13.38 (11.77–14.72) versus 14.01 (12.16–15.13); Bliss 12.56 (10.70–13.55) versus 13.05 (11.66–14.29); Loewe 14.14 (12.73–15.41) versus 14.63 (12.12–16.27); and HSA 11.48 (9.86–12.67) versus 12.24 (11.00–13.43). Only the unadjusted HSA comparison reached nominal significance ($P = 0.043$), and this disappeared after false-discovery-rate correction ($q = 0.260$), which illustrates why uncorrected P values should not be over-interpreted in multi-endpoint designs (Huang & Peddada, 2023). The overall distribution of synergy, additive and indifferent categories did not differ between species (χ^2 , $P = 0.267$), and antagonism was recorded in 0/71 *K. pneumoniae* isolates (0.0%) and 3/74 *P. aeruginosa* isolates (4.1%). The single normalised checkerboard matrix for the whole panel showed a progressive increase in mean growth inhibition with increasing fractional concentration of

either component, with the strongest suppression at paired sub-MICs; normalisation to each isolate's own MIC permitted all isolates to be represented on one common concentration scale.

Table 2: Combination and Synergy Profile by Bacterial Species

Synergy metric	<i>K. pneumoniae</i> median (IQR)	<i>P. aeruginosa</i> median (IQR)	P value	FDR q	Rank-biserial r
FICI	0.39 (0.33–0.45)	0.36 (0.30–0.45)	0.184	0.369	0.128
Ampicillin DRI	8.00 (4.00–16.00)	8.00 (4.00–8.00)	0.549	0.549	0.055
ZIP score	13.38 (11.77–14.72)	14.01 (12.16–15.13)	0.260	0.391	-0.108
Bliss score	12.56 (10.70–13.55)	13.05 (11.66–14.29)	0.149	0.369	-0.139
Loewe score	14.14 (12.73–15.41)	14.63 (12.12–16.27)	0.420	0.504	-0.078
HSA score	11.48 (9.86–12.67)	12.24 (11.00–13.43)	0.043	0.260	-0.195
Interaction profile					
Synergy	66/71 (93.0%)	67/74 (90.5%)			
Additive	1/71 (1.4%)	2/74 (2.7%)			
Indifferent	4/71 (5.6%)	2/74 (2.7%)			
Antagonism	0/71 (0.0%)	3/74 (4.1%)			
Overall category distribution			0.267		

Combination testing showed a mainly synergistic effect for both species. Synergy was recorded in 133 of 145 isolates (91.7%), including 66/71 *K. pneumoniae* isolates (93.0%) and 67/74 *P. aeruginosa* isolates (90.5%). The median FICIs were 0.391 and 0.359 for the two species, respectively, with a median dose-reduction index of 8-fold for ampicillin in both cases. ZIP, Bliss, and Loewe scores also were comparable among species. The difference was not significant when using the false-discovery-rate method ($q = 0.260$), but was significant when the HSA was unadjusted ($P = 0.043$). There was no significant difference in the overall distribution of synergy values, additive and indifferent (χ^2 test, $P = 0.267$) or antagonistic values between species. Overall, the synergy was significant and repeatable for both organisms and there was no indication that one organism caused the synergy systematically.

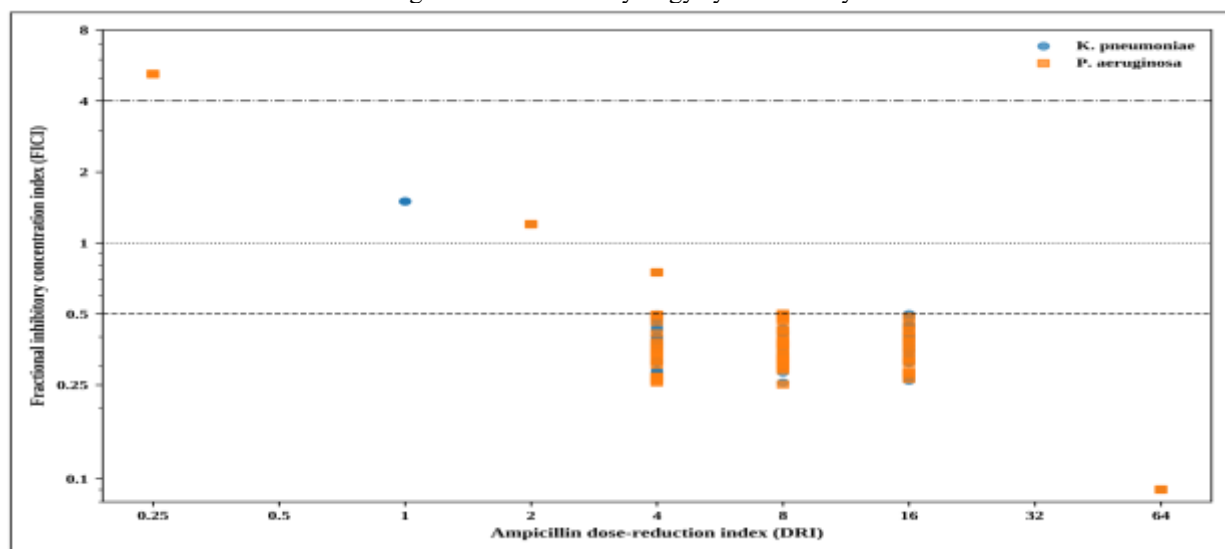


Figure 2: Synergy Profile and Dose-Reduction Index

Combination testing revealed mostly synergistic effects for both species. Synergy was recorded in 133 of 145 isolates (91.7%), including 66/71 *K. pneumoniae* isolates (93.0%) and 67/74 *P. aeruginosa* isolates (90.5%). The median value

of FICI was 0.391 and 0.359 in both species, respectively, and the median ampicillin dose-reduction index was 8-fold in both species. There was also no difference in the scores for ZIPS, Bliss and Loewe between species. The unadjusted HSA comparison resulted in $P = 0.043$, but this difference was not significant when false-discovery-rate corrected ($q = 0.260$). No significant difference was observed in the grand distribution of synergy, additive, indifferent and antagonistic categories between the species (χ^2 test, $P = 0.267$). The combination effect was significant overall and consistent across both organisms, with no apparent systematic evidence that one species consistently drove the synergistic effect.

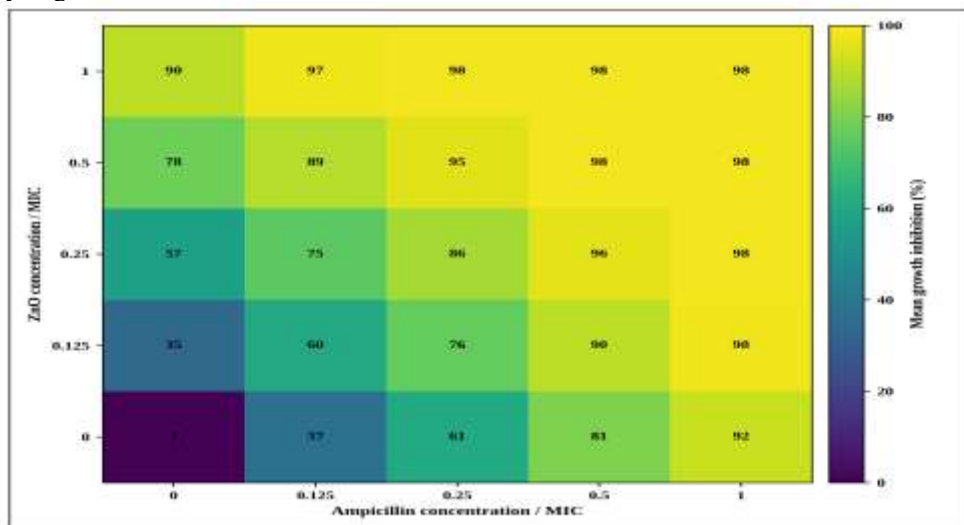


Figure 3: Mean Normalized Checkerboard Synergy Matrix

The single normalized checkerboard matrix shows the concentration-response pattern for the entire panel of isolates, with ZnO and ampicillin concentrations normalized to each isolate's MIC. There was a progressive increase in mean growth inhibition with increasing fractional concentration of either component and highest when both agents were used in higher fractional concentration. Importantly, there was a high amount of suppression observed at paired sub-MICs, and this is reflected visually in the predominance of FICI-defined synergy in the isolate-level analysis. The normalization to the individual MIC values makes it possible to show the checkerboard response in one common matrix while avoiding the possibility of suppressing the concentration scale by isolates with higher absolute MIC values.

