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Synergy with farnesol rejuvenates colistin activity against Colistin-resistant Gram-negative bacteria *in vitro* and *in vivo*

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ABSTRACT

Colistin (COL) is considered the last line of treatment against infections due to multidrug-resistant (MDR) Gram-negative bacteria (GNB). However, the increasing number of colistin-resistant (COL-R) bacteria is a great threat to public health. In this study, a strategy of combining farnesol (FAR), which has anti-inflammatory and antitumor properties, with COL to restart COL activity was proposed. The synergistic effect of FAR combined with COL against COL-R GNB *in vivo* and *in vitro* were investigated. The excellent synergistic antibacterial activity of the COL–FAR combination was confirmed by performing the checkerboard assay, time-killing assay, and LIVE/DEAD bacterial cell viability assay. Crystal violet staining and scanning electron microscopy results showed that COL–FAR prevented biofilm formation and eradicated pre-existing mature biofilm. Cytotoxicity assay showed that FAR at 64 μg/mL was not cytotoxic to RAW264.7 cells. *In vivo* infection experiments showed that COL–FAR increased the survival rate of infected *Galleria mellonella* and decreased the bacterial load in a mouse thigh infection model. These results indicate that COL–FAR is a potentially effective therapeutic option for combating COL-R GNB infections.

1. Introduction

Antibiotic resistance rates of Gram-negative bacteria (GNB) are increasing worldwide, which is a serious global crisis [1,2]. The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) GNB with multiple resistance mechanisms is a huge and increasing threat because these pathogens are resistant to almost all clinically available antibiotics [3–5]. As there are few possible alternatives for treating infections due to drug-resistant pathogens, and the development of new antibiotics is slow, colistin (COL) is usually used and is considered a drug of last resort [6,7].

Colistin is from a structurally distinct class of non-ribosomal, cyclic oligopeptide antimicrobials. The Food and Drug Administration (FDA) approved COL as an antibiotic in 1959 for treating infections due to MDR–GNB [2]. Colistin was then replaced with other, safer, antimicrobials because of the nephrotoxicity and neurotoxicity associated with the drug. However, COL has been used again in recent years because of the emergence of carbapenem resistance in *Enterobacteriaceae*. The reuse of COL has led to the global emergence of colistin-resistant (COL-R) bacteria, thereby further aggravating the current antimicrobial resistance situation [8]. Therefore, new treatment strategies are urgently required to prevent the emergence of drug-resistant pathogens and/or improve the efficacy of COL. The combination of plant extracts, antimicrobial peptides, and other nontraditional antimicrobial drugs and antibiotics is a new treatment strategy for combating bacterial resistance [9–11].

Farnesol (FAR) is a sesquiterpene alcohol that is mainly found in the essential oils of plants. Previous studies have shown that FAR has anticancer and anti-inflammatory properties and can alleviate allergic asthma, gliosis, and edema [12–14]. FAR destroys the integrity of the cell membrane of *Acinetobacter baumannii (A. baumannii)*, changes the morphology of bacterial cells, and attenuates their virulence [15]. The combination of FAR and gentamicin has a synergistic effect against methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *S. aureus* [16]. However, no study has investigated the synergistic antibacterial activity of COL–FAR against four COL-R GNB and their antibiofilms.

In this study, the antibacterial and antibiofilm synergistic activity of the COL–FAR combination against COL-R GNB was investigated *in vitro*, and the potential effects of this combination using the *Galleria mellonella* infection model and mouse thigh infection model were determined *in vivo*.

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2. Materials and Methods

2.1. Antibiotics and Solvents

FAR was purchased from MedChemExpress (MCE) Co., Ltd., NJ, USA and dissolved in dimethyl sulfoxide (DMSO) [2% (vol/vol)] (Sigma-Aldrich, Saint Louis, MO, USA). All antibiotics, including COL, aztreonam (ATM), ceftazidime (CAZ), cefepime (FEP), imipenem (IPM), ciprofloxacin (CIP), levofloxacin (LVX), gentamicin (GEN), and tobramycin (TOB), were purchased from Wenzhou Kangtai Biological Technology Co., Ltd., Zhejiang, China. For the following tests, cation-adjusted Mueller–Hinton broth (CAMHB) medium and Luria-Bertani (LB) broth (Thermo Fisher Scientific, Waltham, MA, USA) were utilized. The LIVE/DEAD BacLight Bacterial Viability Kit was purchased from Invitrogen, USA. The ROS Assay Kit was purchased from Beyotime, Shanghai, China.

2.2. Bacterial Isolates and Growth Conditions

A total of 40 non-duplicate Gram-negative clinical isolates were recovered from the First Affiliated Hospital of Wenzhou Medical University in China, including COL-R *A. baumannii* (n = 10), *Pseudomonas aeruginosa (P. aeruginosa)* (n = 10), *Klebsiella pneumoniae (K. pneumoniae)* (n = 10), and *Escherichia coli (E. coli)* (n = 10). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS; bioMérieux, Lyon, France) was used to identify all isolates. For all subsequent uses, all strains were maintained in LB broth with 30% glycerol at -80°C. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 (the National Center of Clinical Laboratory, NCCL) served as the quality control.

2.3. Antimicrobial Susceptibility Tests

Broth microdilution in CAMHB was used to evaluate the minimum inhibitory concentrations (MICs) of all antibiotics and FAR. Briefly, successive concentrations of antibiotics were prepared in a 96-well plate. The bacteria were adjusted to 0.5 McFarland (0.5 McFarland = 1.5×10^8 CFU/mL) in sterile saline and diluted 1:100 in CAMHB. Then, 100 µL of the bacterial solution was added to the wells with the ultimate bacterial concentration of 7.5×10^5 CFU/mL and the plate was incubated at 37°C for 16–18 h. The lowest concentration of an antibiotic that could completely inhibit the bacterial growth was judged as the MIC. Each experiment was repeated three times independently. The results were interpreted with reference to the latest Clinical and Laboratory Standards Institute (CLSI).

2.4. Checkerboard Assays

The synergistic activity of FAR and COL was evaluated using the checkerboard method as previously described [17], with slight modifications. Colistin was selected as medication A and 2-fold serially diluted along the X-axis, and FAR was diluted 2-fold serially along the Y-axis, creating a 12×8 matrix. The overnight bacterial culture was adjusted to 0.5 McFarland in sterile saline water and then diluted 1:100 in CAMHB. Subsequently, 100 µL of the bacterial suspension was transferred into each well of a 96-well microplate to give a final bacterial concentration of 7.5×10^5 CFU/mL. The microplates were then incubated at 37°C for 16–20 h, and the results were observed. The fractional inhibitory concentration index (FICI) was used to determine the synergistic effect of FAR and COL, where FICI was calculated using the following formula: FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). An FICI of ≤ 0.5 indicated synergy, an FICI of 0.5–4 indicated indifference, and an FICI of > 4 indicated antagonism [18]. Each test was conducted in triplicate.

