

ORIGINAL RESEARCH ARTICLE

Epicatechin gallate disrupts methicillin resistant *Staphylococcus aureus* cell envelope integrity and enhances antibiotic activity

Sze-Tieng Ang¹, Matthew James Cheesman², Tak Kim¹, and Ian Edwin Cock^{1*}

¹School of Environmental and Science, Griffith University, Nathan Campus, Brisbane, Queensland, Australia

²School of Pharmacy and Medical Sciences, Griffith University, Gold Coast Campus, Southport, Queensland, Australia

Abstract

The increasing prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) highlights the need for alternative therapeutic strategies. Natural product-derived resistance-modifying compounds are gaining attention as antibiotic adjuvants to restore drug activity. Epicatechin gallate (EPCG) has antibacterial and anti-virulence properties. This study investigates the antibacterial activity of EPCG and its potential to enhance the efficacy of selected antibiotics against *S. aureus* and MRSA. Standard broth microdilution assays were used to determine the minimum inhibitory concentration (MIC) of EPCG against *S. aureus* and MRSA. Synergistic interactions with selected antibiotics were evaluated using checkerboard and isobologram assays. Furthermore, cytotoxicity was evaluated using *Artemia franciscana* nauplii (brine shrimp) lethality and human dermal fibroblast (HDF) assays. The impact of EPCG, cefotaxime, and their combinations on cells and biofilms was visualised through confocal and cryo-electron microscopy. EPCG demonstrated strong antibacterial activity (MIC = 208 µg/mL against *S. aureus* and 188 µg/mL against MRSA). Isobologram assays also revealed strong synergy for the EPCG–cefotaxime combination (Σ fractional inhibitory concentration = 0.15 for MRSA; 0.25 for *S. aureus*). EPCG exhibited moderate toxicity toward HDF cells (LC₅₀ = 100 µg/mL), whereas the optimised cefotaxime: EPCG (90:10 [v/v]) combination was non-toxic across all assays. Additionally, confocal microscopy revealed reduced bacterial aggregation and diminished biofilm-associated fluorescence following EPCG treatment. Cryo-electron microscopy revealed pronounced ultrastructural disruptions in MRSA cells treated with the combination. In conclusion, EPCG enhances the antibacterial activity of antibiotics such as cefotaxime against *S. aureus* and MRSA. Its synergistic action, lower cytotoxicity at optimal ratios, and confirmed damage to bacterial cells highlight EPCG's potential as a natural antibiotic adjuvant.

Keywords: Methicillin-resistant *Staphylococcus aureus*; Epicatechin gallate; Minimum inhibitory concentration; Synergy; Confocal microscopy; Cryo-electron microscopy

***Corresponding author:**
Ian Cock
(i.cock@griffith.edu.au)

Citation: Ang S-T, Cheesman MJ, Kim T, Cock IE. Epicatechin gallate disrupts methicillin resistant *Staphylococcus aureus* cell envelope integrity and enhances antibiotic activity. *Innov Med Omics*. 2026;3(3):026160018.
doi: 10.36922/IMO026160018

Received: April 16, 2026

Revised: June 8, 2026

Accepted: June 9, 2026

Published online: June 23, 2026

Copyright: © 2026 Author(s). This is an Open-Access article distributed under the terms of the Creative Commons Attribution License, permitting distribution, and reproduction in any medium, provided the original work is properly cited.

Publisher's Note: AccScience Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Antimicrobial resistance is one of the leading global health threats, with bacterial species such as methicillin-resistant *Staphylococcus aureus* (MRSA) causing substantial morbidity and mortality in both healthcare and community settings.^{1,2} MRSA is a leading cause of severe bloodstream, respiratory, and device-associated infections worldwide.³ Clinical outcomes remain suboptimal despite the availability of last-line agents. Therapeutic management is increasingly complicated by reduced susceptibility to glycopeptide antibiotics, heteroresistance, biofilm-associated tolerance, and the relatively slow bactericidal activity of vancomycin.^{2,4–8} Notably, treatment failure and infection relapse are frequently reported, even when isolates are categorised as susceptible *in vitro*, highlighting the limitations of laboratory susceptibility testing in predicting clinical outcomes.

To improve therapeutic outcomes, combination antibiotic regimens have been reported as being capable of enhancing bactericidal activity, accelerating the clearance of bacteraemia, and reducing the development of resistance.^{9–12} However, clinical evidence supporting the use of routine combination therapy remains inconclusive, and concerns about toxicity have limited its widespread use. For instance, a randomized clinical trial by Tong *et al.*¹³ showed limited benefits from combining vancomycin or daptomycin with an anti-staphylococcal β -lactam for the treatment of MRSA infections. Whilst combination therapy decreased microbiological relapse, it also resulted in acute kidney injury in more than 37% of cases.¹³ These findings highlight a fundamental therapeutic dilemma: intensifying antibiotic regimens may improve certain microbiological endpoints, yet such strategies often increase adverse events without conferring a clear survival advantage. These limitations underscore the need for alternative strategies that enhance antimicrobial efficacy without substantially increasing toxicity. Recently, an emerging therapeutic paradigm has focused on restoring or potentiating the activity of existing antibiotics through resistance-modifying compounds.

Natural products represent a potential source of resistance-modifying compounds. Among these, catechins and their galloylated derivatives have attracted considerable attention for their ability to disrupt bacterial cell envelope integrity.^{14–16} Epicatechin gallate (EPCG), a galloylated catechin, exhibits intrinsic antibacterial activity and also enhances the efficacy of selected β -lactams against MRSA.^{14,15,17} Proposed mechanisms include disruption of cytoplasmic membrane integrity, alteration of peptidoglycan structure, interference with penicillin-binding proteins (PBPs), and facilitation of antibiotic

penetration. Nevertheless, most previous investigations have relied primarily on fractional inhibitory concentration (FIC) indices to define synergy, with limited use of quantitative interaction modelling or direct ultrastructural validation. Furthermore, the capacity of EPCG to restore the activity of cephalosporins, such as cefotaxime, against MRSA remains insufficiently characterised.

To systematically investigate the resistance-modifying potential of EPCG, antibiotics representing distinct mechanisms of action were selected to capture a broad spectrum of antibacterial targets. Tetracycline, ciprofloxacin, and cefotaxime were chosen as representatives of protein synthesis inhibitors, DNA replication inhibitors, and β -lactam antibiotics, respectively. This selection allowed assessment of whether EPCG-mediated potentiation is dependent on antibiotic class or reflects a more generalised effect on bacterial susceptibility. In particular, the inclusion of cefotaxime provided a stringent, clinically relevant model, as MRSA exhibits intrinsic resistance to third-generation cephalosporins due to the presence of PBP2a.¹⁸

While conventional checkerboard assays are widely used to evaluate antimicrobial interactions, their interpretation is often limited to empirical indices that may not fully capture the complexity of drug interactions. Quantitative approaches such as isobologram modelling offer a more rigorous framework for distinguishing between additive and truly synergistic effects, particularly across varying concentration ratios. Moreover, although previous studies have proposed membrane and cell wall disruption as key mechanisms underlying catechin activity^{14,15}, direct structural evidence supporting these effects remains limited. Advanced imaging techniques, therefore, provide an important complementary approach for elucidating the cellular basis of antimicrobial interactions. Confocal laser scanning microscopy enables the assessment of bacterial viability, membrane integrity, and potential biofilm-associated changes, whereas cryo-electron microscopy preserves near-native cellular architecture and allows high-resolution visualisation of ultrastructural alterations. Integrating these approaches offers a more comprehensive understanding of how EPCG influences bacterial physiology and antibiotic susceptibility.

The present study aimed to (i) determine the antibacterial activity of EPCG against MRSA and *S. aureus*, (ii) evaluate its interaction with tetracycline, ciprofloxacin and cefotaxime using checkerboard assays complemented by isobologram modelling, and (iii) characterise ultrastructural changes induced by EPCG alone and in combination therapy using confocal and cryo-electron microscopy. By integrating pharmacodynamic modelling with structural validation, this work aims to determine

whether EPCG can serve as an antibiotic adjuvant and to provide mechanistic insights that support its potential translational application against MRSA.

2. Materials and methods

2.1. Materials

Mueller–Hinton broth and agar powders (Oxoid Ltd., Australia) were prepared according to the manufacturer's instructions and used as bacterial growth media. Unless stated otherwise, all additional chemicals used in this study were obtained from Merck Life Science Pty. Ltd. (Australia).

Epicatechin gallate was purchased from Merck Life Science Pty. Ltd. (Australia) as analytical reagent-grade powder and dissolved in 1% dimethyl sulfoxide to a stock concentration of 2,000 µg/mL. The solubilised EPCG was subsequently diluted to the test concentrations using sterile deionised water.

2.2. Antibacterial studies

2.2.1. Bacterial cultures

Reference strains of *S. aureus* (ATCC 25923) and MRSA (ATCC 43300) were purchased from the American Type Culture Collection (United States). Individual bacterial strains were streaked onto Mueller–Hinton agar plates containing 2% sodium chloride and incubated at 35 °C for 24 h to obtain pure cultures. A single bacterial colony was then transferred into freshly prepared Mueller–Hinton broth with 2% sodium chloride. The bacterial broth cultures were incubated at 35 °C until the bacteria reached the log phase of growth. To ensure the purity of bacterial cultures, each was individually re-streaked on Mueller–Hinton agar plates.