3.3 Treatment condition, not species, governed growth inhibition

Growth differed markedly across treatment conditions but not between species. Untreated growth was $98.78 \pm 0.67\%$ for *K. pneumoniae* and $98.74 \pm 0.74\%$ for *P. aeruginosa*. ZnO alone reduced growth to $59.15 \pm 6.07\%$ and $60.37 \pm 7.73\%$ respectively; ampicillin alone to $64.78 \pm 5.27\%$ and $63.32 \pm 7.27\%$; and the combination further reduced growth to $24.05 \pm 8.88\%$ and $24.46 \pm 13.32\%$ (overall mean $24.26 \pm 11.33\%$ versus $98.76 \pm 0.70\%$ untreated, $59.77 \pm 6.97\%$ for ZnO alone and $64.04 \pm 6.39\%$ for ampicillin alone). The pump-inhibitor condition produced the lowest growth ($13.07 \pm 9.34\%$ and $13.36 \pm 13.55\%$; overall $13.22 \pm 11.64\%$), and the reference strain control gave $18.06 \pm 9.24\%$ and $18.28 \pm 12.91\%$ (overall $18.17 \pm 11.23\%$). Two-way mixed-design ANOVA showed a very large treatment effect (Greenhouse-Geisser $\epsilon = 0.341$; $F = 5396.507$; $P < 0.001$; partial $\eta^2 = 0.974$), no species main effect ($F = 0.008$; $P = 0.931$; partial $\eta^2 = 0.000$) and no Species $\times$ Treatment interaction ($F = 0.924$; $P = 0.385$; partial $\eta^2 = 0.006$). Holm-adjusted paired contrasts confirmed that combination growth was significantly lower than untreated, ZnO-only and ampicillin-only growth (all adjusted $P < 0.001$), and also lower than the pump-inhibitor condition (adjusted $P < 0.001$).

Table 3: Species × Treatment Factorial Analysis

Treatment/control condition	<i>K. pneumoniae</i> mean ± SD	<i>P. aeruginosa</i> mean ± SD	Overall mean ± SD
Untreated	98.78 ± 0.67	98.74 ± 0.74	98.76 ± 0.70
ZnO only	59.15 ± 6.07	60.37 ± 7.73	59.77 ± 6.97
Ampicillin only	64.78 ± 5.27	63.32 ± 7.27	64.04 ± 6.39
ZnO+Ampicillin	24.05 ± 8.88	24.46 ± 13.32	24.26 ± 11.33
Pump inhibitor	13.07 ± 9.34	13.36 ± 13.55	13.22 ± 11.64
Reference strain	18.06 ± 9.24	18.28 ± 12.91	18.17 ± 11.23
Factorial effect	F	P value	Partial η^2
Species	0.008	0.931	0.000
Treatment (GG ϵ =0.341)	5396.507	<0.001	0.974
Species × Treatment	0.924	0.385	0.006
Key paired contrasts (Holm-adjusted)			
Combo vs Untreated		<0.001	
Combo vs ZnO only		<0.001	
Combo vs Ampicillin only		<0.001	
Combo vs Pump inhibitor		<0.001	

Growth varied significantly under the 6 treatment/control conditions while the 2 species were similar under these conditions. Untreated growth was around 99% in both species, where the mean growth in ZnO alone and ampicillin alone was about 59–60% and 63–65%, respectively. The combination of ZnO and ampicillin resulted in further decrease of mean growth to ~24% in both species. The two-way mixed analysis showed a very large treatment effect (Greenhouse–Geisser-corrected $P < 0.001$; partial $\eta^2 = 0.974$), but no main effect of species ($P = 0.931$) and no Species × Treatment interaction ($P = 0.385$; partial $\eta^2 = 0.006$). Holm adjusted paired comparisons were performed and revealed that combination growth was significantly below the untreated, ZnO-only and ampicillin-only growth (all adjusted $P < 0.001$). Therefore, treatment condition was a major factor as opposed to the species of bacteria.

3.4 Cytotoxicity was concentration-dependent but far above the synergistic range

Both healthy cell lines showed concentration-dependent loss of viability, and the four-parameter logistic models described the pooled data well, with R^2 values at or above 0.9999 for the combination curves and narrow 95% confidence intervals. For *K. pneumoniae*-associated combinations, mean IC_{50} was 558.8 $\mu\text{g/mL}$ (95% CI 540.4–577.2; Hill slope 1.415) in WI-38 cells and 613.0 $\mu\text{g/mL}$ (95% CI 593.0–632.9; Hill slope 1.391) in HaCaT cells. For *P. aeruginosa*-associated combinations, the corresponding values were 542.8 $\mu\text{g/mL}$ (95% CI 529.3–556.4; Hill slope 1.421) and 598.2 $\mu\text{g/mL}$ (95% CI 564.3–632.1; Hill slope 1.338). Hill slopes clustered between 1.34 and 1.42 in all four species–cell-line combinations, indicating a consistent sigmoidal response. For ampicillin alone, 50% loss of viability was not reached within 0–1000 $\mu\text{g/mL}$; extrapolated values for this condition were not treated as experimentally observed endpoints.

Table 4: Cytotoxicity and Dose–Response Parameters

Species	Cell line	4PL IC_{50} ($\mu\text{g/mL}$)	95% CI	Hill slope	R^2
<i>K. pneumoniae</i>	WI-38	558.8	540.4–577.2	1.415	1.0000
<i>K. pneumoniae</i>	HaCaT	613.0	593.0–632.9	1.391	1.0000
<i>P. aeruginosa</i>	WI-38	542.8	529.3–556.4	1.421	1.0000
<i>P. aeruginosa</i>	HaCaT	598.2	564.3–632.1	1.338	1.0000
Ampicillin-only note	Both cell lines	IC_{50} not reached within 0–1000 $\mu\text{g/mL}$			

The MTT dose–response data indicated that both healthy cell lines suffered from concentration-dependent loss of viability, and the dose–response data for all cell lines were well described by the four-parameter logistic models with $R^2 \geq 0.9999$ for the combination curves. The fitted IC₅₀ for the combination of *K. pneumoniae* was 558.8 $\mu\text{g/mL}$ in WI-38 cells and 613.0 $\mu\text{g/mL}$ in HaCaT cells. The values corresponding to *P. aeruginosa* associated combinations were 542.8 and 598.2 $\mu\text{g/mL}$, respectively. The 95% confidence intervals were fairly small, and the Hill slopes were around 1.34–1.42, suggesting a similar sigmoidal response among species and cell lines. In the ampicillin-only treatment, a concentration level of 0–1000 $\mu\text{g/mL}$ was not sufficient to achieve a 50% loss of viability - extrapolated values for this treatment were not considered as experimentally observed points.