2.5. Time-Kill Assays

The time-kill assay was performed according to published methodology, with some minor changes [18]. Briefly, 8 strains were selected as the experimental strains: COL-R *A. baumannii* (n = 2), *P. aeruginosa* (n = 2), *K. pneumoniae* (n = 2), and *E. coli* (n = 2). The ultimate concentrations of COL and FAR were 0.5–2 µg/mL and 16–128 µg/mL, respectively, according to the checkerboard assay. The bacteria were exposed to FAR and COL either alone or in combination at a concentration of 1×10^6 CFU/mL, with the tube containing CAMHB alone serving as the growth control. The tubes were incubated at 37°C, and viable cells were counted by plating 100 µL of the samples on antibiotic-free Mueller–Hinton agar plates at 0, 2, 4, 6, 12, and 24 h, after appropriate dilutions with saline. After 16–18 h of incubation at 37°C, the bacterial colonies were enumerated. For the two-drug combination compared with either drug alone, a reduction in the number of colonies of 3 $\log_{10}$ by 24 h was considered to indicate a bactericidal action, and a 2 $\log_{10}$ reduction by 24 h was considered to indicate a synergistic action [19]. Next, the means and standard deviations (SDs) of viable CFU were estimated and plotted on a semilogarithmic graph.

2.6. LIVE/DEAD bacterial cell viability assay

The LIVE/DEAD BacLight Bacterial Viability Kit was used the LIVE/DEAD bacterial cell viability assay. After treating bacteria with FAR (32 µg/mL) and COL (2 µg/mL) separately or in combination at 37°C for 4 h, the bacteria were stained with propidium iodide (PI) and SYTO9 as per the manufacturer's instructions. The samples were excited at wavelengths of 488 nm and 561 nm for emission at 530 nm (green) and 617 nm (red), respectively, and a confocal microscope (Nikon A1R-SIM-STORM, Japan) was used to view the samples [20].

2.7. Biofilm Formation and Eradication Assay

The synergy of FAR and COL on the formation and eradication of biofilms was investigated [21]. At the start, 0.5 McFarland of suspension was prepared, and 10^5 CFU of bacteria was treated with FAR and COL at final concentrations of 8–64 µg/mL and 0.5–2 µg/mL, respectively. After incubating for 24 h at 37°C, the wells were rinsed with phosphate-buffered saline (PBS) to remove planktonic bacteria. Next, 1% solution of crystal violet (Beijing Solarbio Biotechnology Co., Ltd., China) was added to an air-dried plate and incubated at 37°C for 15 min. After washing in sterile PBS and air-drying, 200 µL of 95% ethanol + 5% acetic acid was added to the 96-well plates to dissolve the crystal violet. Then, a fresh 96-well plate was used to transfer the dissolved crystal violet. The biomass of the biofilm was calculated by measuring the absorbance at 595 nm. At least three separate experiments were conducted. The drug was added before the biofilm had formed in the biofilm-formation experiments and after the biofilm had matured in the eradication experiments.

2.8. Scanning Electron Microscope (SEM)

Silicon wafers (3×3 mm) were placed into 24-well plates to create a biofilm-forming surface for SEM investigations. Then, 10 µL of the fresh cell suspension was added to 990 µL of LB broth containing FAR and/or COL to achieve a cell density of 10^6 CFU/mL, which was then incubated for 24 h. The silicon wafers were cleaned three times with PBS, separately fixed with 2.5% glutaraldehyde, and dehydrated for 5 min using a gradient series of ethanol (30, 50, 70, 80, 90, 95, and 100%). The final samples were gold-sprayed, air-dried, and then SEM-evaluated (Hitachi SU8010, Japan).

2.9. The Study of Antimicrobial Mechanism

To detect reactive oxygen species (ROS), 500 µL of bacterial suspension (10^6 CFU/mL) containing 10 µM 2'-7'dichlorofluorescin diacetate (DCFH-DA) was incubated at 37°C for 45 min. After loading with DCFH-DA, the cells were treated with FAR (16 µg/mL) and/or COL (2 µg/mL) for 2 h at 30°C. Sterile distilled water was selected as the blank control. With excitation at 488 nm and emission at 535 nm, all samples were scanned by a flow cytometer (BioTek Synergy NEO2, USA).

2.10. In Vitro *Cytotoxicity Assays*

The cytotoxicity assay was conducted on RAW264.7 cells (ATCC, Manassas, VA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. A total of 1×10^5 cells in 100 µL of cell suspension were added to each well of a 96-well microplate. Then, the media was supplemented with 10 µL FAR with the final concentration of 8, 16, 32, and 64 µg/mL and 10 µL of the mixture of FAR and COL (1, 2 µg/mL). The plate was incubated for 12 h, followed by the addition of 10 µL of CCK-8 (Dojindo Laboratories, Japan) to each well. The plate was then incubated for 1 h in the dark at room temperature, and the absorbance was measured at 450 nm using a microplate reader. The experiment was conducted in triplicate.

2.11. Galleria mellonella *Infection Model*

The synergistic effect of FAR and COL was evaluated by measuring the survival rate using the modified version of the *Galleria mellonella* infection model *in vivo* [22,23]. Milky white *G. mellonella* larvae weighing 250–300 mg were chosen for this experiment. Four strains (TL2314, FK6556, BM2431, and DC5286) were selected. Overnight cultures were diluted to 1.5×10^5 CFU/mL, 1.5×10^7 CFU/mL, 1.5×10^5 CFU/mL, and 1.5×10^7 CFU/mL, respectively. The study included the control, FAR monotherapy, COL monotherapy, and combination groups. Each group was injected with 10 µL bacterial suspension in the rear left proleg of *G. mellonella* using a microinjector. After 2 h, 10 µL of sterile saline water was administered to the control group, and COL (2 µg/mL × 7) and/or FAR (64 µg/mL × 7) were administered to the monotherapy/combination groups. Larvae were incubated in the dark at 37°C, and survival rates were recorded for 7 days. Larvae that repeatedly failed to respond to physical stimuli were considered dead. The primary outcome measure for the insect model was the rapidity and extent of mortality of *G. mellonella*, which was assessed by Kaplan–Meier analysis and log-rank test.