2.2.2. Liquid microdilution assay

Liquid microdilution assays were used to quantify the antibacterial susceptibility by determining the minimum inhibitory concentration (MIC) of EPCG against the tested bacterial strains, as previously described.^{19–21} A 100 µL volume of EPCG or antibiotic solution (2,000 µg/mL for EPCG or 40 µg/mL for antibiotics) was added to the first row of sterile flat-bottomed 96-well plastic plates, followed by 100 µL of sterile broth to achieve a final volume of 200 µL. All other wells received 100 µL of sterile broth. From the first row, 100 µL was transferred to the next row and mixed thoroughly. This process was repeated down the plate to create a two-fold serial dilution series. After dilution, 100 µL of the bacterial suspension, adjusted to approximately 10⁸ cells/mL (0.5 McFarland standard), was added to each well. The negative control wells contained only broth and bacterial inoculum, whilst the sterility

control wells contained only broth. The plates were incubated at 35 °C for 24 h before determining the MIC using ρ -iodonitrotetrazolium (INT) colorimetric assays (Sigma-Aldrich, Australia). INT solution was prepared at 0.4 mg/mL in sterile deionised water. The INT solution turns red when microbial growth occurs. The MIC is the lowest concentration of EPCG or an antibiotic that does not cause a colour change. MIC measurements were performed in triplicate across three separate experiments ($n = 9$).

2.2.3. Fractional inhibitory concentration determination

The interaction between EPCG and selected conventional antibiotics was also evaluated. The combinational effects were initially examined at a 1:1 ratio and analysed by calculating the sum of FICs (Σ FIC): $\text{FIC (EPCG)} = (\text{MIC of EPCG in combination with antibiotic}) / (\text{MIC of EPCG alone})$, and $\text{FIC (antibiotic)} = (\text{MIC of antibiotic in combination with EPCG}) / (\text{MIC of antibiotic alone})$; thus: $\Sigma\text{FIC} = \text{FIC (EPCG)} + \text{FIC (antibiotic)}$. Interactions with ΣFIC values ≤ 0.5 were classified as synergistic; > 0.5 – ≤ 1.0 as additive; > 1.0 – ≤ 4.0 as indifferent; and > 4.0 as antagonistic.

Any combinations that produced synergistic or additive interactions were subsequently examined across nine different ratios ranging from 10:90 to 90:10 (antibiotic: EPCG [v/v]), with 10% increasing increments. All tests were evaluated in three independent experiments, each with internal triplicates ($n = 9$). Isobolograms were generated from the FIC for each ratio to identify synergistic ratios between EPCG and the antibiotic. Values at or below the 0.5:0.5 line indicate synergy; points between the 0.5:0.5 and 1.0:1.0 lines show an additive effect; and those above the 1.0:1.0 line were classified as indifferent interactions.

2.3. Toxicity studies

2.3.1. *Artemia franciscana* nauplii lethality assay

Standard *Artemia franciscana* nauplii lethality assays were used as an initial evaluation of EPCG toxicity. EPCG concentrations that caused $\geq 50\%$ mortality were considered toxic, and further dilutions were used to determine the concentration producing 50% mortality (LC_{50}) by linear regression. Approximately 50 *A. franciscana* nauplii (North America Brine Shrimp, LLC, United States) were placed into each well of a 48-well plate, with 400 µL of artificial saltwater (32 g/L, Red Sea Pharm Ltd., Israel) to standardise the assay environment and 400 µL of EPCG. For controls, separate wells received either potassium dichromate (2 mg/mL, Chem-Supply, Australia) as a positive control (dissolved in sterile deionised water), or artificial seawater

as a negative control. The positive control was used at a concentration of 1 mg/mL in the assay. The plates were incubated at room temperature for 24 h, and the number of dead nauplii in each well was determined to calculate the concentration that induced 50% mortality. The results are expressed as the mean of three independent experiments, each with triplicate ($n = 9$).

2.3.2. MTS cellular viability assay

Human primary dermal fibroblasts (HDFs; ATCC PCS-201-012) were purchased from American Type Culture Collection (United States) and were used to evaluate the toxicity of EPCG. Aliquots of HDFs (70 μ L) containing approximately 5,000 cells were used in all tests. Individual 30 μ L volumes of EPCG, with an initial concentration ranging from 20 to 250 μ g/mL, were added to each well containing 70 μ L of cells. The plates were then incubated at 37 °C in a humidified 5% CO₂ atmosphere for 24 h. To prevent interference from the sample colour, control wells were prepared by mixing 30 μ L of EPCG or antibiotic with 70 μ L of cell-free media, and the values were subtracted from the test results. Wells containing cells and media, as well as media only (without cells), served as negative and blank controls, respectively. After the 24-h incubation, 20 μ L of MTS reagent (Abcam, Australia) was added to each well, followed by an additional four-hour incubation at 37 °C. Absorbance was measured at 490 nm using a CLARIOstar plus multi-plate reader (BMG Labtech, Australia). All tests were repeated three times, each with internal triplicates ($n = 9$). The % cellular viability of each replicate was calculated using Equation 1:

$$\text{Percentage of cellular viability} = \frac{A_{\text{treatment}} - A_{\text{treatment control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100\% \quad (1)$$

where A = absorbance at 490 nm. Cellular viability below 50% indicates toxicity.

2.4. Confocal imaging

The combination of EPCG and cefotaxime showed strong synergy against *S. aureus* and MRSA. Therefore, this combination was used in imaging techniques.

2.4.1. Sample preparation

A cell concentration of approximately 10⁸ cells/mL (0.5 McFarland turbidity) was used to ensure consistent data collection. Two fluorescent dyes, Hoechst 33342 and fluorescein isothiocyanate (FITC), were used to stain the bacterial cells. A mixture of EPCG and cefotaxime in a 90:10 ratio (100 μ L) was added to the bacterial well (100 μ L) in a 96-well plate. Two positive controls were included: EPCG (1,600 μ g/mL) and cefotaxime (40 μ g/mL), along

with a negative control of untreated bacterial cells. The mixture was incubated for three hours, then centrifuged at 2,200 $\times$ g for ten minutes. The supernatant was removed, and 80 μ L of phosphate-buffered saline (PBS) was added to the pellet. The centrifugation step was repeated to remove the supernatant.

Hoechst 33342 dye (40 μ L) was added to the pellet and incubated for 30 min at 35 °C. After this incubation, the second dye, FITC (60 μ L), served as a counterstain for the cells. The mixture underwent two centrifugation steps to remove excess dyes. The cells were then fixed with 4% paraformaldehyde for 60 min, after which the paraformaldehyde was removed by centrifugation. The cells were resuspended in PBS, the plate was sealed with parafilm, covered with aluminium foil, and stored at 4 °C until imaging.

2.4.2. Imaging protocol

Images were obtained using a high-content imaging system, the Operetta CLS (PerkinElmer, Australia), equipped with a 40 $\times$ /1.1 numerical aperture objective. Each well was captured in Brightfield, Hoechst 33342 (excitation 310–350 nm; emission 460–500 nm), and FITC (excitation 460–490 nm; emission 500–550 nm). The images were processed using ImageJ software (version 1.54p).

2.5. Cryo-electron microscopy imaging

2.5.1. Sample preparation

A cell concentration of approximately 10⁸ cells/mL (equivalent to 0.5 McFarland turbidity) was used to ensure reliable data collection. In a 96-well plate, 100 μ L of a cefotaxime–EPCG mixture (90:10) was added to each well containing 100 μ L of bacterial cells. Two positive controls, EPCG at 500 μ g/mL and cefotaxime at 40 μ g/mL, were included, along with a negative control of untreated bacterial cells. The mixture was incubated for 24 h, then centrifuged at 2,200 $\times$ g for 10 min. The supernatant was removed, and 80 μ L of PBS was added to the pellet, followed by a repeat centrifugation to remove residual supernatant. The cells were resuspended in PBS for imaging.

2.5.2. Imaging protocol

Lacey carbon films served as sample carriers on 300 mesh copper grids (Electron Microscopy Sciences (United States). Immediately before use, the grids were glow-discharged for 30 s with a Diener Femto TEM system set at 30% power (Diener Electronic, Germany). The samples were cryo-fixed in liquid ethane at –183 °C using a Leica GP-EM2 (Leica Microsystems, Austria). Freezing was performed on a Leica GP-EM2 with environmental chamber conditions set at 25 °C and 95% relative humidity, applying 2.5 μ L of

sample and blotting for seven seconds. The samples were then stored in liquid nitrogen until further analysis.