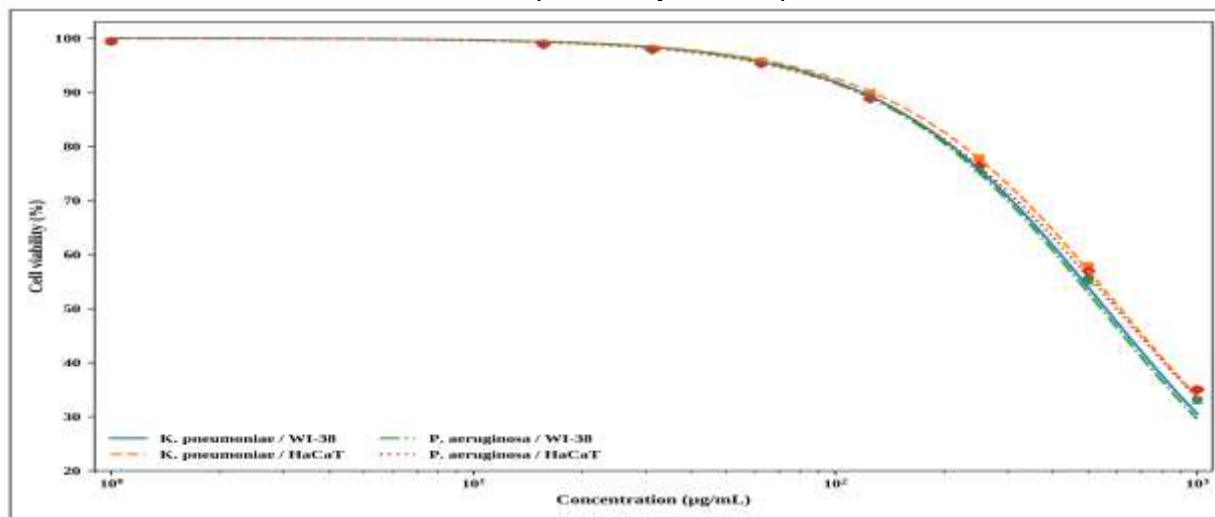


Figure 4: MTT Dose–Response Curves for WI-38 and HaCaT Cells

The concentration-dependent loss of viability in both cell lines from the MTT dose–response data, and the excellent fits of the four-parameter logistic models to the pooled raw observations ($R^2 \geq 0.9999$ for the combination curves), confirm that the combined data are sufficient for the construction of a single model. The concentration-dependent loss of viability observed in both cell lines in the MTT dose–response data, and the excellent fits of the four-parameter logistic models to the pooled raw observations ($R^2 \geq 0.9999$ for the combination curves) confirm that the combined data are sufficient for the construction of a single model. The fitted IC₅₀ values for *K. pneumoniae*-associated combinations were 613.0 $\mu\text{g/mL}$ for HaCaT cells and 558.8 $\mu\text{g/mL}$ for WI-38 cells. The values were 542.8 and 598.2 $\mu\text{g/mL}$, respectively for *P. aeruginosa* associated combinations. The 95% confidence intervals were relatively narrow and the Hill slopes were reasonably close to 1.34–1.42, which would indicate a consistent sigmoidal response for all species and cell lines. The ampicillin-only IC₅₀ values were not considered to be experimentally observed endpoints, as the 0–1000 $\mu\text{g/mL}$ range did not result in 50% loss of viability.

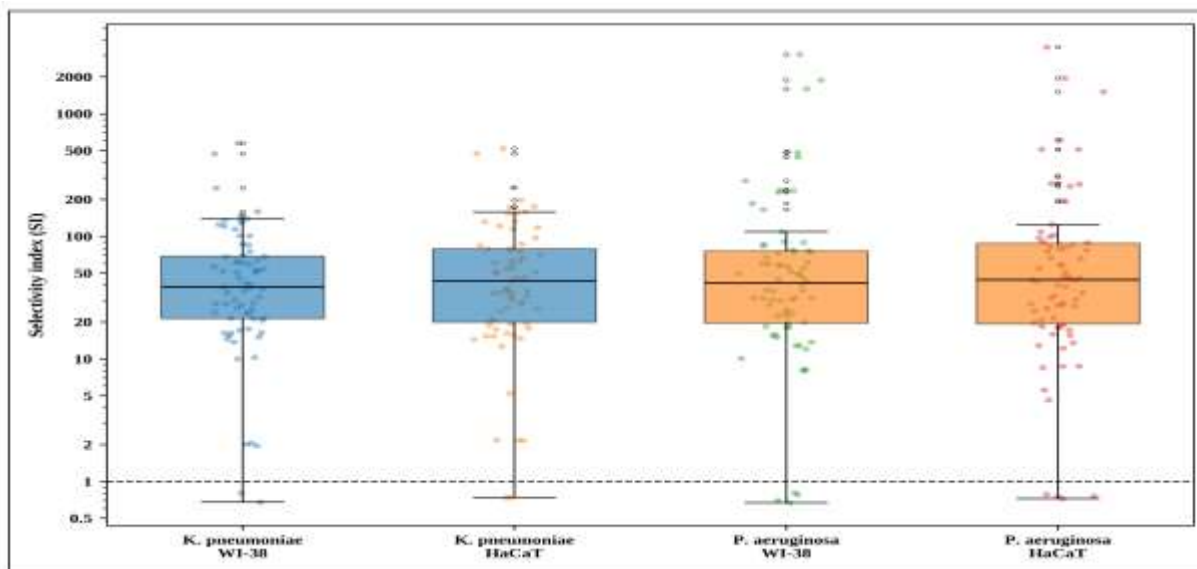
3.5 A selectivity window was present in the great majority of combinations

The effective concentrations of the combination lay well below the cytotoxic midpoint of both cell lines. Median effective ZnO concentration was 15.12 $\mu\text{g/mL}$ (IQR 7.78–27.69) for *K. pneumoniae* and 13.61 $\mu\text{g/mL}$ (IQR 7.58–27.00) for *P. aeruginosa* ($P = 0.777$); median ampicillin concentration in the combination was 32.00 $\mu\text{g/mL}$ (IQR 16.00–64.00) in both species ($P = 0.223$); and median ampicillin DRI was 8.00 in both ($P = 0.549$). Median selectivity index on WI-38 cells was 38.57 (IQR 21.37–68.78) for *K. pneumoniae* and 41.52 (IQR 19.60–75.62) for *P. aeruginosa* ($P = 0.851$); on HaCaT cells it was 43.27 (IQR 19.92–78.92) and 44.19 (IQR 19.33–87.31) respectively ($P = 0.814$). Isolates with SI > 1 numbered 69/71 (97.2%) for *K. pneumoniae* and 70/74 (94.6%) for *P. aeruginosa* on both cell lines. The minority of combinations with SI ≤ 1 are reported explicitly rather than averaged into the group summary, since they define the boundary of the window.

Table 5: Selectivity Index and Therapeutic Safety Window

Metric	K. pneumoniae	P. aeruginosa	P value
Effective ZnO concentration in combination (µg/mL)	15.12 (7.78–27.69)	13.61 (7.58–27.00)	0.777
Ampicillin concentration in combination (µg/mL)	32.00 (16.00–64.00)	32.00 (16.00–64.00)	0.223
Ampicillin DRI	8.00 (4.00–16.00)	8.00 (4.00–8.00)	0.549
SI on WI-38	38.57 (21.37–68.78)	41.52 (19.60–75.62)	0.851
SI on HaCaT	43.27 (19.92–78.92)	44.19 (19.33–87.31)	0.814
Isolates with SI > 1 on WI-38	69/71 (97.2%)	70/74 (94.6%)	—
Isolates with SI > 1 on HaCaT	69/71 (97.2%)	70/74 (94.6%)	—

The selectivity analysis showed that the concentrations of the bacterial combination were in general not very close to the cytotoxic midpoint, as determined in healthy cell lines. The median selectivity indices of *K. pneumoniae* combinations were 38.57 and 43.27 in WI-38 and HaCaT cells, respectively, while for *P. aeruginosa* combinations they were 41.52 and 44.19, respectively. No significant difference was found in either selectivity index, effective ZnO concentration, ampicillin concentration in the combination or dose-reduction index among species. For all healthy cell lines, 97.2% of the combinations of *K. pneumoniae* and 94.6% of the combinations of *P. aeruginosa* had an SI > 1. These data suggest the existence of an in-vitro selectivity window for most of the combinations tested, and the small number of combinations having SI ≤ 1 should be kept in the foreground, rather than being averaged together and covered over.