2.12. Mouse Infection Model

To establish the thigh infection model of neutropenic mice, female BALB/c mice (5–6 weeks old, Charles River, Hangzhou, China) were utilized in a manner consistent with the Chinese National Standards for Laboratory Animals (GB 14925–2010). The Zhejiang Association for Science and Technology SYXK approved these analyses (ID: SYXK[Zhejiang] 2018-0017) and were consistent with the Wenzhou Laboratory Animal Welfare and Ethics standards.

Briefly, mice were intraperitoneally injected with 150 mg/kg and 100 mg/kg cyclophosphamide 4 days and 1 day before the bacterial injection. TL2314 was selected as the experimental strain, and the mice were assigned to four groups of three mice each. Then, 100 µL of 1.5×10^7 CFU/mL bacterial suspension was injected into the thigh muscle of each mouse. At 2 h post-bacterial inoculation, the mice received intraperitoneal injections of the following treatments: 1) sterile saline water (untreated group), 2) COL (5 mg/kg), 3) FAR (50 mg/kg), and 4) COL–FAR combination. After 24 h, the mice were euthanized via cervical dislocation, and the posterior thigh tissues were collected, weighed, homogenized, diluted in PBS, and spread on Mueller–Hinton agar plates for CFU quantification.

2.13. Statistical Analysis

All experiments were performed in triplicate, and the results were evaluated using Prism 8 (GraphPad Software Inc., CA, USA). The data were presented as the mean ±SD. Statistical significance was determined using two-sample *t*-tests and log-rank tests and indicated as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Result

3.1. Antimicrobial Susceptibility Tests

The results showed that many COL-resistant strains had MDR phenotypes. As shown in Table S1, MICs of COL ranged from 4 to ≥128 µg/mL. MICs of FAR were ≥256 µg/mL for all strains, indicating that FAR did not show antibacterial activity against any tested strains.

3.2. Checkerboard Assays

The checkerboard assay was performed to determine the synergistic effects of COL–FAR on 40 COL-R clinical isolates. The MICs of the tested strains to COL decreased 4–256 times in the presence of FAR (Table 1). Checkerboard assay showed that the combination of COL–FAR showed significant synergistic activity (FICI ≤ 0.5) against all tested strains.

3.3. Time-killing Assays

To further investigate the effect of the COL–FAR combination on clinical isolates, 8 COL-R experimental strains [*A. baumannii* (n = 2), *P. aeruginosa* (n = 2), *K. pneumoniae* (n = 2), and *E. coli* (n = 2)] were selected for the time-killing assay. The drug concentration of the time-killing assay was selected from the checkerboard assay with FICI ≤ 0.5. Colistin concentrations were 0.5, 1, and 2 µg/mL, and FAR concentrations were 16 and 32 µg/mL. The results showed that bacteria exhibited rapid growth without inhibition in the absence of drug treatment and in the presence of FAR alone (Figure 1). *P. aeruginosa* TL2314 treated with COL showed an inhibitory effect during the first 6 h, after which the effect of COL on bacteria was negligible. The COL–FAR combination significantly decreased bacterial growth (by >2 $\log_{10}$ CFU/mL) during the first 12 h compared with the monotherapy group. The growth of most strains was well inhibited after 12 h, particularly that of *A. baumannii* BM2412 and *A. baumannii* BM2431, which demonstrated good synergistic and bactericidal activity within 24 h. The antibacterial activity of COL–FAR against most experimental strains was maintained for 24 h after increasing the concentration of FAR (128 µg/mL).

3.4. LIVE/DEAD bacterial cell viability assay

To further determine the synergistic effect of COL–FAR on bacteria, *P. aeruginosa* TL2314 isolates were selected for LIVE/DEAD bacterial cell viability assay. SYTO9 stains all bacterial cells and exhibits green fluorescence, whereas PI only penetrates ruptured cells and exhibits red fluorescence (Figure 2). In the absence of drug treatment or in the presence of FAR alone, almost no red fluorescence was observed, indicating that most bacteria were still alive.

Table 1
FICI values for colistin/farnesol combinations against colistin-resistant GNB

Species	Strains	Monotherapy (μg/mL)		Combination (μg/mL)		FICI	Interpretation
		colistin	farnesol	colistin	farnesol		
	BM1539	4	≥256	0.5	8	≤0.15625	Synergistic
A. baumannii	BM1595	32	≥256	8	16	≤0.3125	Synergistic
	BM2349	4	≥256	0.25	4	≤0.0781	Synergistic
	BM2370	8	≥256	0.5	8	≤0.09375	Synergistic
	BM2390	4	≥256	0.25	4	≤0.0781	Synergistic
	BM2412	16	≥256	0.25	16	≤0.0781	Synergistic
	BM2431	8	≥256	2	8	≤0.28125	Synergistic
	BM2622	8	≥256	0.125	8	≤0.047	Synergistic
	BM7225	4	≥256	0.5	8	≤0.15625	Synergistic
	BM8090	16	≥256	0.125	16	≤0.07	Synergistic
P. aeruginosa	TL1671	32	≥256	8	16	≤0.3125	Synergistic
	TL1722	≥128	≥256	2	16	≤0.0781	Synergistic
	TL1736	8	≥256	0.5	16	≤0.125	Synergistic
	TL1744	64	≥256	2	16	≤0.09375	Synergistic
	TL2314	8	≥256	2	16	≤0.3125	Synergistic
	TL2294	16	≥256	2	32	≤0.25	Synergistic
	TL2917	8	≥256	0.5	16	≤0.125	Synergistic
	TL2967	8	≥256	0.5	16	≤0.125	Synergistic
	TL3008	≥128	≥256	32	32	≤0.375	Synergistic
	TL3086	≥128	≥256	16	32	≤0.25	Synergistic
K. pneumoniae	FK169	≥128	≥256	0.5	16	≤0.0664	Synergistic
	FK610	≥128	≥256	16	32	≤0.25	Synergistic
	FK1342	8	≥256	2	32	≤0.375	Synergistic
	FK1913	≥128	≥256	0.5	16	≤0.0664	Synergistic
	FK2066	4	≥256	0.5	8	≤0.15625	Synergistic
	FK3810	≥128	≥256	32	32	≤0.375	Synergistic
	FK3994	64	≥256	8	16	≤0.1875	Synergistic
	FK6556	4	≥256	0.125	16	≤0.09375	Synergistic
	FK6663	4	≥256	0.5	8	≤0.15625	Synergistic
	FK6696	64	≥256	2	16	≤0.09375	Synergistic
	DC90	16	≥256	1	16	≤0.125	Synergistic
	DC3539	4	≥256	0.5	8	≤0.15625	Synergistic
E. coli	DC3599	4	≥256	1	8	≤0.28125	Synergistic
	DC3737	8	≥256	2	32	≤0.375	Synergistic
	DC3806	8	≥256	1	32	≤0.25	Synergistic
	DC3846	16	≥256	4	16	≤0.3125	Synergistic
	DC4887	16	≥256	4	32	≤0.375	Synergistic
	DC5262	64	≥256	2	16	≤0.09375	Synergistic
	DC5286	4	≥256	0.125	16	≤0.15625	Synergistic
	DC7333	8	≥256	1	16	≤0.1875	Synergistic