For imaging of frozen-hydrated bacteria, a JEOL CryoARM 300 JEM-Z300 FSC (JEOL, Japan) equipped with a Gatan K3 direct electron detector (Gatan, United States) was used. The microscope operated at 300 kV with a 20 eV slit width on the omega energy filter. SerialEM^{22,23} (version 4.2) was used for image acquisition. Samples were imaged at -180°C under low-dose conditions, with each exposure lasting four seconds and comprising 40 subframes in counting mode. Movie frames were motion-corrected using SerialEM's frame alignment feature and combined into a single image of 40 subframes. The images were saved as TIFF files via the Snapshot function in SerialEM. To determine the effects of EPCG–cefotaxime on membrane thickness and structural damage, six representative cells were randomly selected; their thicknesses and gaps were measured, and the mean values were reported.

2.4. Statistical analysis

Whilst statistical tests were not possible with the broth microdilution assays, the MIC values were determined three times on separate days, with three technical replicates per assay. This was done to help ensure the reliability and reproducibility of the MIC value determinations.

3. Results

3.1. Liquid microdilution assay

Epicatechin gallate was tested at a maximum concentration of 500 $\mu\text{g/mL}$, with equivalent concentrations expressed in μM for reference (Table 1). EPCG demonstrated notable antibacterial activity against *S. aureus* and MRSA, with MIC values of 208.33 $\mu\text{g/mL}$ (470.94 μM) and 187.50 $\mu\text{g/mL}$ (423.85 μM), respectively. Both bacterial strains were susceptible to tetracycline. Varying levels of resistance to cefotaxime and ciprofloxacin were also observed.

3.2. Fractional inhibitory concentration determination

Combination assays of EPCG with three conventional antibiotics were conducted to evaluate the antibacterial potential of EPCG and characterise its combinatorial effects. Combinations were tested at a 1:1 ratio, and the ΣFIC was calculated to determine the nature of their interactions. Notably, the combination of EPCG and cefotaxime, an antibiotic to which both strains exhibited resistance, produced a synergistic effect (Table 2). The ΣFIC values of 0.25 calculated for this combination against *S. aureus* and 0.15 for MRSA indicate strong synergy. A synergistic interaction was also observed in the combination of EPCG and tetracycline against *S. aureus* ($\Sigma\text{FIC} = 0.5$). All other

Table 1. Minimum inhibitory concentration values for epicatechin gallate and the reference antibiotics against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*

Chemical or antibiotic	Minimum inhibitory concentration, $\mu\text{g/mL}$ (μM)	
	<i>Staphylococcus aureus</i>	Methicillin-resistant <i>Staphylococcus aureus</i>
Epicatechin gallate	208.33 (470.94)	187.50 (423.85)
Cefotaxime	2.5 (5.24)	12.5 (26.18)
Ciprofloxacin	1.25 (3.77)	2.5 (7.55)
Tetracycline	1.46 (3.04) ^a	0.52 (1.08) ^a

Note: ^a indicates bacteria are susceptible to the tested antibiotic, based on Clinical and Laboratory Standards Institute M100 and M45 guidelines.²⁴ The results are expressed as the mean of three independent experiments, each with internal triplicates ($n = 9$).

Table 2. Sum of fractional inhibitory concentration values at a 1:1 ratio for epicatechin gallate in combination with reference antibiotics

Bacterial species	Fractional inhibitory concentration (ΣFIC)		
	Cefotaxime	Ciprofloxacin	Tetracycline
<i>Staphylococcus aureus</i>	0.25 ^a	2.0	0.5 ^a
Methicillin-resistant <i>Staphylococcus aureus</i>	0.15 ^a	2.5	0.75 ^b

Note: ΣFIC values were evaluated in triplicate ($n = 3$). ΣFIC values ≤ 0.5 are classed as synergy; $>0.5 - \leq 1.0$ are classed as additive; and $>1.0 - \leq 4.0$ as indifferent. ^a and ^b values indicate synergy and additive, respectively. The results are expressed as the mean of three independent experiments, each with internal triplicates ($n = 9$).

tested combinations demonstrated either additive or indifferent interactions, and no antagonistic effects were detected.

3.3. Isobologram analysis

The three initial combinations showing synergistic interactions were further tested across eight additional concentration ratios, from 90:10 to 10:90 (v/v) in 10% increments. All these ratios continued to exhibit synergy. Notably, every EPCG–cefotaxime combination ratio demonstrated strong synergy against MRSA, consistently yielding low Σ FIC values across the range. This emphasises the powerful synergistic effect of this pairing against resistant Gram-positive bacteria (Figure 1).

3.4. Quantification of toxicity

Epicatechin gallate was tested against *Artemia franciscana nauplii* at concentrations of 125 to 250 μ g/mL for an initial toxicity evaluation. The highest concentration (250 μ g/mL) was chosen because the maximum MIC observed was

208.33 μ g/mL (Table 1). Mean mortality percentages from triplicate tests were used to determine LC_{50} values. EPCG showed no toxicity to *A. franciscana* at 250 μ g/mL (Table 3).

Additionally, cytotoxicity against HDFs was assessed across a concentration range of 25–250 μ g/mL. Samples causing $\leq 50\%$ cell viability at a given concentration were deemed toxic. The LC_{50} for EPCG was 100 μ g/mL (226.06 μ M), as shown in Table 3. The 90:10 cefotaxime–EPCG combination did not exhibit cytotoxicity in either the *Artemia nauplii* lethality test or the HDF test, with cell viability remaining above 50% at all tested concentrations. No LC_{50} value is provided for this mixture because it involves a mixture ratio rather than a single compound.

3.5. Imaging analysis

The combination of cefotaxime and EPCG exhibited the strongest synergistic effect against both *S. aureus* and MRSA strains. Therefore, this combination was selected for microscopy analysis. To visualise the cellular effects

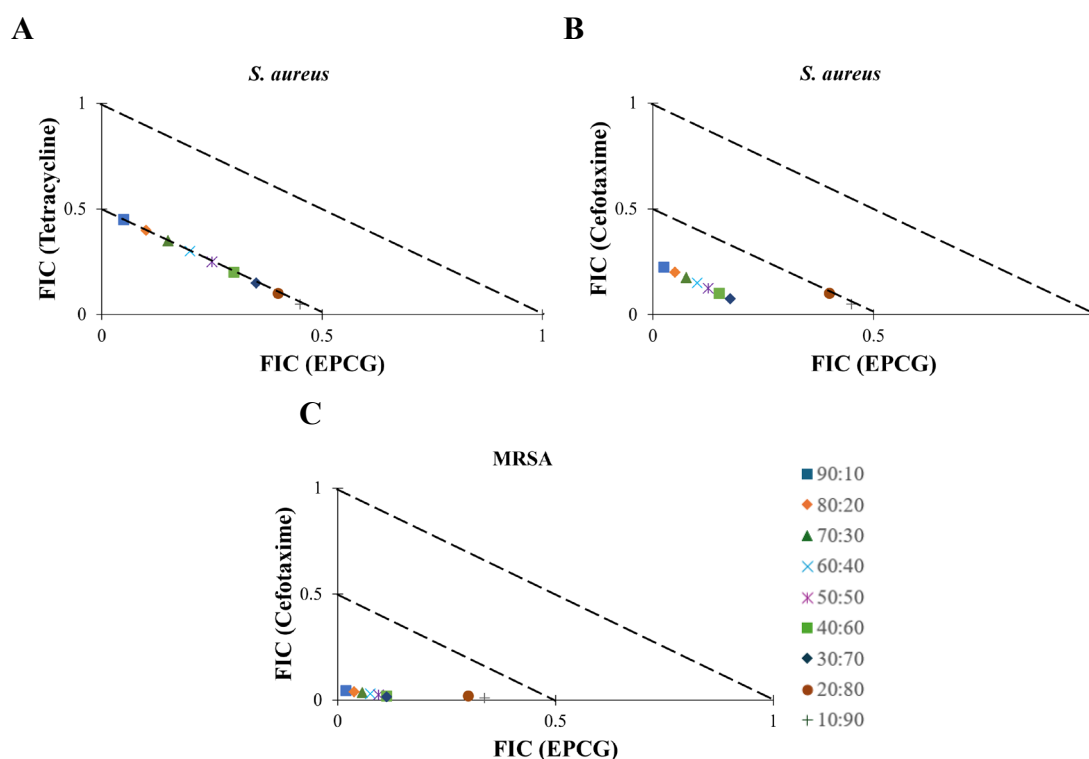


Figure 1. Isobolograms of EPCG in combination with cefotaxime and tetracycline against *S. aureus* (A & B) and MRSA (C). The ratios are expressed as the Σ FIC values for the antibiotic relative to EPCG. Ratio = % antibiotic: % EPCG (v/v). FIC values are displayed as the means of three repeats in three independent experiments ($n = 9$). Σ FIC values below the 0.5-line represent synergy; Σ FIC values between the 0.5 and 1 lines are classified as additive interactions. Only the synergistic and additive ratios are shown in these figures.

Abbreviations: EPCG: Epicatechin gallate; FIC: Fractional inhibitory concentration; MRSA: Methicillin-resistant *Staphylococcus aureus*.