Figure 5: Selectivity Index and Safety Window

The selectivity analysis showed that in general, the bacterial combination concentrations were at levels much lower than the cytotoxic midpoint of the healthy cell lines. The median selectivity indices for *K. pneumoniae* combinations were 38.57 and 43.27 on WI-38 and HaCaT cells, respectively and 41.52 and 44.19 on WI-38 and HaCaT cells for *P. aeruginosa* combinations. No significant species difference was observed for either of the selectivity indices, effective ZnO concentration or ampicillin concentration in the combination and dose-reduction index. For each healthy cell line, 97.2% (94.6%) of *K. pneumoniae* (*P. aeruginosa*) combinations had an SI > 1. These data would confirm the existence of an in-vitro selectivity window for most combinations tested and would highlight that a few combinations (SI ≤ 1) should not be hidden from the view but rather be clearly visible.

3.6 Phenotypic, transcriptional and computational evidence converged on an efflux/permeability mechanism

Efflux-related phenotypes did not differ between species. Median EtBr retention was 1.94-fold (IQR 1.82–2.09) in *K. pneumoniae* and 1.96-fold (IQR 1.81–2.13) in *P. aeruginosa* ($P = 0.765$), and median membrane permeability was 1.64-fold (IQR 1.51–1.73) and 1.59-fold (IQR 1.50–1.73) respectively ($P = 0.700$). Pump-gene expression was below unity for all four targets: *acrA* 0.69 (0.60–0.75), *acrB* 0.66 (0.61–0.72), *mexA* 0.68 (0.60–0.74) and *mexB* 0.67 (0.60–0.74), with significant treatment-related down-regulation of all targets on the raw ΔC_t scale ($P < 0.001$ for each gene). Docking scores clustered between –6.3 and –6.4 kcal/mol: *acrA* –6.44 (–6.78 to –6.17), *mexA* –6.43 (–6.71 to –6.15), *acrB* –6.30 (–6.56 to –6.04) and *mexB* –6.34 (–6.65 to –6.16). Increased EtBr retention, increased membrane permeability, reduced pump-gene transcription and favourable predicted binding energies therefore point in the same direction.

Cellular injury markers were correspondingly low and species-independent. Median LDH release was 10.20% (IQR 8.30–12.65) for *K. pneumoniae* and 10.30% (IQR 8.72–12.57) for *P. aeruginosa* ($P = 0.752$; $r = -0.031$); median apoptosis was 6.90% (IQR 5.90–8.20) and 7.40% (IQR 5.50–8.78) ($P = 0.972$; $r = 0.004$); median necrosis was 3.30% (IQR 2.70–3.95) and 3.40% (IQR 3.02–4.00) ($P = 0.347$; $r = -0.091$); and MMP was maintained at 89.40% (IQR 85.65–91.50) and 87.90% (IQR 85.53–91.57) of control ($P = 0.570$; $r = 0.055$). All effect sizes were small, and no species-dependent difference in host-cell toxicity was detected under combination conditions.

Table 6: Efflux Pump Activity, Membrane Permeability and Gene Expression

Domain	Endpoint	<i>K. pneumoniae</i> / <i>acr</i>	<i>P. aeruginosa</i> / <i>mex</i>	P value
Phenotypic	EtBr retention (fold)	1.94 (1.82–2.09)	1.96 (1.81–2.13)	0.765
Phenotypic	Membrane permeability (fold)	1.64 (1.51–1.73)	1.59 (1.50–1.73)	0.700
qPCR	<i>acrA</i> / <i>mexA</i> fold change	0.69 (0.60–0.75)	0.68 (0.60–0.74)	<0.001 within each gene
Docking	<i>acrA</i> / <i>mexA</i> score (kcal/mol)	–6.44 (–6.78–6.17)	–6.43 (–6.71–6.15)	—
qPCR	<i>acrB</i> / <i>mexB</i> fold change	0.66 (0.61–0.72)	0.67 (0.60–0.74)	<0.001 within each gene
Docking	<i>acrB</i> / <i>mexB</i> score (kcal/mol)	–6.30 (–6.56–6.04)	–6.34 (–6.65–6.16)	—

The efflux-related phenotypes and molecular measurements did not differ between the two species. The medians of the retention of EtBr and the permeability of the membrane were of the order of 1.94–1.96 and 1.59–1.64, respectively, and were not significantly different among the various species. The median pump-gene expression was <1 for the following four targets: *acrA* (0.69), *acrB* (0.66), *mexA* (0.68) and *mexB* (0.67) at the level of transcription. The raw qPCR ΔC_t values showed significant treatment-related down-regulation of all targets (all $P < 0.001$). The docking scores for the four target molecules associated with the pump were clustered around the average score of –6.3 to –6.4 kcal/mol. The internal consistency of the phenotypic, molecular and computational evidence for an efflux/permeability component in the combination response is supported by the increased EtBr retention, increased membrane permeability, and reduced pump-gene expression along with the favorable docking scores.

Table 7: Cellular Mechanisms of Cytotoxicity

Endpoint	<i>K. pneumoniae</i> median (IQR)	<i>P. aeruginosa</i> median (IQR)	P value	Rank-biserial r
LDH release (%)	10.20 (8.30–12.65)	10.30 (8.72–12.57)	0.752	–0.031
Apoptosis (%)	6.90 (5.90–8.20)	7.40 (5.50–8.78)	0.972	0.004
Necrosis (%)	3.30 (2.70–3.95)	3.40 (3.02–4.00)	0.347	–0.091
MMP (% of control)	89.40 (85.65–91.50)	87.90 (85.53–91.57)	0.570	0.055

Both species-linked combination groups had similar cellular toxicity markers. Median LDH release was 10.2% and 10.3% for the combinations of *K. pneumoniae* and *P. aeruginosa*, respectively, while apoptosis was 6.9% and 7.4%, respectively, and necrosis 3.3% and 3.4%, and mitochondrial membrane potential was close to 88–89% of the control. There were no significant differences among the endpoints, and all the rank biserial effect sizes were small. In

consequence, the measured cellular injury profile did not reveal any species-dependent difference in host-cell toxicity as tested for the combination conditions.