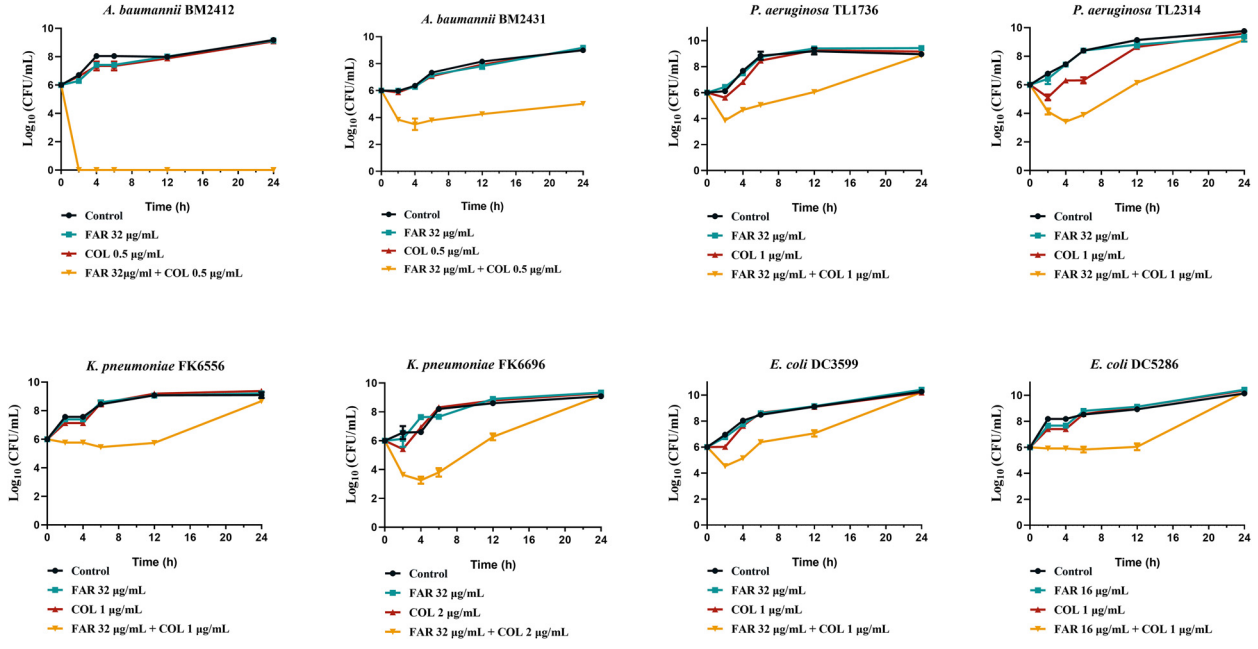

Figure 1. Time-kill curves of the treatments and control against colistin-resistant Gram-negative bacteria (GNB).

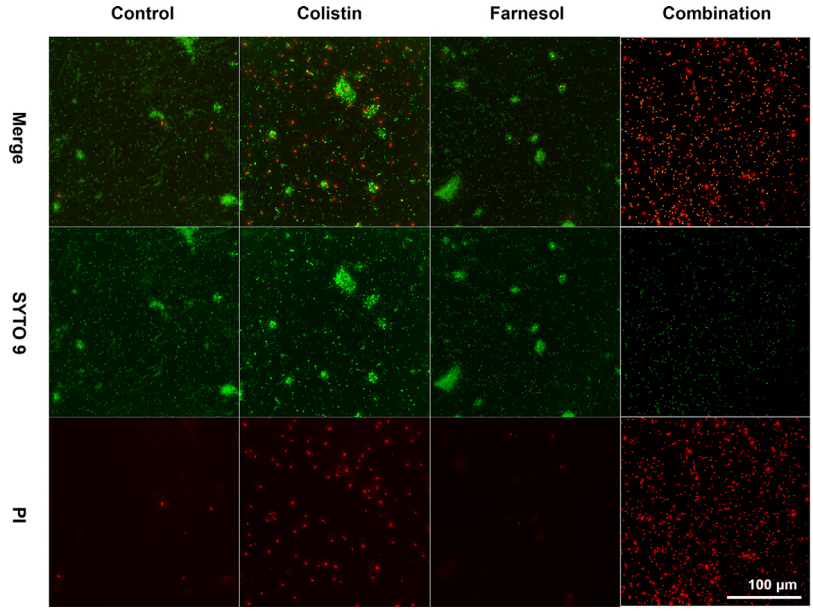

Figure 2. Fluorescence images of colistin-resistant *P. aeruginosa* TL2314 after incubation with natural saline(NS) and COL and/or FAR using LIVE/DEAD bacterial cell viability assay.

Compared with the control group, the COL group showed a small amount of red fluorescence, indicating a certain antibacterial activity. However, a large amount of green fluorescence indicated that many bacteria still survived. However, COL–FAR combined treatment showed a high intensity of red fluorescence and weakened green fluorescence, indicating that cells underwent cell permeability changes after combination treatment.

3.5. Effect of COL–FAR on Bacterial Biofilm

The effects of the COL–FAR combination on biofilm formation and mature biofilm eradication were investigated using crystal violet staining. The combination of COL–FAR significantly inhibited biofilm formation in all experimental strains compared with the control group and the single-drug group ($P < 0.5$) (Fig. 3). Fig. 4 shows the results of the mature biofilm-eradicating experiment. The combination of COL–FAR had a strong effect on eradicating the biofilm formed by all experimental strains ($P < 0.5$). Notably, the biofilm-forming ability of some strains was also decreased in the presence of FAR alone, indicating that FAR had an inhibitory effect on biofilm formation. These results are consistent with those of previous studies [15].

3.6. Scanning Electron Microscopy

Biofilm structures affected by COL, FAR, or COL–FAR were visualized by SEM (Fig. 5). SEM images showed that the control group and the single-drug treatment group formed a complete and dense biofilm. However, under COL–FAR, the biofilm was significantly damaged, the number and density of biofilm decreased, and the bacterial cells showed distinct morphological damages at 7000 × magnification.