Table 3. LC₅₀ values of epicatechin gallate and the cefotaxime–epicatechin gallate (90:10) combination

Compounds	LC ₅₀ value, µg/mL (µM)	
	<i>Artemia nauplii</i>	Human dermal fibroblasts
Epicatechin gallate	>250 (>564.14)	100 (226.06)
Cefotaxime: Epicatechin gallate (90:10)	Non-toxic ^a	Non-toxic ^a

Note: ^a indicates that the combination resulted in >50% cell viability.

of this combination, bacterial cells treated with EPCG, cefotaxime, or their combination were imaged using two techniques: confocal laser scanning microscopy at 40× magnification with counterstaining and high-resolution electron microscopy to examine nanoscale ultrastructural changes.

3.5.1. Confocal imaging

A counterstaining method was used with two fluorescent dyes: Hoechst 33342 and FITC. Hoechst 33342 selectively binds to DNA and is frequently employed to stain nuclei in both live and dead cells, emitting blue fluorescence.²⁵ Conversely, FITC attaches to proteins and other molecules with primary amines, causing them to emit green fluorescence.²⁶ Dual staining with Hoechst 33342 and FITC enables visualisation of nucleic acid- and membrane-associated components in *S. aureus* and MRSA. When the fluorescence channels were combined in ImageJ, overlapping areas of Hoechst 33342 (blue) and FITC (green) appeared teal, indicating co-localisation of the two fluorophores within the same region, as expected from additive blending of blue and green channels.^{27,28} In this study, the untreated control groups for both *S. aureus* and MRSA showed higher bacterial cell density, as evidenced by the large, blue-stained areas representing intact or fragmented bacterial DNA (Figures 2A and 3A). The observed green fluorescence is thought to indicate extracellular protein material, likely associated with secreted proteins or broken cellular components.²⁶ The lack of teal fluorescence (a mix of blue and green signals) in the negative control samples indicates no biofilm formation, as expected in untreated cells (Figures 2C and 3C). Conversely, samples treated with cefotaxime alone (Figures 2F and 3F) and with both cefotaxime and EPCG (Figures 2L and 3L) showed dense cell clusters with teal staining, with more noticeable clumping in the cefotaxime-only MRSA group (Figure 3F). This teal colour may suggest the co-localisation of DNA and proteins, likely representing extracellular polymeric substances or cellular aggregates typical of biofilms. In comparison, EPCG-treated samples (Figures 2I and 3I) displayed minimal green fluorescence and no teal-stained clusters, implying limited protein secretion and no biofilm-like cell aggregation. All treated

samples had a smaller, blue-stained surface area, reflecting a reduced bacterial cell density relative to the control.

3.5.2. Cryo-electron microscopy imaging

Confocal imaging revealed significant differences between MRSA cells treated with cefotaxime and those treated with cefotaxime–EPCG, prompting further analysis of their ultrastructural changes by cryo-electron microscopy. Figure 4A,B display untreated MRSA cells with a normal average membrane thickness of about 32.5 nm (measured between the blue and red lines), showing intact cytoplasm and structural integrity. After cefotaxime treatment, a β-lactam antibiotic that inhibits PBPs involved in peptidoglycan synthesis, three of four MRSA cells demonstrated a reduced membrane thickness of approximately 10 nm (Figure 4C,D). Despite MIC data indicating MRSA resistance to cefotaxime, structural alterations were apparent. Most treated cells retained membrane integrity, though one cell showed lysis, marked by the teal arrow in Figure 4C.

Epicatechin gallate-treated MRSA cells showed a different morphological response (Figure 4E,F). There was a clear detachment of the cytoplasmic membrane from both the peptidoglycan layer and the cytoplasm. The gap between the peptidoglycan and the membrane was 45 to 52.5 nm, and the space between the membrane and cytoplasm ranged from 50 to 142.5 nm. This detachment and cytoplasmic shrinkage suggest cytoplasmic lysis, despite the outer membrane remaining mostly intact. Notably, MRSA cells exposed to a combination of cefotaxime and EPCG had ultrastructural damage affecting both the cell wall and internal components (Figure 4G,H). In Figure 4G, membrane thickness varied from 28.6 to 57.2 nm, showing features similar to those observed with individual treatments. Severe stress characteristics included membrane deformation, destruction of the peptidoglycan layer, and significant cytoplasmic shrinkage. Figure 4G shows partial cell lysis, whilst Figure 4H shows marked curvature of the cell envelope and cytoplasmic disintegration, without full lysis. These results reveal a synergistic effect at the ultrastructural level, where the combination of cefotaxime and EPCG induces greater cellular disruption in MRSA than either compound alone.

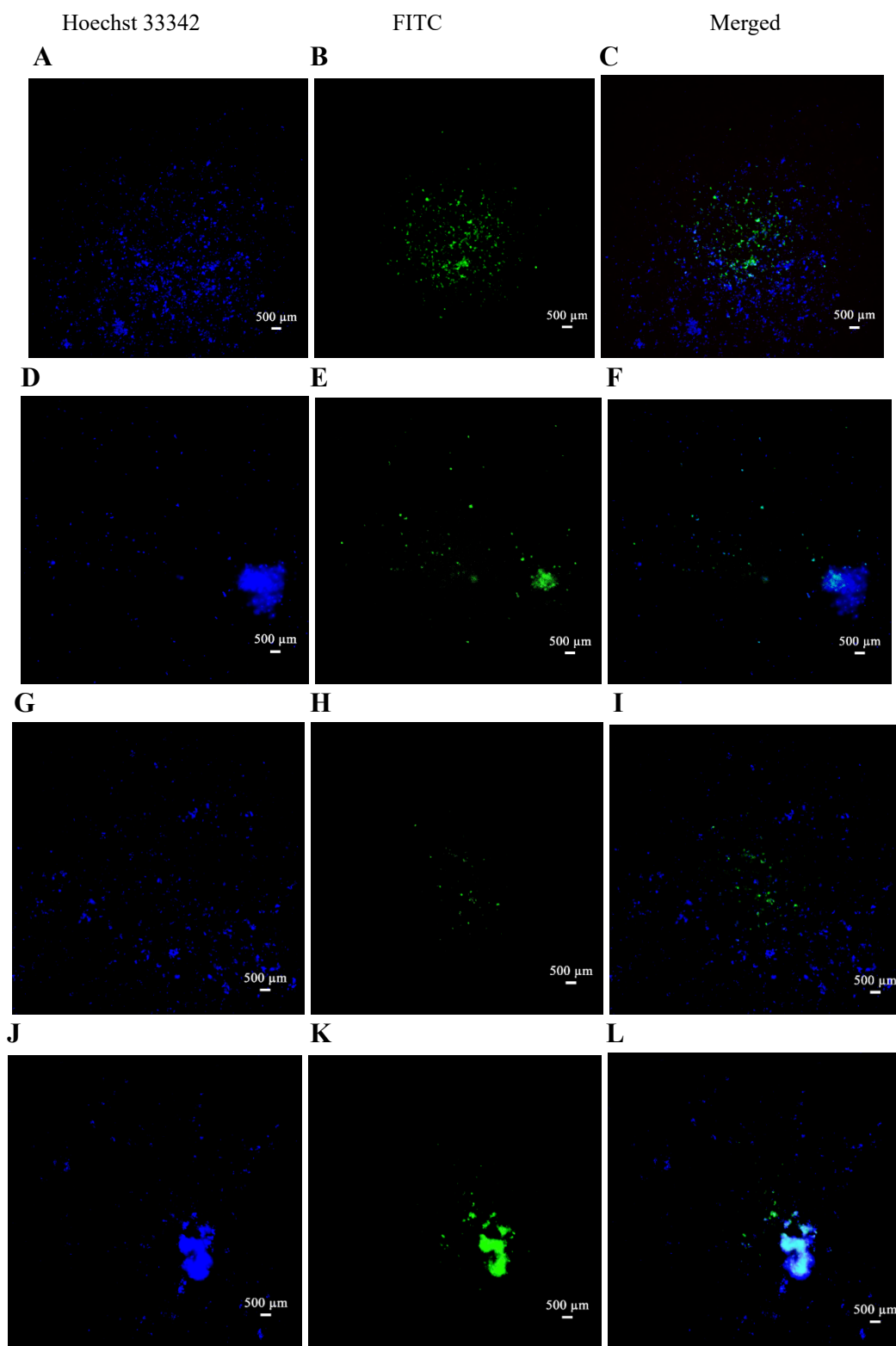


Figure 2. Confocal microscopy images of untreated *S. aureus* cells (A–C) and *S. aureus* cells treated with cefotaxime (D–F), epicatechin gallate (EPCG) (G–I) and 90:10 cefotaxime–EPCG combination (v/v) (J–L) counterstained with Hoechst 33345 and fluorescein isothiocyanate (FITC) and imaged at 40× magnification. Scale bar: 500 μm.