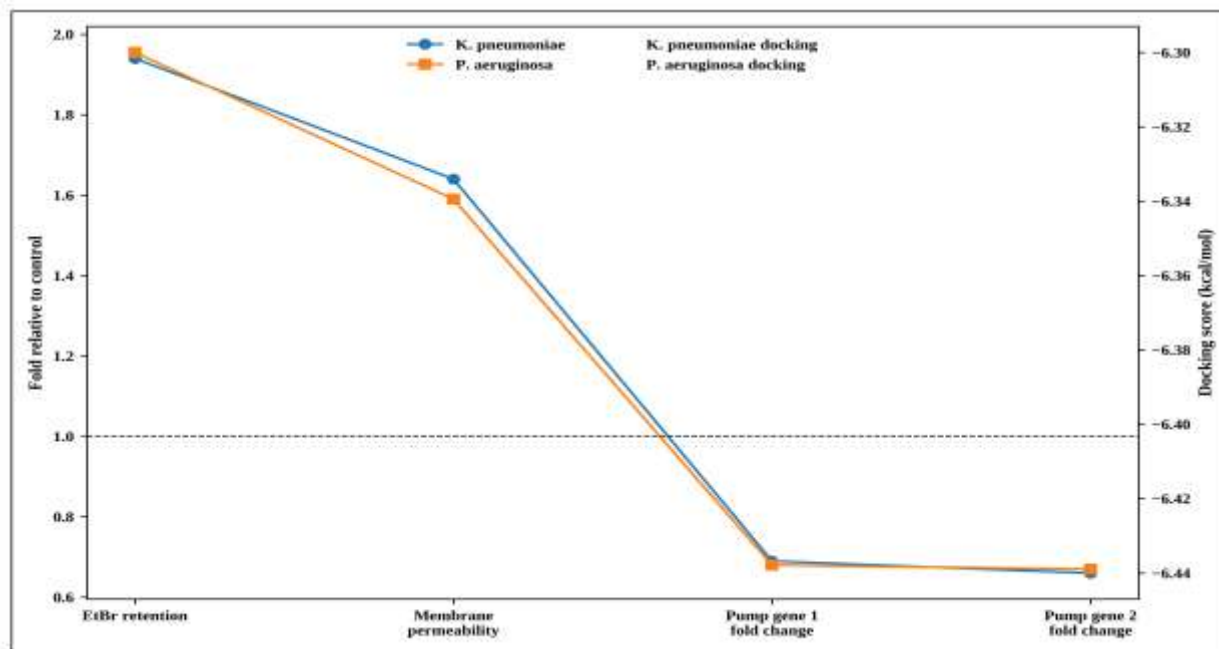


Figure 6: Efflux Pump and Molecular Response Profile

The efflux related phenotypes and molecular measurements were comparable between the two species. There were no significant difference between the species with regard to median EtBr retention, which was around 1.94–1.96 fold and membrane permeability, which was around 1.59–1.64 fold. Median pump-gene expression at the transcriptional level was found to be below unity for all four targets: *acrA* (0.69), *acrB* (0.66), *mexA* (0.68) and *mexB* (0.67). The raw qPCR ΔC_t values were analysed and showed significant down-regulation in all treatments (all $P < 0.001$). The docking scores of the four targets associated with the pump were roughly similar in the center of the range from -6.3 to -6.4 kcal/mol. All the above three factors give internally consistent phenotypic, molecular and computational information that the combination response results from an efflux/permeability component.

3.7 Low-dose exposure stimulated bacterial community phenotypes

A clear biphasic dose–response was observed for all three low-dose endpoints, in both species. Biofilm biomass rose from $102.09 \pm 2.06\%$ and $102.76 \pm 2.31\%$ of control at 0.2% of the ZnO MIC, through $103.82 \pm 3.82\%$ and $104.71 \pm 4.12\%$ at 0.4%, to $107.06 \pm 7.10\%$ and $108.69 \pm 7.83\%$ at 0.8%. Metabolic activity rose from $101.53 \pm 1.84\%$ and $101.18 \pm 1.79\%$, through $102.94 \pm 3.18\%$ and $102.22 \pm 3.41\%$, to $105.64 \pm 5.84\%$ and $104.18 \pm 5.95\%$. EPS production rose from $102.59 \pm 2.49\%$ and $102.63 \pm 2.49\%$, through $105.19 \pm 4.67\%$ and $105.21 \pm 4.43\%$, to $109.41 \pm 8.73\%$ and $109.49 \pm 8.23\%$. The dose effect was significant for all three endpoints (Greenhouse–Geisser-corrected $P < 0.001$) with large partial η^2 values: 0.505 for biofilm, 0.410 for metabolic activity and 0.554 for EPS. The species main effect and the Species $\times$ Dose interaction were non-significant for every endpoint (species $P = 0.158$, 0.164 and 0.958; interaction $P = 0.281$, 0.119 and 0.955 for biofilm, metabolic activity and EPS respectively), indicating that the low-dose stimulation was shared rather than species-specific.

Table 8A: Low-Dose Hormesis Analysis

Outcome	Dose (% MIC)	K. pneumoniae mean $\pm$ SD	P. aeruginosa mean $\pm$ SD	Dose P	Species P	Interaction P	Partial η^2 (dose)
Biofilm	0.2	102.09 $\pm$ 2.06	102.76 $\pm$ 2.31	<0.001	0.158	0.281	0.505
Biofilm	0.4	103.82 $\pm$ 3.82	104.71 $\pm$ 4.12				
Biofilm	0.8	107.06 $\pm$ 7.10	108.69 $\pm$ 7.83				
Metabolic activity	0.2	101.53 $\pm$ 1.84	101.18 $\pm$ 1.79	<0.001	0.164	0.119	0.410
Metabolic activity	0.4	102.94 $\pm$ 3.18	102.22 $\pm$ 3.41				
Metabolic activity	0.8	105.64 $\pm$ 5.84	104.18 $\pm$ 5.95				
EPS	0.2	102.59 $\pm$ 2.49	102.63 $\pm$ 2.49	<0.001	0.958	0.955	0.554
EPS	0.4	105.19 $\pm$ 4.67	105.21 $\pm$ 4.43				
EPS	0.8	109.41 $\pm$ 8.73	109.49 $\pm$ 8.23				

A clear hormetic dose-response curve was observed for the low-dose series. Biofilm biomass, metabolic activity and EPS for 0.2%, 0.4% and 0.8% of the ZnO MIC were 102%, 101% and 103% of control, while increases were 107–109%, 104–106% and 109% of control, respectively. The dose effect was significant for all three endpoints (all Greenhouse–Geisser-corrected $P < 0.001$), with large partial η^2 values of 0.505 for biofilm, 0.410 for metabolic activity and 0.554 for EPS. This low-dose stimulation was observed in both species (no Species $\times$ Dose interaction) and was not significant. The integrated correlations also revealed a closer relationship between the efflux/permeability response and the lower FICI values with higher EtBr retention ($\rho = -0.627$, FDR $q < 0.001$), higher membrane permeability ($\rho = -0.528$, $q < 0.001$) and lower mean pump-gene expression ($\rho = 0.685$, $q < 0.001$). Additionally, with increasing dose reduction, pump expression was reduced ($\rho = -0.286$, $q < 0.001$). In contrast, the selectivity indexes did not significantly correlate with LDH release or apoptosis after FDR correction, distinguishing the bacterial synergy signal from the cells' injury markers, indicating healthy cells. Thereby in contrast, the selectivity indexes did not show significant correlation with LDH release or apoptosis after FDR correction, separating the bacterial synergy signal from the cells' injury markers and indicating healthy cells.

Table 8B: Integrated Cross-Domain Associations

Variable 1	Variable 2	Spearman ρ	P value	FDR q
FICI	DRI	-0.253	0.002	0.003
FICI	EtBr retention	-0.627	<0.001	<0.001
FICI	Membrane permeability	-0.528	<0.001	<0.001
FICI	Mean pump-gene fold change	0.685	<0.001	<0.001
DRI	Mean pump-gene fold change	-0.286	<0.001	<0.001
Mean docking score	Mean pump-gene fold change	0.586	<0.001	<0.001
SI WI-38	LDH release	0.043	0.611	0.667
SI HaCaT	Apoptosis	0.059	0.479	0.602
EtBr retention	Mean pump-gene fold change	-0.497	<0.001	<0.001
Membrane permeability	Mean pump-gene fold change	-0.479	<0.001	<0.001
FICI	Low-dose biofilm	-0.056	0.501	0.602
FICI	Low-dose EPS	0.021	0.800	0.800

There was a clear hormetic trend in the low dose series. For the 0.2%, 0.4% and 0.8% of the ZnO MIC, the biofilm biomass was approximately 102%–107–109% of control, metabolic activity was approximately 101%–104–106% of control and EPS was approximately 103%–109–106% of control. All three endpoints showed a dose effect (all Greenhouse–Geisser-corrected $P < 0.001$) and large partial η^2 values for biofilm (0.505), metabolic activity (0.410) and EPS (0.554). There was no significant species main effect and no significant Species $\times$ Dose interaction, suggesting that this stimulation at the low dose level was similar for both species. The integrated correlations further correlated efflux/permeability response with higher lower FICI values ($\rho = -0.627$, FDR $q < 0.001$), higher membrane permeability ($\rho = -0.528$, $q < 0.001$) and lower mean pump-gene expression ($\rho = 0.685$, $q < 0.001$). A reduction in the dose was also correlated with lower expression of the pump genes ($\rho = -0.286$, $q < 0.001$). In contrast, after FDR correction, selectivity indices were not significantly related with LDH release or apoptosis, allowing the separation of the bacterial synergy signal from the measured markers of injury of healthy cells.