3.7. Mechanisms for Drug Synergy

The COL-FAR combination significantly elevated ROS levels (Fig. 6), which may have played a role in the antibacterial activity.

3.8. In Vivo Cytotoxicity Assays

Cytotoxicity tests were performed to determine whether FAR alone and in combination with COL can be used safely *in vivo*. The results showed that COL–FAR is not toxic to cells and has application prospects *in vivo* at the concentration used in this experiment (Fig. 7).

3.9. Evaluation of Antibacterial Effect In Vivo

The *G. mellonella* survival experiment was established to verify the antibacterial activity of the COL–FAR combination *in vivo*. *A. baumannii* BM2431, *P. aeruginosa* TL2314, *K. pneumoniae* FK6556, and *E. coli* DC5286 were selected as experimental strains. For the untreated and monotherapy groups, almost no survival was observed after 168 h. For *A. baumannii* BM2431, *P. aeruginosa* TL2314, and *E. coli* DC5286, the survival rate with the COL–FAR combination reached 80% after 168 h ($P < 0.5$). For *K. pneumoniae* FK6556, no statistical difference was found between the combination group and the single-drug group; however, 50% of the combination group was alive on day 7, whereas all the monotherapy and the control group had died (Fig. 8).

A mouse thigh infection model of *P. aeruginosa* TL2314 was also established. Monotherapy with 5 mg/kg COL or 50 mg/kg FAR only slightly inhibited the growth of *P. aeruginosa* TL2314, whereas the combination of COL and FAR reduced the bacterial count in the thigh muscles by 0.4 $\log_{10}$ CFU/g ($P < 0.5$) (Fig. 9). These results indicated that the COL-FAR combination could effectively prevent COL-R GNB infection, thereby demonstrating potential clinical application.

4. Discussion

Increasing antibiotic resistance in MDR–GNB due to drug overuse and misuse is causing serious health problems worldwide. Colistin was discontinued because of its nephrotoxicity and neurotoxicity; however, it has been reactivated as a drug of last resort for treating MDR-GNB-associated infections [24]. The use of COL has led to the emergence of COL-R bacteria, and there is an urgent need to develop new, effective, broad-spectrum antimicrobial

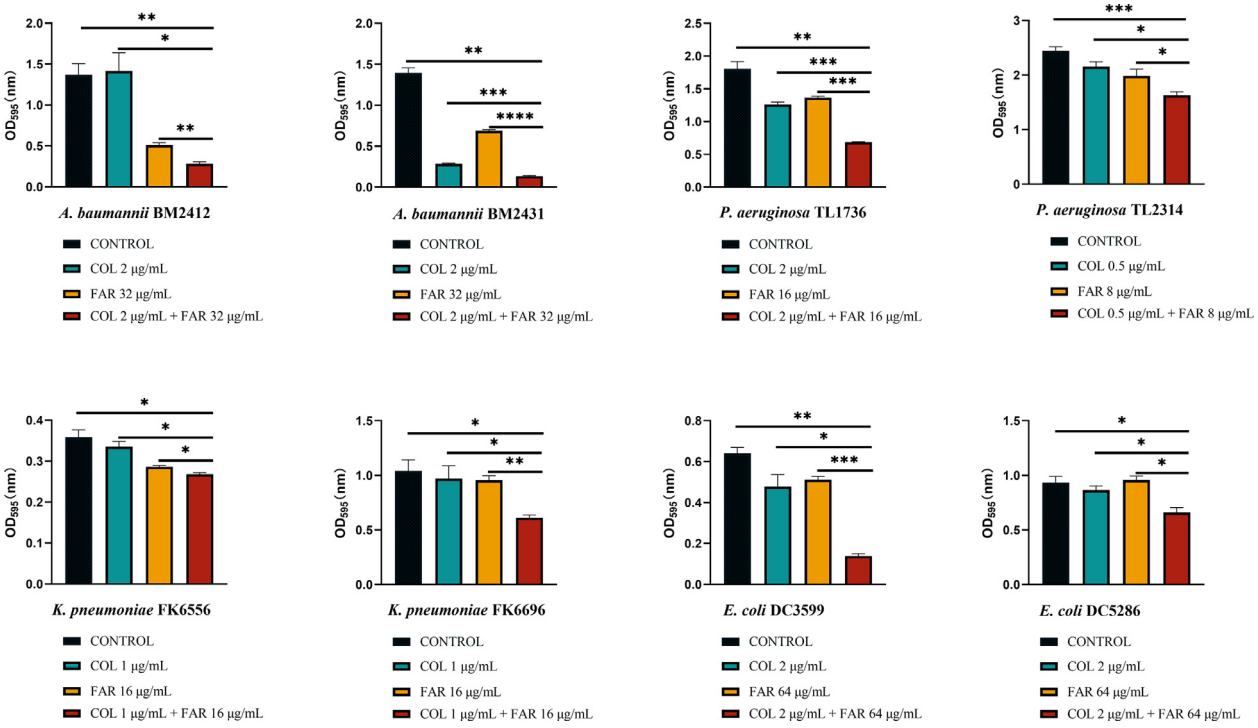

Figure 3. Colistin (COL), farnesol (FAR), and the COL–FAR combination inhibited biofilm formation of GNB. The concentration of drugs was derived from the checkerboard assay. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were analysed using the Student' s *t*-test. The experiments were performed three times.