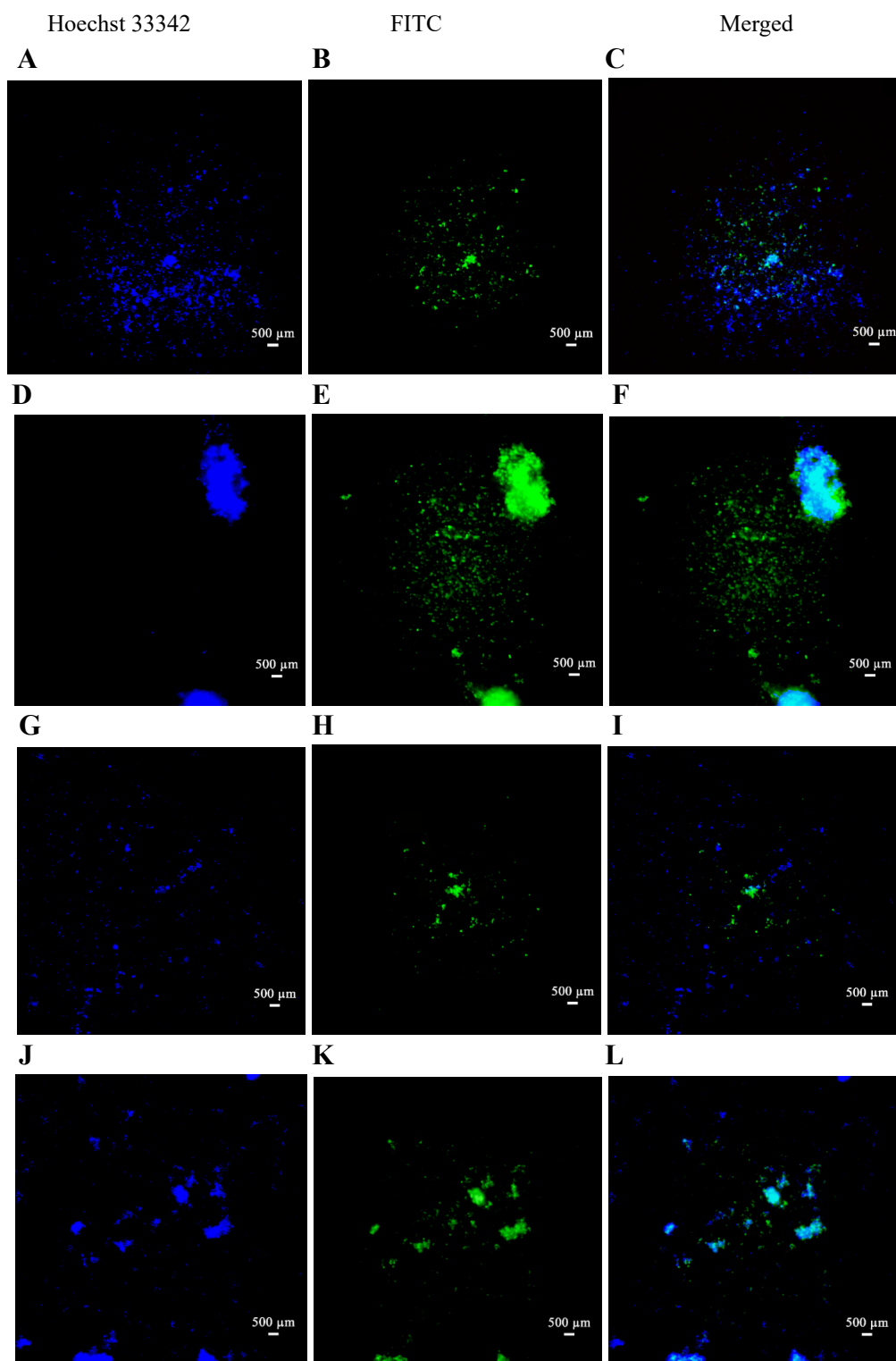


Figure 3. Confocal microscopy images of untreated methicillin-resistant *Staphylococcus aureus* (MRSA) cells (A–C) and MRSA cells treated with cefotaxime (D–F), epicatechin gallate (EPCG) (G–I) and 90:10 cefotaxime–EPCG combination (v/v) (J–L) counterstained with Hoechst 33345 and fluorescein isothiocyanate (FITC) and imaged at 40× magnification. Scale bar: 500 μm.

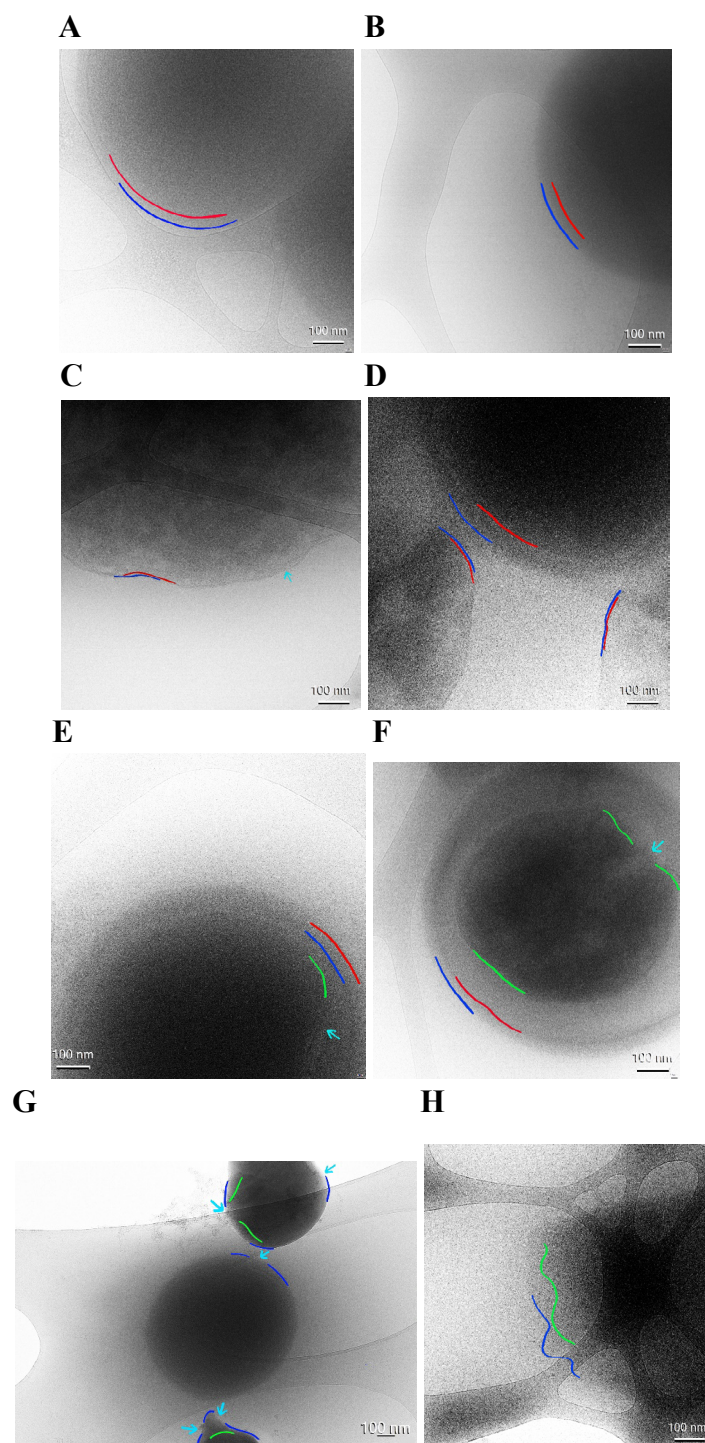


Figure 4. Cryo-electron microscopy imaging showing changes in the ultrastructure of methicillin-resistant *Staphylococcus aureus* (MRSA) cells. Blue line = peptidoglycan layer; red line = cytoplasmic membrane; green line = cytoplasm; teal arrowhead = rupture of cell envelope and release of cytoplasmic contents. (A & B) Representative images of intact MRSA cells are shown. (C & D) MRSA cells treated with cefotaxime. (C) A cell with a destroyed cell envelope leading to rupture. (D) A top right cell displaying membrane layer changes, a decreased periplasmic space, and cytoplasm shrinkage. (E & F) MRSA cells exposed to epicatechin gallate (EPCG). These cells exhibited cytoplasmic shrinkage and lysis, while the peptidoglycan layer stayed mostly intact. (G and H) MRSA cells treated with a 90:10 cefotaxime-to-EPCG ratio. (G) Multiple forms of damage, including cell envelope destruction, membrane thinning, cytoplasmic shrinkage, and lysis, are observed. (H) Peptidoglycan layer destruction, cell envelope curvature, cytoplasm shrinkage, and no cell lysis are noted. Scale bar: 100 nm; magnification: 30,000 $\times$.

4. Discussion

The ongoing therapeutic difficulties associated with MRSA highlight the need for approaches that endeavour to improve the effectiveness of existing antibiotics rather than relying exclusively on the discovery of new antimicrobial agents. In this regard, naturally derived compounds capable of modifying bacterial susceptibility have attracted increasing interest as antibiotic adjuvants.^{29,30} Galloylated catechins, including EPCG, have emerged as particularly promising candidates due to their ability to interact with bacterial membranes and cell wall structures.^{14–17,31} The findings of the present study reinforce this concept, demonstrating that EPCG can alter bacterial susceptibility and enhance the antibacterial performance of conventional antibiotics against both *S. aureus* and MRSA.