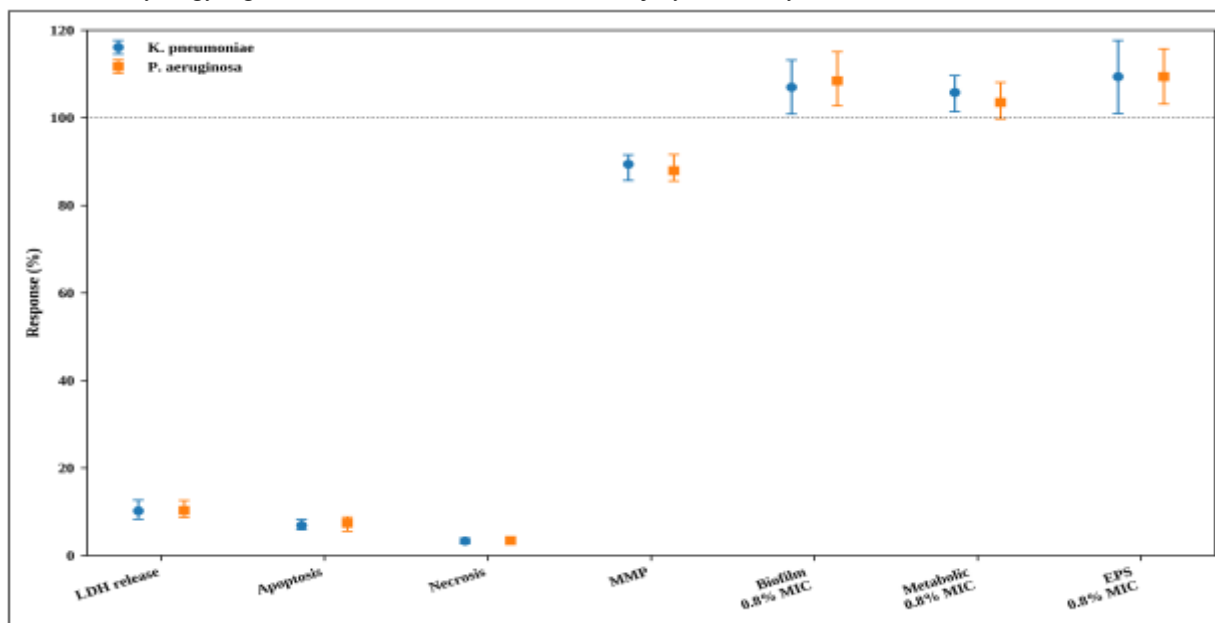


Figure 7: Cellular and Low-Dose Biological Response Profile

The single response-profile plot compares the injury indicators in the host cells with the major low-dosage bacterial responses in a common percentage format. In both species linked combination groups, LDH release, apoptosis and necrosis were not significantly elevated and the mitochondrial membrane potential was maintained at approximately 88–89% of control. At the low dose endpoint (0.8% of the ZnO MIC), bacterial endpoints differed from the control in opposite ways, with an increase in biofilm biomass and metabolic activity and EPS production for both organisms. The similarity of the two species for all seven endpoints is consistent with the formal analyses that did not find a meaningful species effect for the cellular toxicity endpoints and a lack of Species $\times$ Dose interaction for the low-dose responses. The plot thus emphasizes the difference between the limited (tested) combination conditions and the measurable stimulation of the bacteria in the very-low-dose range.

3.8 Cross-domain correlations separated the antibacterial signal from the cytotoxicity signal

Integration of the prespecified correlation set clarified the structure of the dataset. Lower FICI values, that is stronger synergy, were associated with higher EtBr retention ($\rho = -0.627$; FDR $q < 0.001$), higher membrane permeability ($\rho = -0.528$; $q < 0.001$) and lower mean pump-gene expression ($\rho = 0.685$; $q < 0.001$). Greater ampicillin dose reduction correlated with lower pump-gene expression ($\rho = -0.286$; $q < 0.001$), and the mean docking score correlated with mean pump-gene expression ($\rho = 0.586$; $q < 0.001$). FICI and DRI were themselves inversely related ($\rho = -0.253$; $P = 0.002$; $q = 0.003$). By contrast, neither selectivity index correlated significantly with LDH release ($\rho = 0.043$; $P =$

0.611; $q = 0.667$) or with apoptosis ($\rho = 0.059$; $P = 0.479$; $q = 0.602$), and FICI did not correlate with either low-dose biofilm ($\rho = -0.056$; $P = 0.501$; $q = 0.602$) or low-dose EPS ($\rho = 0.021$; $P = 0.800$; $q = 0.800$). The statistical separation of the synergy axis from the host-cell injury axis, and its disconnection from the hormetic axis, is the central structural finding of this analysis.

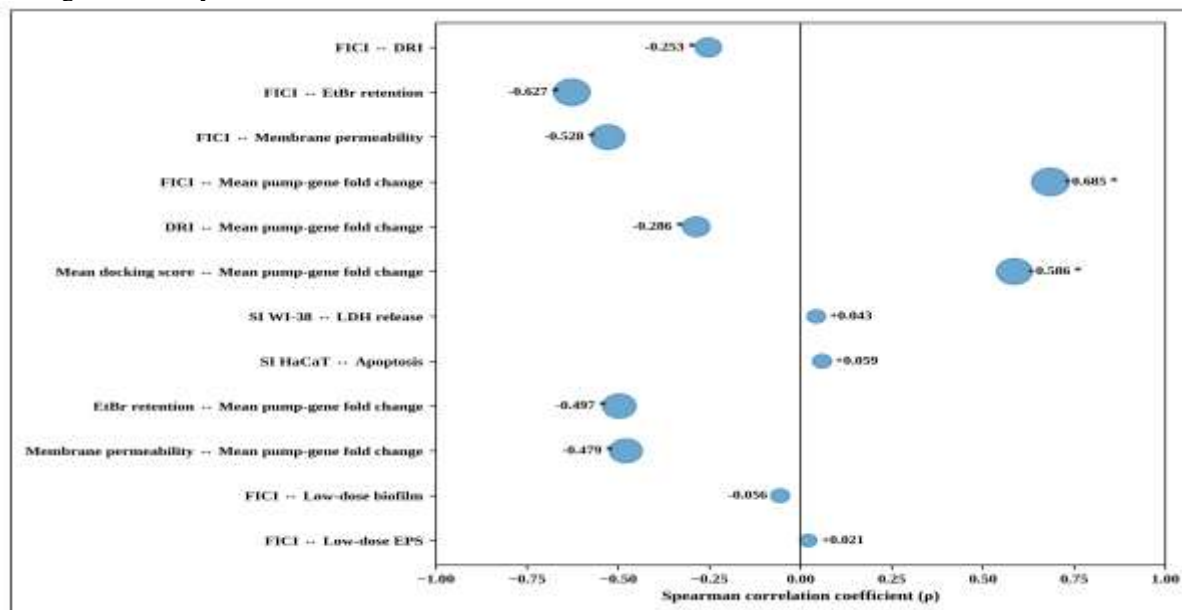


Figure 8: Integrated Cross-Domain Correlation Bubble Plot

The correlation bubble plot is a summary of the direction and strength of the pre-specified cross-domain relationships, but without the heatmap. Lower FICI values were associated with higher EtBr retention ($\rho = -0.627$, FDR $q < 0.001$), higher membrane permeability ($\rho = -0.528$, $q < 0.001$) and lower mean pump-gene expression ($\rho = 0.685$, $q < 0.001$). There were also associations between greater dose reduction of ampicillin and lower pump-gene expression ($\rho = -0.286$, $q < 0.001$); and between mean docking score and mean pump-gene expression ($\rho = 0.586$, $q < 0.001$). On the other hand, the selectivity indices were not significantly related to LDH release or apoptosis at FDR $p < 0.05$ and the FICI index was not significantly correlated with the low-dose biofilm nor the EPS measures. The size of the bubbles indicates the strength of the correlation, and the x-axis indicates the sign and strength of the Spearman correlation.