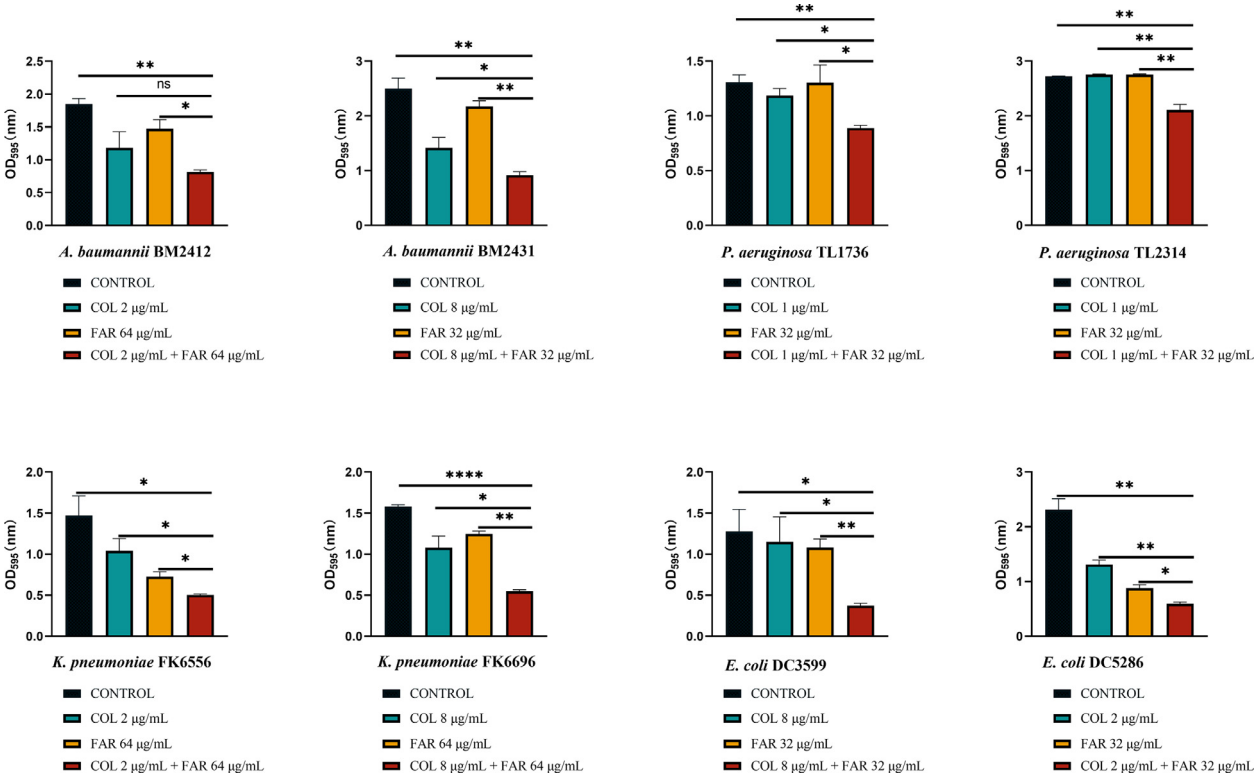

Figure 4. Colistin (COL), farnesol (FAR), and the COL–FAR combination eradicated mature biofilm of GNB. The concentration of drugs was derived from the checkerboard assay. ns, not statistically significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ analysed using the Student's *t*-test. The experiments were performed three times.

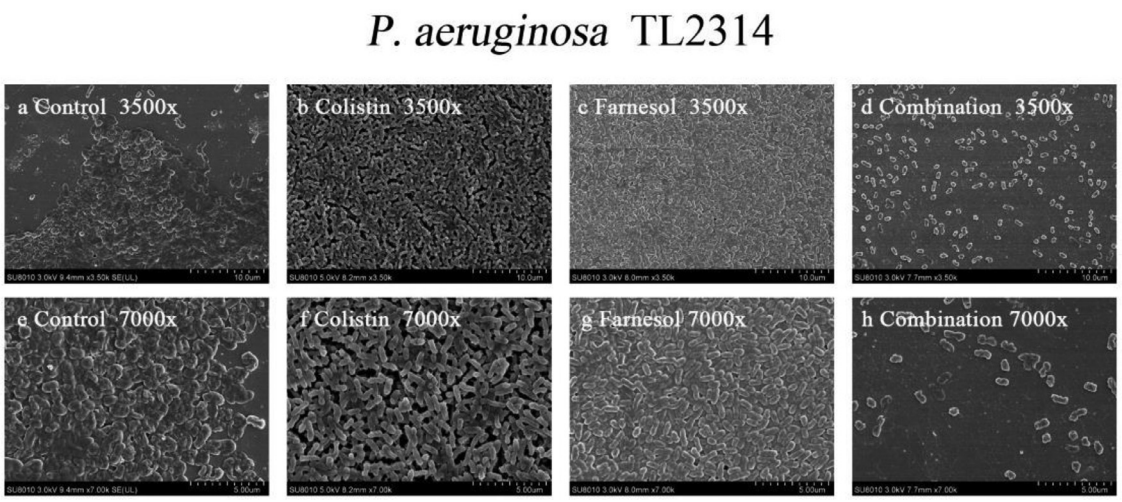

Figure 5. SEM image of *P. aeruginosa* TL2314 depicting the result of biofilm and bacterial morphology between various groups. (a) LB broth-control, 3500 ×, (b) COL (2 μg/mL), 3500 ×, (c) FAR (32 μg/mL), 3500 ×, (d) COL (2 μg/mL) + FAR (32 μg/mL), 3500 ×, (e) LB broth-control, 7000 ×; (f) COL (2 μg/mL), 7000 ×, (g) FAR (32 μg/mL), 7000 ×; (h) COL (2 μg/mL) + FAR (32 μg/mL), 7000 ×.

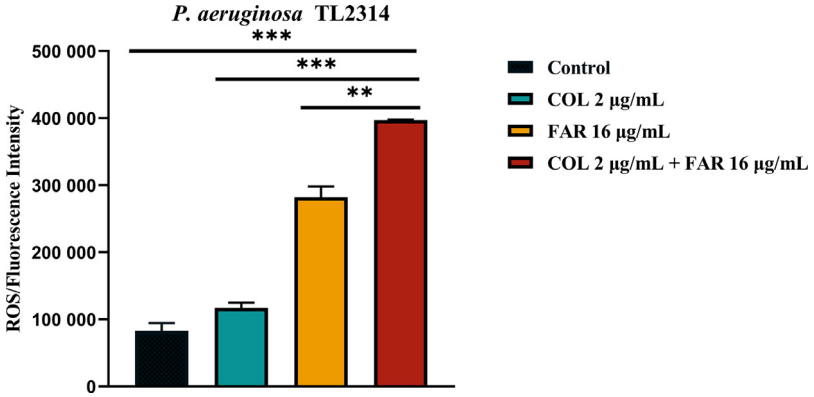

Figure 6. Reactive oxygen species (ROS) level of colistin *P. aeruginosa* TL2314 after different treatments. COL, colistin; FAR, farnesol.