Epicatechin gallate exhibited MICs of 208.33 µg/mL (470.94 µM) against *S. aureus* and 187.50 µg/mL (423.85 µM) against MRSA, indicating moderate intrinsic antibacterial activity against both strains. Catechins derived from green tea, including EPCG and related galloylated analogues, have been extensively reported to inhibit the growth of Gram-positive pathogens such as *S. aureus*, MRSA and *Bacillus cereus*.^{31–34} Much like the structurally related catechin epigallocatechin-3-gallate (EGCG), EPCG is believed to exert antibacterial effects through multiple mechanisms, including disruption of cytoplasmic membrane integrity, inhibition of key bacterial enzymes, and modulation of virulence-associated gene expression.^{16,35,36}

Previous studies have also demonstrated that catechins can exert differential antibacterial activity depending on bacterial cell envelope structure. For example, EGCG preferentially affects Gram-positive bacteria, compared with Gram-negative organisms.³⁷ In *S. aureus*, EGCG has been reported to induce aggregation within the bacterial cell envelope, ultimately leading to membrane destabilisation and cell lysis.³⁷ Notably, this effect differs from oxidative stress-mediated killing mechanisms, as similar aggregation is not observed when cells are exposed to reactive oxygen species such as hydrogen peroxide.³⁷ In contrast, bactericidal effects against Gram-negative bacteria appear to rely more on hydrogen peroxide generation, suggesting that the mode of action of catechins is influenced by differences in cell envelope composition and oxidative stress sensitivity. The selective activity observed in the present study, in which EPCG demonstrated antibacterial activity against Gram-positive strains, is therefore consistent with these previous reports.^{14–17} Collectively, these findings support the view that galloylated catechins exert antibacterial activity through a combination of direct structural disruption and interference with key bacterial physiological processes,

effects that are particularly pronounced in Gram-positive organisms with accessible peptidoglycan-rich cell walls.

Whilst EPCG demonstrated intrinsic antibacterial activity against *S. aureus* and MRSA, the relatively moderate MIC values suggest that its therapeutic potential may be more effectively realised when used in combination with conventional antibiotics. Accordingly, the capacity of EPCG to function as a resistance-modifying agent was further explored through combinational analyses. Isobologram evaluations revealed that EPCG can potentiate the activity of several antibiotics, most notably β-lactams and tetracyclines, at defined concentration ratios. These interactions were assessed using both *S. aureus* and MRSA to enable comparison between an antibiotic-susceptible strain and a β-lactam-resistant phenotype. Examining the combinations across these two strains provided insights into how EPCG influences antibiotic performance under different resistance conditions. The consistent synergistic interactions observed in these analyses suggest that EPCG may act as a natural resistance-modifying compound capable of enhancing the efficacy of existing antibiotics against Gram-positive pathogens.

Among the combinations evaluated, the EPCG–cefotaxime pairing produced the most pronounced synergistic effects. The ΣFIC values reached as low as 0.15 against MRSA and 0.25 against *S. aureus*, both well below the established synergy threshold of 0.5. These findings are particularly significant given that MRSA is characteristically resistant to β-lactam antibiotics due to alterations in PBPs.³⁸ The marked synergy observed therefore suggests that EPCG may enhance cefotaxime activity by partially overcoming or destabilising these resistance mechanisms. Several mechanisms may contribute to this interaction, including EPCG-induced disruption of membrane integrity, increased antibiotic permeability, interference with PBP localisation, or modulation of bacterial regulatory pathways involved in virulence and resistance.^{17,35,39}

Previous work by Stapleton *et al.*¹⁴ provides additional insights into the interaction between EPCG and β-lactam antibiotics. Their study showed that EPCG increased MRSA susceptibility to oxacillin and promoted the aggregation of staphylococcal cells, whilst also inducing changes in cell wall structure.¹⁴ Importantly, this enhanced β-lactam activity was not linked to reduced expression of PBP2a or direct binding of EPCG to peptidoglycan. Instead, only a modest decrease (<10%) in peptidoglycan cross-linking was observed. Although this level of reduction does not appear sufficient to cause structural collapse of the cell wall, it may still destabilise cellular homeostasis. Such alterations may influence bacterial stress responses,

including the downregulation of efflux pump activity, thereby allowing greater intracellular accumulation of antibiotics and ultimately enhancing antibacterial activity. Further mechanistic investigations, particularly using imaging approaches such as confocal and cryo-electron microscopy, are therefore valuable for clarifying how EPCG influences membrane permeability, cell wall structure, and intracellular dynamics.

In the present study, EPCG consistently potentiated the antibacterial activity of cefotaxime against both *S. aureus* and MRSA across all tested ratios (Figure 1B,C), whilst also enhancing the efficacy of tetracycline against *S. aureus* (Figure 1A). The synergistic effect likely arises from complementary mechanisms of action between the two agents. EPCG has been shown to perturb bacterial membranes and increase permeability^{14,15,35}, whereas cefotaxime inhibits peptidoglycan synthesis and disrupts cell wall formation.¹⁸ Simultaneous targeting of multiple structural components of the bacterial envelope can amplify antibacterial effects, even when each compound is present at sub-inhibitory concentrations. This cooperative interaction provides a plausible explanation for the strong synergy observed in the checkerboard and isobologram analyses (Table 2 and Figure 1). Importantly, such interactions may enable the therapeutic use of lower EPCG concentrations, thereby reducing the likelihood of host-cell toxicity whilst maintaining antibacterial efficacy. However, this remains to be verified in future investigations, particularly through *in vivo* studies.

To evaluate the safety profile of EPCG, cytotoxicity was assessed using the *Artemia nauplii* lethality assay and a HDF cell model. In the *Artemia nauplii* assay, EPCG produced less than 50% mortality, even at the highest concentration tested (250 µg/mL), a value exceeding the MICs determined against *S. aureus* and MRSA (Table 1). These results indicate an absence of acute toxicity at concentrations relevant to antibacterial activity. In contrast, testing in HDF cells revealed an LC₅₀ of 100 µg/mL (226.06 µM), indicating a narrower margin between antibacterial potency and host-cell toxicity. Indeed, these results equate to a safety index (calculated by dividing the MIC by the LC₅₀) of approximately 2 for both *S. aureus* and the MRSA strain (based on HDF toxicity data). Nevertheless, this LC₅₀ remains approximately twofold higher than the MIC values observed against both *S. aureus* and MRSA. Whilst this indicates a moderate therapeutic window, it also highlights potential limitations of EPCG as a standalone antimicrobial at higher concentrations.

These observations emphasise the advantage of combination strategies that maintain antibacterial efficacy, whilst reducing the required EPCG dose. Consistent

with this rationale, the cefotaxime: EPCG combination at a 90:10 (v/v) ratio showed no detectable toxicity in either the *Artemia nauplii* or HDF assays. Because the formulation consists of two compounds administered together, calculation of an LC₅₀ for the mixture was not feasible. However, both assays showed cell viability above 50%, indicating that the combination treatment does not compromise host cell survival under the tested conditions. This finding suggests that incorporating EPCG at a relatively low proportion in the combination not only enhances cefotaxime activity but also mitigates the cytotoxic effects observed with EPCG alone. Therefore, this combination therapy approach may have several benefits over conventional antibiotic therapies: it has greater antibacterial potency and allows for lower therapeutic doses, which not only increases therapeutic safety but may also reduce the induction of further antibiotic resistance in the target pathogens. Consequently, this ratio was considered appropriate for further mechanistic evaluation.

Given the strong synergistic interaction and favourable toxicity profile observed at the optimised ratio, the cefotaxime–EPCG combination was selected for subsequent imaging analyses. Confocal microscopy was first employed to visualise bacterial cell density and potential biofilm formation using dual fluorescent staining (Figures 2 and 3). Untreated control samples of both *S. aureus* (Figure 2A) and MRSA (Figure 3A) showed intense blue fluorescence from Hoechst 33342 staining, indicating a high bacterial cell density. Simultaneously, widespread green fluorescence from FITC staining suggested the presence of extracellular proteinaceous material associated with early biofilm development (Figures 2B and 3B). These findings align with the well-documented ability of *S. aureus* and MRSA to form biofilms as a survival strategy, particularly under environmental stress or suboptimal growth conditions.⁴⁰ However, the proposed effects of the cefotaxime–EPCG combination on biofilm formation should be confirmed in future studies using crystal violet biomass assays, viable biofilm CFU counts, COMSTAT analysis, extracellular polymeric substance staining, and other established biofilm models.

When treated with cefotaxime alone, *S. aureus* samples displayed only small clusters of teal-coloured fluorescence resulting from overlapping blue and green signals, indicating partial inhibition of bacterial growth with residual biofilm activity (Figure 2F). In contrast, MRSA cells treated with cefotaxime formed larger, more intense, teal-stained aggregates, accompanied by stronger green fluorescence signals (Figure 3F). This observation is consistent with the lower susceptibility of MRSA to cefotaxime (MIC = 12.5 µg/mL compared with 2.5 µg/mL

for *S. aureus*). The increased aggregation and extracellular protein signal may reflect persistent biofilm-associated activity, which is known to involve surface proteins, such as fibrinogen-binding proteins, and extracellular DNA.⁸ These findings further highlight the limited ability of cefotaxime alone to overcome the intrinsic resistance mechanisms of MRSA.