4. Discussion

4.1 Principal findings

Three results define this study. First, the combination of ZnO-NPs with ampicillin was synergistic against 133 of 145 MDR clinical isolates (91.7%), with median FICI values of 0.391 and 0.359 and a median eight-fold reduction in the effective ampicillin concentration, and with no evidence that either species systematically drove the effect. Second, the synergistic concentrations sat far below the cytotoxic midpoint of both healthy cell lines, giving median selectivity indices of 38.57–43.27 for *K. pneumoniae* and 41.52–44.19 for *P. aeruginosa*, with $SI > 1$ in 97.2% and 94.6% of combinations. Third, and tempering the first two, exposure at 0.2–0.8% of the ZnO MIC increased biofilm biomass, metabolic activity and EPS by up to approximately 109% of control in both species. A narrow but real therapeutic corridor therefore exists, bounded above by cytotoxicity and below by hormesis.

4.2 The synergy is mechanistically coherent

The convergence of phenotypic, transcriptional and computational endpoints is the strongest argument that the observed synergy is not an artefact of the checkerboard format. EtBr retention rose approximately two-fold while membrane permeability rose approximately 1.6-fold, indicating both impaired extrusion and a more permeable

envelope. Pump-gene transcription fell to 0.66–0.69 of control for *acrA*, *acrB*, *mexA* and *mexB*, and docking scores for the same four proteins clustered tightly between –6.3 and –6.4 kcal/mol. Crucially, the correlation structure is internally consistent: stronger synergy (lower FICI) accompanied higher EtBr retention, higher permeability and lower pump-gene expression, and greater ampicillin dose reduction accompanied lower pump-gene expression. This is the pattern predicted when a nanoparticle acts as an efflux-pump inhibitor and envelope disruptor, allowing an otherwise expelled β -lactam to accumulate at its target. It also matches independent evidence that polyol-coated metal oxide nanoparticles at sub-inhibitory concentrations promote EtBr accumulation in MDR *P. aeruginosa* and act synergistically with meropenem and ciprofloxacin (Eleftheriadou *et al.*, 2021), and that nanoparticles can circumvent efflux either by intracellular delivery or by pump blockade (Modi *et al.*, 2023). Because the RND transporters AcrAB-TolC and MexAB-OprM are principal determinants of multidrug resistance in Enterobacteriaceae and *P. aeruginosa* (Alenazy, 2022), and because efflux inhibition also attenuates biofilm adhesion and maturation (Musioł, 2023), the mechanism identified here is of general rather than species-specific significance.

Two cautions belong with this interpretation. First, the docking energies reported here are modest in absolute terms: binding modes of RND pump substrates and inhibitors obtained with a flexible binding site differ substantially from rigid-docking estimates, and the AcrB and AdeB pockets accommodate chemically diverse ligands through different interaction patterns (Ornik-Cha *et al.*, 2021). Scores between –6.3 and –6.4 kcal/mol are therefore best read as evidence of plausible interaction, not as proof of inhibition. Second, because PA β N itself permeabilises the outer membrane at higher doses while retaining efflux-inhibitory activity at lower doses (Al-Marzooq *et al.*, 2023), the efflux and permeability components of the mechanism cannot be fully separated by phenotype alone; the convergence of four independent readouts in the same direction is what gives the interpretation weight.

The envelope-directed component is equally consistent with the ZnO literature. ZnO-NPs act through ROS generation with lipid peroxidation and membrane rupture, and through Zn²⁺ release with thiol-group oxidation and inhibition of glycolytic enzymes (Gomaa, 2022), with surface-area-dependent ROS generation (Hadi *et al.*, 2025). Independent work has shown that ZnO-NPs are active against β -lactamase-producing Gram-negative pathogens, with MICs of 0.04–0.08 mg/mL and MBCs of 0.12–0.24 mg/mL, and that this activity involves membrane leakage, DNA damage and apparent β -lactamase inhibition (Krishnamoorthy *et al.*, 2022). A membrane-permeabilising and efflux-inhibiting nanoparticle combined with a cell-wall-directed β -lactam attacks two independent barriers at once, which is a plausible explanation for FICI values as low as 0.36–0.39 rather than the additivity that simple co-administration would predict.

4.3 The selectivity window is real but should be read quantitatively

The cytotoxicity data are unambiguous in direction and large in magnitude. Combination IC₅₀ values of 542.8–613.0 μ g/mL were two orders of magnitude above the median effective ZnO concentration of 13.61–15.12 μ g/mL in the combination. Ampicillin alone did not reach IC₅₀ within 0–1000 μ g/mL, so the cytotoxic signal is attributable to the nanoparticle component rather than to the antibiotic. Hill slopes of 1.34–1.42 and R² at or above 0.9999 indicate that the fitted curves were not model artefacts, and the low and species-independent injury markers — LDH release of 10.2–10.3%, apoptosis of 6.9–7.4%, necrosis of 3.3–3.4% and MMP maintained at 87.9–89.4% of control — indicate that at the effective combination concentrations the host-cell response remained sub-lethal across several independent mechanisms of injury.

This picture should nevertheless be read in the context of the nanoparticle literature, which reports wide variation in ZnO cytotoxicity according to particle size, formulation and cell type. Micrometric ZnO has been shown to be more cytotoxic than 100 nm and 50 nm particles, with keratinocytes more affected than lung epithelial cells (Mitjans *et al.*, 2023); ZnO-NPs have been reported to cause approximately 50% HaCaT death at combination exposures involving 10–12.5 μ g/mL (Wang *et al.*, 2024); and exogenous zinc concentrations of 21–31 μ g/mL have been associated with keratinocyte cytotoxicity (Khabir *et al.*, 2021). Conversely, green-synthesised ZnO-NPs have been reported with an IC₅₀ of 568.59 μ g/mL against WI-38 fibroblasts (Naiel *et al.*, 2022), a value very close to the 558.8 μ g/mL obtained here for *K. pneumoniae*-associated combinations on the same cell line. The most economical interpretation is that particle physicochemical characteristics, which can differ substantially between synthesis routes, dominate the cytotoxicity outcome, and that this is why selectivity must be measured rather than assumed for each preparation (Chumachenko *et al.*, 2023). Reporting the selectivity index alongside MIC or FICI is therefore not optional: the index

formalises the requirement that the pathogen be killed before the host cell is damaged (Ye *et al.*, 2023), and the related therapeutic index should exceed unity for an agent to be considered usable (Al-Hakkani *et al.*, 2022).

4.4 Sub-inhibitory exposure is the practical constraint

The hormesis data are the most operationally important negative finding. At 0.2% of the ZnO MIC, biofilm biomass, metabolic activity and EPS were already at 101–103% of control; by 0.8% of the MIC, biofilm had risen to 107.06% (*K. pneumoniae*) and 108.69% (*P. aeruginosa*), metabolic activity to 105.64% and 104.18%, and EPS to 109.41% and 109.49%, with large dose effects (partial η^2 0.410–0.554) and no Species $\times$ Dose interaction. This reproduces, in *K. pneumoniae* as well as *P. aeruginosa*, the biphasic response previously described for low-dose ZnO-NPs in *P. aeruginosa*, where growth and biofilm formation were stimulated at low concentrations (Al-Momani *et al.*, 2024), and for ZnO-NPs inducing biofilm formation in *K. pneumoniae* and *S. aureus* at sub-inhibitory concentrations (Sreekanth *et al.*, 2023). Comparable stimulation has been reported for the lowest dose of copper-containing nanoparticles, which enhanced biofilm formation of *S. aureus*, *P. aeruginosa* and *C. albicans* by $11.3 \pm 1.7\%$, $11.7 \pm 1.5\%$ and $14.2 \pm 0.9\%$ (Almahdy *et al.*, 2024). More broadly, community-associated functions such as biofilm formation and quorum sensing are affected in 50% of nanomaterial studies conducted at sub-inhibitory concentrations, compared with only 10–20% of studies at inhibitory concentrations (Mortimer *et al.*, 2021).