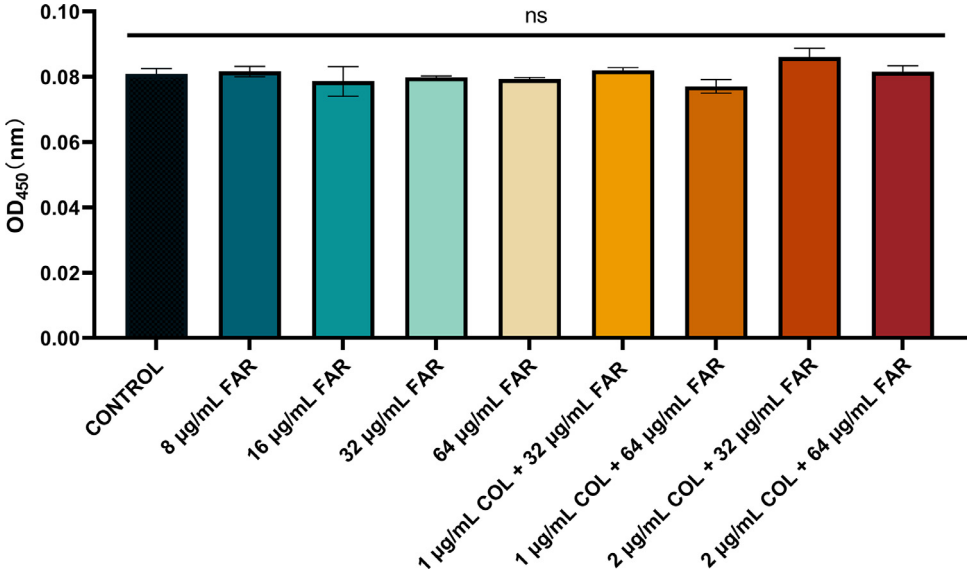

Figure 7. Cytotoxic effect of colistin (COL) and/or farnesol (FAR) at different concentrations against RAW 264.7 murine macrophage cell line. ns, not statistically significant. The results are presented as the mean and standard deviation of three independent experiments.

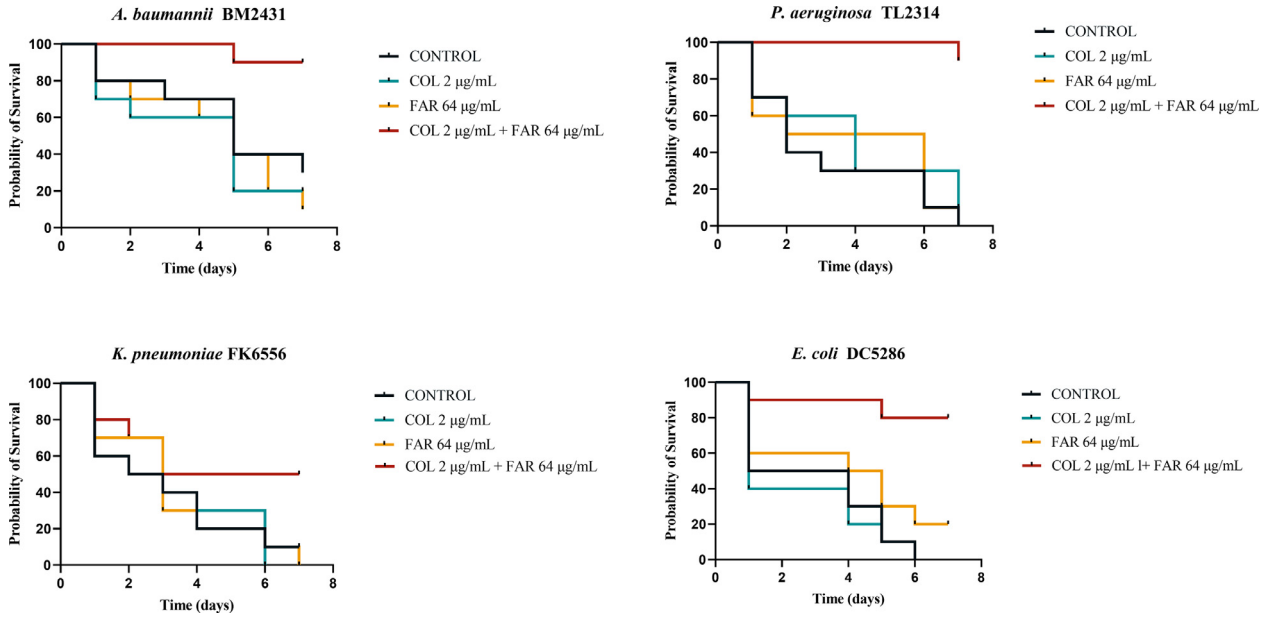

Figure 8. Survival rate of *Galleria mellonella* for different therapies. TL2314, FK6556, BM2431, and DC5286 as the experimental strains. The survival rate of *G. mellonella* was recorded for 7 days. COL, colistin; FAR, farnesol.

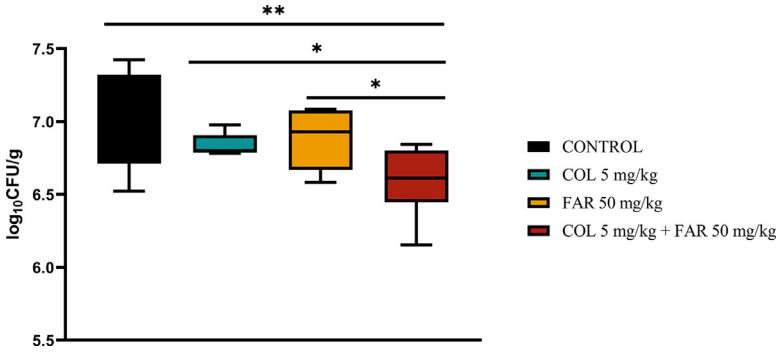

Figure 9. Quantified $\log_{10}$ CFU/g in mice 24 h after different treatments. Changes in the thigh muscles ($\Delta\log_{10}$ CFU/g) of mice after single or combined treatment of colistin-resistant *P. aeruginosa* TL2314 strain (n = 6) with different doses for 24 h. COL, colistin; FAR, farnesol.

strategies to prevent and treat COL-R GNB infection [2]. Combination antibiotic therapy is considered a potential option. Herein is described a novel strategy that was developed to restore the effect of COL on COL-R GNB through a combination of COL–FAR.

FAR is a sesquiterpene alcohol with antioxidant, anti-inflammatory, chemoprophylaxis, anti-anxiety, antidepressant, analgesic, and neuroprotective effects [25–27]. Studies have shown that FAR may be a promising adjuvant for antimicrobial drugs. High concentrations of FAR showed antimicrobial potential against *S. aureus* biofilms [28] and disrupted the cell membrane of *A. baumannii* [15]. However, the effect of FAR on COL has not been completely reported.