In contrast, treatment with EPCG resulted in a pronounced reduction in green fluorescence intensity and the disappearance of teal-stained aggregates, suggesting a substantial decrease in extracellular matrix production and biofilm formation (Figures 2I and 3I). The bacterial cells were also more dispersed compared with untreated controls, indicating reduced intercellular adhesion. Such dispersion may reflect disruption of early biofilm formation processes, which rely on cell–cell interactions and extracellular matrix development. Given that biofilms provide protective barriers against environmental stress and antibiotic exposure, the suppression of biofilm formation observed in EPCG-treated samples highlights the potential of this compound as an anti-virulence and anti-biofilm agent.

Notably, samples treated with the combined EPCG–cefotaxime formulation exhibited an even greater reduction in teal-stained aggregates compared with cefotaxime treatment alone (Figures 2L and 3L). The diminished size and intensity of these clusters correspond well with the synergistic interactions observed in the Σ FIC and isobologram analyses. This suggests that EPCG not only enhances the direct antibacterial activity of cefotaxime, but may also disrupt the structural stability of biofilms, thereby facilitating improved antibiotic penetration into bacterial communities. Such effects further support the role of EPCG as a promising antibiotic adjuvant for the treatment of biofilm-associated infections caused by resistant Gram-positive pathogens.

To further investigate structural changes induced by these treatments, cryo-electron microscopy was employed to visualise bacterial ultrastructure (Figure 4). Whilst confocal microscopy provided an overview of bacterial distribution and extracellular matrix formation, electron microscopy allowed detailed examination of cellular architecture, including membrane integrity and cytoplasmic organisation. Untreated MRSA cells exhibited intact cell envelopes with a peptidoglycan layer approximately 32.5 nm thick (Figure 4A,B), consistent with previously reported dimensions for Gram-positive bacterial cell walls, which typically range between 20 and 40 nm.^{39,41} No abnormalities were observed in the membrane or cytoplasmic compartments, confirming normal cellular integrity under control conditions.

Exposure of MRSA cells to cefotaxime resulted in noticeable structural alterations. Although the MIC data confirmed resistance to cefotaxime according to Clinical and Laboratory Standards Institute guidelines (MIC = 12.50 μ g/mL), electron microscopy revealed a substantial reduction in peptidoglycan thickness to approximately 10 nm compared with the untreated control. Additionally, distorted membrane contours and occasional localised membrane disruption were observed (Figure 4C,D). However, no widespread cytoplasmic leakage or extensive cellular rupture was detected, indicating that the antibiotic induced partial structural damage without achieving complete bactericidal effects. Such sublethal responses have been reported previously for β -lactam antibiotics in resistant bacteria, where altered PBPs or β -lactamase activity prevent full inhibition of cell wall synthesis while still triggering stress responses.^{42,43} The persistence of closely associated cells further suggests ongoing biofilm-related interactions despite antibiotic exposure.

Previous studies have suggested that EPCG integrates into the cytoplasmic membrane of MRSA, disrupting lipid organisation and altering cell wall synthesis pathways.¹⁵ In particular, EPCG has been reported to reduce levels of lysyl-phosphatidylglycerol, a membrane lipid involved in bacterial stress responses and virulence regulation. Reduction of this lipid may impair the ability of MRSA to adapt to environmental stress and may therefore weaken its pathogenic potential.¹⁵ The ultrastructural observations in the present study are consistent with this mechanism. EPCG-treated MRSA cells displayed a pronounced expansion of the periplasmic space between the peptidoglycan layer and the cytoplasmic membrane, measuring between 45 and 52.5 nm (Figure 4E,F). In addition, significant cytoplasmic shrinkage created a secondary gap ranging from approximately 50 to 142.5 nm between the membrane and the cytoplasmic contents. In some cells, collapse of internal structures was evident despite preservation of the outer cell wall, indicating cytoplasmic lysis. These observations suggest that EPCG disrupts intracellular homeostasis and osmotic balance without immediately causing catastrophic membrane rupture.

The combination of cefotaxime and EPCG (in a 90:10 [v/v] ratio) produced distinctive ultrastructural alterations consistent with a dual mechanism of antibacterial action (Figure 4G,H). Electron microscopy revealed pronounced morphological distortions, including S-shaped curvature of the peptidoglycan layer and underlying cytoplasmic membrane. The periplasmic space appeared irregular, ranging from approximately 28.6 to 57.2 nm, indicating significant destabilisation of the cell envelope. Evidence of

cytoplasmic lysis was observed in two of the four analysed cells, accompanied by disrupted intracellular organisation. Interestingly, the pronounced cytoplasmic shrinkage characteristic of EPCG monotherapy was less evident in samples treated with the combination. This difference may reflect the dominance of cefotaxime activity at the applied 90:10 (v/v) cefotaxime:EPCG ratio. Overall, the structural observations support the conclusion that the combined treatment exerts complementary antibacterial effects, whereby EPCG compromises membrane integrity and intracellular stability whilst cefotaxime inhibits peptidoglycan synthesis. Together, these mechanisms enhance antibacterial activity and reinforce the synergistic potential of this combination against resistant Gram-positive pathogens, such as MRSA. However, it is noteworthy that our study tested EPCG against a limited panel of reference bacterial strains. Future studies should screen against an expanded bacterial panel that includes additional antibiotic-resistant strains. EPCG should be tested (alone and in combination with conventional antibiotics) against further bacterial species to provide a more complete understanding of its spectrum of activity. Additionally, future studies using membrane permeability assays, peptidoglycan analysis, PBP2a expression/binding assays, and β -lactamase testing are required to validate the envelope disruption, PBP-related effects, permeability changes, and mechanisms of resistance modification postulated in our study. Furthermore, transcriptomic/proteomic analyses may provide additional insights into mechanisms of resistance modification.

5. Conclusion

Overall, the findings of this study reveal the potential of EPCG as a natural resistance-modifying compound that enhances the antibacterial efficacy of conventional antibiotics against Gram-positive pathogens, particularly by inducing structural changes in the cell envelope. The pronounced synergistic interaction observed between EPCG and cefotaxime, particularly against MRSA, suggests that galloylated catechins may partially overcome β -lactam resistance mechanisms by destabilising bacterial membranes and altering cell wall-associated processes. These functional interactions were further supported by confocal and cryo-electron microscopy, which revealed significant alterations in bacterial structure, reduced cell aggregation, and diminished biofilm-associated features following combination treatment. Importantly, the optimised cefotaxime-EPCG combination ratio demonstrated minimal cytotoxicity in both the *Artemia nauplii* and human dermal fibroblast models, indicating that synergistic combinations may improve antibacterial efficacy whilst maintaining host cell safety. Future studies

should confirm this low toxicity across additional assay systems, particularly *in vivo* models. Collectively, these findings support the model in which EPCG functions as an antibiotic adjuvant, restoring or amplifying the activity of existing drugs and offering a promising strategy for addressing persistent infections caused by resistant Gram-positive bacteria, including MRSA. These results provide a foundation for developing EPCG-based adjuvant therapies to restore the clinical utility of conventional antibiotics. Future investigations should include clinical isolate panels, biofilm-forming strains, and *in vivo* infection models to further validate the applicability of EPCG-based combinations.

Acknowledgments

None.

Funding

None.

Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: Ian Edwin Cock, Matthew James Cheesman

Formal analysis: Sze-Tieng Ang, Tak Hyun Kim

Investigation: Sze-Tieng Ang, Tak Hyun Kim

Methodology: Sze-Tieng Ang, Tak Hyun Kim

Writing—original draft: Sze-Tieng Ang

Writing—review & editing: Matthew James Cheesman, Ian Edwin Cock

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data related to the results presented herein are available from the corresponding author upon reasonable request.

Further disclosure

Section 3.5.2 has been presented at the 5th Global Congress on Pharmaceutical Sciences and Clinical Research (GCPSCR 2026) on the 27th March 2026 in Kyoto, Japan.