The therapeutic implication is that for this combination the relevant parameter is not the minimum effective concentration but the minimum concentration that remains inhibitory in the target compartment. Because topical and wound applications inevitably generate concentration gradients, sub-MIC regions at the periphery of an applied dose are a realistic clinical scenario; the fact that FICI did not correlate with low-dose biofilm ($\rho = -0.056$; $P = 0.501$) or EPS ($\rho = 0.021$; $P = 0.800$) indicates that synergy and hormesis behave as independent axes, so a strongly synergistic pair is not automatically protected against low-dose stimulation.

4.5 Comparison with the wider ZnO–antibiotic literature

The direction of the present result agrees with earlier work combining ZnO-NPs with β -lactams and other antibiotics. ZnO-NPs with piperacillin–tazobactam enhanced activity against MDR *E. coli* and *P. aeruginosa* (Yu *et al.*, 2024); ZnO-NPs with ciprofloxacin or ceftazidime increased activity against resistant *A. baumannii* through membrane damage and intracellular accumulation (Abdelghani *et al.*, 2022); FICI-documented synergy has been reported with colistin, ciprofloxacin and meropenem (Irede *et al.*, 2024); and meropenem-loaded ZnO nanoflakes achieved an average inhibition zone of 15.2 mm against metallo- β -lactamase-producing clinical isolates (Singh *et al.*, 2023). Direct conjugation of ZnO-NPs to clinically approved antibiotics, ampicillin included, has also enhanced antibacterial efficacy while producing minimal cytotoxicity against human cell lines (Akbar *et al.*, 2021). What the present study adds is not the existence of synergy — that is now well documented — but its quantification against a controlled, species-matched, multidrug-resistant baseline in parallel with a formal selectivity analysis and a mechanistic correlation structure, applied to 145 clinical isolates rather than to reference strains. The species-matched baseline is a methodological point of some weight: because the two organisms entered the interaction experiments with statistically indistinguishable MIC and MBC distributions, the similarity of their synergy responses is a genuine finding rather than a consequence of unequal starting susceptibility.

4.6 Limitations

Design and scope. The work is an in vitro study, and neither intracellular bacterial survival nor biofilm-embedded killing was evaluated, so the observed growth inhibition of planktonic cells cannot be extrapolated directly to biofilm-associated infection. The 145 isolates came from a single hospital in Anbar Province, which limits generalisability to other Iraqi or non-Iraqi settings even though it strengthens relevance to the local resistance epidemiology (Al-Fahad *et al.*, 2024). The combination was characterised with a fixed 1:1 design in the growth experiments, so the ratio dependence of synergy was not explored, and only two human cell lines were used, both immortalised; primary respiratory epithelial or macrophage models would strengthen the cytotoxicity assessment. The ampicillin-only IC₅₀ was not reached within the tested range, so no direct cytotoxic reference point for the antibiotic component is available.

Material characterisation. The ZnO-NP preparation was not characterised in-house by X-ray diffraction, electron microscopy, dynamic light scattering or zeta potential, so particle size, morphology, agglomeration state in the assay medium and surface charge are not documented. Because particle size strongly influences ZnO cytotoxicity (Mitjans *et al.*, 2023), the absolute IC₅₀ values reported here should be regarded as specific to the material lot used, and batch-to-batch reproducibility cannot be verified from these data. This limitation applies to the absolute concentrations rather than to the internal comparisons: because the same stock, dispersion protocol and medium were used throughout, the relative comparisons between species, between treatment conditions and between concentrations remain internally valid. Nevertheless, any attempt to reproduce these values with a different ZnO preparation should expect the antibacterial and cytotoxic thresholds to shift, and physicochemical characterisation of the material is the first requirement for any subsequent formulation work.

Mechanistic evidence. The mechanistic evidence is in part associative. Docking scores of −6.3 to −6.4 kcal/mol indicate plausible binding rather than confirmed inhibition, and were not validated by efflux-pump knockout or by a protonophore control; rigid-receptor docking is known to overstate the stability of inhibitor binding relative to flexible-receptor simulation (Ornik-Cha *et al.*, 2021). Gene expression was measured at a single time point, and the efflux and outer-membrane permeabilisation components of the mechanism cannot be fully separated by phenotype, since efflux inhibitors of the PAβN class themselves permeabilise the outer membrane at higher doses (Al-Marzooq *et al.*, 2023).

Safety endpoints. The cytotoxicity endpoints measured at the synergistic concentrations were sub-lethal, but longer exposures, repeated dosing and evaluation of zinc accumulation and clearance were not examined, and safety in vivo is outside the scope of this work. No time-kill kinetics were performed, so the combination is characterised as inhibitory rather than as bactericidal on a kinetic basis; time-kill data would be required to establish whether the eight-fold dose reduction translates into sustained killing, and comparative in vitro work on nanomaterial–antibiotic combinations identifies time-kill as one of the more informative endpoints for this purpose.

4.7 Implications for development

If this combination is to be taken forward, the data argue for three priorities. First, formulation: because cytotoxicity in ZnO systems is strongly dependent on particle size (Mitjans *et al.*, 2023), the immediate engineering objective is to widen the selectivity window by shifting the cytotoxic threshold upward rather than by increasing antibacterial potency. Second, dosing: the hormesis threshold must be avoided, which favours formulations that release ZnO or Zn²⁺ in a controlled manner above 0.8% of the MIC at the site of infection, and makes sustained-release or surface-immobilised systems more attractive than free nanoparticle suspensions. Third, translation: nanoparticle–antibiotic combinations face unresolved obstacles in reproducibility, scalability, characterisation and regulatory classification, and the absence of dedicated regulatory pathways for such materials remains a recognised barrier alongside cost of production and excretion concerns (Doolan *et al.*, 2022) (Huang *et al.*, 2024) (Mamun *et al.*, 2021). Early engagement with these questions — full physicochemical characterisation, standardised batch-to-batch reproducibility, and explicit safety documentation — materially accelerates the path to clinical evaluation (Muir *et al.*, 2025) (Blackman *et al.*, 2022).

5. Conclusions

ZnO nanoparticle–ampicillin combinations were synergistic against 133 of 145 multidrug-resistant clinical isolates (91.7%), comprising 93.0% of *K. pneumoniae* and 90.5% of *P. aeruginosa* isolates, with median FICI values of 0.391 and 0.359 and a median eight-fold reduction in the effective ampicillin concentration. The synergy was accompanied by a favourable in vitro selectivity window: IC₅₀ values of 542.8–613.0 µg/mL against WI-38 and HaCaT cells, median selectivity indices of 38.57–44.19, and SI > 1 in 97.2% and 94.6% of combinations. Phenotypic, transcriptional and computational evidence converged on an efflux/permeability mechanism, with lower FICI values associated with higher EtBr retention ($\rho = -0.627$), higher membrane permeability ($\rho = -0.528$) and lower pump-gene expression ($\rho = 0.685$). Host-cell injury markers remained low, with LDH release of 10.2–10.3%, apoptosis of 6.9–7.4%, necrosis of 3.3–3.4% and mitochondrial membrane potential at 87.9–89.4% of control. However, exposure at 0.2–0.8% of the ZnO MIC stimulated biofilm biomass, metabolic activity and EPS by up to approximately 109% of control in both species, establishing a lower boundary to the useful dose range that is statistically independent of the synergy axis.

The combination therefore merits further development as a dose-controlled, mechanism-informed strategy rather than as a low-dose or uncontrolled-release preparation.

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