To the best of our knowledge, this is the first study to show the synergistic effect of COL–FAR on different COL-R GNB strains (*A. baumannii, P. aeruginosa, K. pneumoniae*, and *E. coli*) *in vitro*. The checkerboard assay showed that the MICs of COL decreased 4–256 times when it was combined with FAR. The results of the time-killing assay also indicated that the COL–FAR combination had significant synergistic antibacterial activity in all tested strains. The experimental results of some strains in the time-killing assay showed that the drug combination had a good inhibitory effect on the growth of bacteria during the first 12 h. However, the inhibitory effect was attenuated after 12 h, and there was no distinct inhibitory effect after 24 h for the majority of the experimental strains. The effective 24 h synergistic antibacterial effect of this drug combination against *A. baumannii* is consistent with previous reports [15]. Notably, the difference in the inhibitory effect with time may be related to the presence of persistent bacteria [15,29]. Therefore, further investigations are required to determine the reason for the weakened antibacterial effect of the COL–FAR combination after 12 h. The time-killing assay was repeated with increased concentrations of FAR and no change in COL concentration. The results indicated that higher concentrations of FAR without a change in the COL concentration significantly increased the inhibitory activity and drug duration. This finding indicates that the same dose should be administered again within 12–24 h after administration, as recommended in clinical practice [30,31], or the concentration of the combined drug should be increased when using this drug combination therapeutically.

Biofilms increase the adaptability of bacteria to the environment, making it difficult for conventional antibiotics to penetrate. They are also one of the main causes of repeated infections [32,33]. FAR is an excellent inhibitor of *Candida albicans, S. aureus,* and *P. aeruginosa* biofilms [28,34,35]. In this study, COL–FAR inhibited the formation of bacterial biofilms and showed a good eradication effect on mature biofilms. SEM results showed that COL–FAR distinctly damaged the bacterial biofilm, and the bacterial morphology. FAR monotherapy did not show a significant effect on

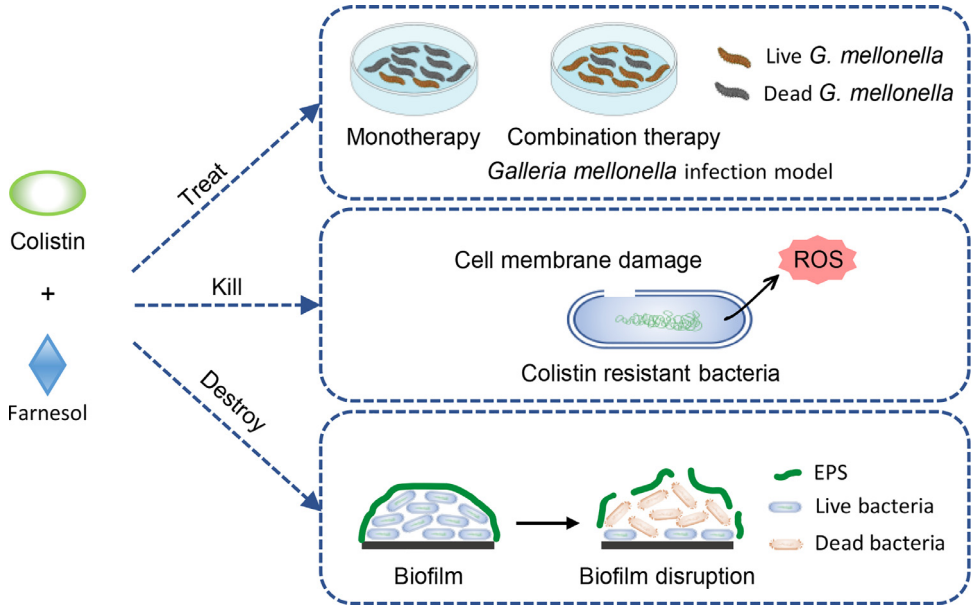

Figure 10. Mechanism of FAR–COL combination eradication of colistin-resistant GNB. EPS, Extracellular Polymeric Substances.

biofilms, perhaps because the concentration used was significantly lower than that used in previous studies. However, the results indicated that the COL–FAR combination can be used to inhibit biofilm formation at an early stage and eradicate biofilm at a later stage.

Studies have shown that FAR interferes with membrane structure and affects bacterial cell division [15]. FAR is lipophilic and may accumulate in bacterial membranes, which may lead to perturbations in membrane permeability and mobility [15,36]. Many sesquiterpenes can also destroy the barrier function of the cell membrane [37]. The results of the LIVE/DEAD bacterial cell viability assay showed that the PI uptake of cells in the combined drug treatment group was significantly increased and the cell integrity was damaged compared with those of the control group. These results indicated that FAR can increase COL activity by destroying the membrane, thereby increasing COL binding and antimicrobial ability.

Cells continuously produce ROS during aerobic metabolism and protect against ROS overproduction through antioxidant defense systems. Oxidative stress is induced when ROS production overwhelms the natural antioxidant defenses of the cell. Studies have shown that ROS act as signaling molecules in various pathways regulating cell survival and death. The COL–FAR combination may cause bacterial cell damage and apoptosis by significantly increasing the level of ROS (Fig. 10). However, the specific reasons behind this deserve further research [38–40].

The safety of the COL–FAR combination was evaluated using cytotoxicity assays in the current study. The results indicated that 64 μg/mL FAR alone was not cytotoxic to RAW264.7 cells nor were the combined concentrations used in this study. Although FAR has not yet been used in clinical trials, no adverse reactions have been observed in experimental animals in related *in vivo* experiments [41], and its analogue, perillyl alcohol, has been used in phase I and II clinical trials [38].

The *G. mellonella* infection model and mouse infection model were used in the current study to determine the antibacterial efficacy of the COL–FAR combination *in vivo*. The COL–FAR combination significantly decreased the number of bacteria in mice and increased the survival rate of *G. mellonella* compared with that of the control group.

To conclude, the synergistic antibacterial effect of the COL–FAR combination was confirmed in various *in vitro* and *in vivo* experiments, and indicates a potential new treatment strategy for infections due to MDR bacteria. The safety and efficacy of currently developed FAR-related nanomaterials on the development of drug resistance also provide more possibilities for their clinical use [42,43].

Declarations

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Ethical Approval: SYXK[Zhejiang] 2018-0017.

Sequence Information: Not applicable.

Author contributions

Yijia Han conceived, designed, and supervised the study, and wrote the drafts of the manuscript. Yi Zhang supervised and assisted in the experiment, and collected the data. Weiliang Zeng provided guidance and assisted with the experiment. Zeyu Huang collected data and assisted in the experiment. Haojun Cheng and Jingchun Kong collected data. Chunquan Xu analysed the data. Mengxin Xu provided ideas. Tie-Li Zhou commented on and revised drafts of the manuscript. Jianming Cao commented on and revised drafts of the manuscript. All authors read and approved the final manuscript.

Data availability statement

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.106899.

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