References

1. World Health Organization. *Antimicrobial Resistance*.

- World Health Organization; 2024. Accessed April 9, 2026. <https://www.who.int/europe/news-room/fact-sheets/item/antimicrobial-resistance>
2. World Health Organization. *WHO Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance*. World Health Organization; 2024. Accessed April 9, 2026. <https://www.who.int/publications/i/item/9789240093461>
3. Centers for Disease Control and Prevention. *Methicillin-Resistant Staphylococcus aureus (MRSA) Basics*. Centers for Disease Control and Prevention; 2025. Accessed April 9, 2026. <https://www.cdc.gov/mrsa/about/index.html>
4. Ventola CL. The antibiotic resistance crisis, part 1: Causes and threats. *Pharm. Ther.* 2015;40(4):277-283.
5. Uddin TM, Chakraborty AJ, Khusro A, *et al.* Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. *J Infect Public Health.* 2021;14(12):1750-1766.
doi: 10.1016/j.jiph.2021.10.020
6. Shlaes DM, Bradford PA. Antibiotics-From there to where?: How the antibiotic miracle is threatened by resistance and a broken market and what we can do about it. *Pathog Immun.* 2018;3(1):19-43.
doi: 10.20411/pai.v3i1.231
7. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: Past, present and future. *Curr Opin Microbiol.* 2019;51:72-80.
doi: 10.1016/j.mib.2019.10.008
8. McCarthy H, Rudkin JK, Black NS, *et al.* Methicillin resistance and the biofilm phenotype in *Staphylococcus aureus*. *Front Cell Infect Microbiol.* 2015;5:1.
doi: 10.3389/fcimb.2015.00001
9. Dilworth TJ, Ibrahim O, Hall P, *et al.* β -Lactams enhance vancomycin activity against methicillin-resistant *Staphylococcus aureus* bacteremia compared to vancomycin alone. *Antimicrob Agents Chemother.* 2014;58(1):102-109.
doi: 10.1128/AAC.01204-13
10. Worthington RJ, Melander C. Combination approaches to combat multidrug-resistant bacteria. *Trends Biotechnol.* 2013;31(3):177-184.
doi: 10.1016/j.tibtech.2012.12.006
11. Davis JS, Sud A, O'Sullivan MVN, *et al.* Combination of vancomycin and β -lactam therapy for methicillin-resistant *Staphylococcus aureus* bacteremia: A pilot multicenter randomized controlled trial. *Clin Infect Dis.* 2016;62(2):173-180.
doi: 10.1093/cid/civ808
12. Basavegowda N, Baek KH. Combination strategies of different antimicrobials: An efficient and alternative tool for pathogen inactivation. *Biomedicines.* 2022;10(9):2219.
doi: 10.3390/biomedicines10092219
13. Tong SYC, Lye DC, Yahav D, *et al.* Effect of vancomycin or daptomycin with vs without an antistaphylococcal β -lactam on mortality, bacteremia, relapse, or treatment failure in patients with MRSA bacteremia: A randomized clinical trial. *JAMA.* 2020;323(6):527-537.
doi: 10.1001/jama.2020.0103
14. Stapleton PD, Shah S, Ehlert K, *et al.* The β -lactam-resistance modifier (-)-epicatechin gallate alters the architecture of the cell wall of *Staphylococcus aureus*. *Microbiology.* 2007;153(7):2093-2103.
doi: 10.1099/mic.0.2007/007807-0
15. Bernal P, Lemaire S, Pinho MG, *et al.* Insertion of epicatechin gallate into the cytoplasmic membrane of methicillin-resistant *Staphylococcus aureus* disrupts penicillin-binding protein (PBP) 2a-mediated β -lactam resistance by delocalizing PBP2. *J Biol Chem.* 2010;285(31):24055-24065.
doi: 10.1074/jbc.M110.114793
16. Zhao Y, Qu Y, Tang J, *et al.* Tea catechin inhibits biofilm formation of methicillin-resistant *S. aureus*. *J Food Qual.* 2021;2021:1-7.
doi: 10.1155/2021/8873091
17. Teng F, Wang L, Wen J, *et al.* Epicatechin gallate and its analogues interact with sortase A and β -lactamase to suppress *Staphylococcus aureus* virulence. *Front Cell Infect Microbiol.* 2025;15:1537564.
doi: 10.3389/fcimb.2025.1537564
18. Carmine AA, Brogden RN, Heel RC, *et al.* Cefotaxime: A review of its antibacterial activity, pharmacological properties and therapeutic use. *Drugs.* 1983;25(3):223-289.
doi: 10.2165/00003495-198325030-00001
19. Hübsch Z, Van Zyl RL, Cock IE, *et al.* Interactive antimicrobial and toxicity profiles of conventional antimicrobials with Southern African medicinal plants. *South African Journal of Botany.* 2014;93:185-197.
doi: 10.1016/j.sajb.2014.04.005
20. Eloff JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica.* 1998;64(8):711-713.
doi: 10.1055/s-2006-957563
21. Ang ST, Kim TH, Cheesman MJ, *et al.* Antibacterial and synergistic effects of *Terminalia citrina* leaf extracts against gastrointestinal pathogens: Insights from metabolomic analysis. *Antibiotics.* 2025;14(6).
doi: 10.3390/antibiotics14060593
22. Mastronarde DN. SerialEM: a program for automated tilt series acquisition on Tecnai microscopes using prediction

- of specimen position. *Microsc Microanal.* 2003;9(Suppl 2):1182-1183.
doi: 10.1017/S1431927603445911
23. Mastronarde DN. Automated electron microscope tomography using robust prediction of specimen movements. *J Struct Biol.* 2005;152(1):36-51.
doi: 10.1016/j.jsb.2005.07.007
24. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing*. CLSI supplement M100. 30th ed. Clinical and Laboratory Standards Institute; 2020. Accessed June 30, 2025. <https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf>
25. Bucevičius J, Lukinavičius G, Gerasimaitė R. The use of Hoechst dyes for DNA staining and beyond. *Chemosensors.* 2018;6(2).
doi: 10.3390/chemosensors6020018
26. Hermanson GT. Fluorescent Probes. In: *Bioconjugate Techniques*. Elsevier; 2013:395-463.
doi: 10.1016/b978-0-12-382239-0.00010-8
27. Lichtman JW, Conchello J-A. Fluorescence microscopy. *Nat Methods.* 2005;2(12):910-919.
doi: 10.1038/nmeth817
28. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Methods.* 2005;2(12):905-909.
doi: 10.1038/nmeth819
29. Gao T, Wang Y, Zhu J, *et al.* Antibacterial activity of a plant natural polyphenol against zoonotic *Streptococcus suis*. *Microb Pathog.* 2025;205:107655.
doi: 10.1016/j.micpath.2025.107655
30. Dhanda G, Acharya Y, Haldar J. Antibiotic adjuvants: A versatile approach to combat antibiotic resistance. *ACS Omega.* 2023;8(12):10757-10783.
doi: 10.1021/acsomega.3c00312
31. Caturla N, Vera-Samper E, Villalain J, *et al.* The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radic Biol Med.* 2003;34(6):648-662.
doi: 10.1016/s0891-5849(02)01366-7
32. Yoda Y, Hu ZQ, Zhao WH, *et al.* Different susceptibilities of *Staphylococcus* and Gram-negative rods to epigallocatechin gallate. *J Infect Chemother.* 2004;10(1):55-58.
doi: 10.1007/s10156-003-0284-0
33. Kitichalermkiat A, Katsuki M, Sato J, *et al.* Effect of epigallocatechin gallate on gene expression of *Staphylococcus aureus*. *J Glob Antimicrob Resist.* 2020;22:854-859.
doi: 10.1016/j.jgar.2020.06.006
34. Zhao WH, Hu ZQ, Okubo S, *et al.* Mechanism of synergy between epigallocatechin gallate and β -lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother.* 2001;45(6):1737-1742.
doi: 10.1128/AAC.45.6.1737-1742.2001
35. Shah S, Stapleton P, Taylor PW. The polyphenol (-)-epicatechin gallate disrupts the secretion of virulence-related proteins by *Staphylococcus aureus*. *Lett Appl Microbiol.* 2008;46(2):181-185.
doi: 10.1111/j.1472-765X.2007.02296.x
36. Friedman M, Henika PR, Levin CE, *et al.* Antimicrobial activities of tea catechins and theaflavins and tea extracts against *Bacillus cereus*. *J Food Prot.* 2006;69(2):354-361.
doi: 10.4315/0362-028x-69.2.354
37. Cui Y, Oh YJ, Lim J, *et al.* AFM study of the differential inhibitory effects of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against Gram-positive and Gram-negative bacteria. *Food Microbiol.* 2012;29(1):80-87.
doi: 10.1016/j.fm.2011.08.019
38. Stapleton PD, Taylor PW. Methicillin resistance in *Staphylococcus aureus*: mechanisms and modulation. *Sci Prog.* 2002;85(1):57-72.
doi: 10.3184/003685002783238870
39. Dmitriev BA, Toukach FV, Holst O, *et al.* Tertiary structure of *Staphylococcus aureus* cell wall murein. *J Bacteriol.* 2004;186(21):7141-7148.
doi: 10.1128/JB.186.21.7141-7148.2004
40. Rodriguez-Lazaro D, Alonso-Calleja C, Oniciuc EA, *et al.* Characterization of biofilms formed by foodborne methicillin-resistant *Staphylococcus aureus*. *Front Microbiol.* 2018;9:3004.
doi: 10.3389/fmicb.2018.03004
41. Romaniuk JA, Cegelski L. Bacterial cell wall composition and the influence of antibiotics by cell-wall and whole-cell NMR. *Philos Trans R Soc Lond B Biol Sci.* 2015;370(1679):20150024.
doi: 10.1098/rstb.2015.0024
42. Brand C, Newton-Foot M, Grobelaar M, *et al.* Antibiotic-induced stress responses in Gram-negative bacteria and their role in antibiotic resistance. *J Antimicrob Chemother.* 2025;80(5):1165-1184.
doi: 10.1093/jac/dkaf068
43. Sethuvel DPM, Bakthavatchalam YD, Karthik M, *et al.* β -Lactam resistance in ESKAPE pathogens mediated through modifications in penicillin-binding proteins: an overview. *Infect Dis Ther.* 2023;12(3):829-841.
doi: 10.1007/s40121-023-00771-8