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**Synergy of PMBN and closantel against *mcr-1*-carrying
multidrug-resistant *E. coli* and *Salmonella* via membrane
permeabilization-driven accumulation and metabolic
disruption**

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Abstract

Background

The rapid dissemination of plasmid-mediated colistin resistance gene *mcr-1*, especially within livestock settings over the past decade, has progressively undermined colistin, a cornerstone antimicrobial in certain veterinary indications. Drug repurposing and antimicrobial combination therapy offer promising approaches to enhance the activity of existing antimicrobials. Here, we investigated whether polymyxin B nonapeptide (PMBN)-mediated outer membrane (OM) permeabilization could restore the antibacterial activity of closantel (CLO) against multidrug-resistant (MDR) GNB and sought to elucidate the underlying mechanism.

Results

The CLO-PMBN exhibited potent synergistic bactericidal activity against all of 30 tested strains, including *mcr-1*-carrying MDR isolates, with fractional inhibitory concentration indices (FICI) ranging from 0.012 to 0.250. Time-kill assays demonstrated a $> 3 \lg$ CFU/mL reduction within 4 h and complete eradication by 24 hours (h) for all four *E. coli* strains tested, whereas single agents were ineffective. Notably, this combination effectively curtailed resistance development and, more importantly, significantly improved therapeutic outcomes *in vivo*, while maintaining a favorable hemolytic safety profile. LC-MS/MS demonstrated that PMBN markedly enhanced intracellular accumulation of CLO by overcoming the OM barrier of *E. coli*. Elevated intracellular CLO exposure led to adenosine triphosphate (ATP) depletion, efflux impairment, reactive oxygen species (ROS) accumulation, and widespread metabolic reprogramming, including energy metabolism, redox balance, and membrane-associated pathways.

Conclusions

PMBN permeabilizes the OM to drive intracellular CLO accumulation, culminating in bioenergetic and metabolic collapse that restores CLO's bactericidal activity against *mcr-1*-carrying MDR *E. coli* and *Salmonella*.

Key words: Closantel; Polymyxin B nonapeptide; *Escherichia coli*; Synergistic

53 antibacterial effect; Metabolic disruption

Introduction

The global proliferation of multidrug-resistant (MDR) Gram-negative bacteria (GNB) has drastically curtailed therapeutic options in both human and veterinary medicine[1, 2]. In particular, the rapid dissemination of plasmid-mediated colistin resistance gene *mcr-1*, especially within livestock settings over the past decade, has progressively undermined colistin, a cornerstone antimicrobial in certain veterinary indications[3-5]. Coupled with the slow pace of antibiotic discovery, these challenges have stimulated growing interest in drug repurposing and combination therapies as alternative strategies to combat antimicrobial resistance[6, 7].

Closantel (CLO) is a halogenated salicylanilide anthelmintic that exerts its antiparasitic activity by uncoupling oxidative phosphorylation and disrupting cellular energy metabolism [8]. Recent studies have demonstrated that CLO and structurally related salicylanilides exhibit potent antibacterial activity against Gram-positive bacteria (GPB), including methicillin-resistant *Staphylococcus aureus*, highlighting their potential for antimicrobial repurposing [9]. However, this activity is largely abolished against GNB because the lipopolysaccharide (LPS)-containing outer membrane (OM) constitutes a highly selective permeability barrier that markedly restricts the intracellular accumulation of hydrophobic compounds such as CLO [10]. Interestingly, CST combined CLO could against GNB, including *mcr-1*-carrying resistant isolates [11-13]. Nevertheless, the mechanistic basis of this synergy remains elusive. One possibility is that CST disrupts the OM, thereby promoting intracellular CLO accumulation and restoring its antibacterial effect. Alternatively, CLO may potentiate CST activity by attenuating bacterial polymyxin resistance mechanisms, such as MCR-1-mediated lipid A modification, PmrAB-dependent OM remodeling, or other adaptive responses associated with polymyxin resistance [14-16]. Given that CST possesses both OM permeabilizing and bactericidal activities, it remains challenging to discern whether the observed synergy primarily arises from enhanced intracellular CLO accumulation or from CST-mediated bacterial killing.

Polymyxin B nonapeptide (PMBN) provides an ideal tool to address this question. As

a deacylated derivative of polymyxin B, PMBN retains high affinity for lipid A and efficiently permeabilizes the Gram-negative OM, but lacks intrinsic bactericidal activity and the systemic toxicity associated with polymyxins [10, 17]. PMBN has therefore been widely used as an OM permeabilizer to potentiate the activity of antibiotics and hydrophobic compounds against GNB [18, 19]. By functionally separating OM permeabilization from bacterial killing, PMBN provides a well-defined experimental system to determine whether OM permeabilization alone is sufficient to restore the antibacterial activity of CLO.

In this study, we systematically investigated the antibacterial activity of CLO in combination with PMBN against MDR *Escherichia coli* (*E. coli*) and *Salmonella*, including *mcr-1*-carrying strains, using both *in vitro* and *in vivo* infection models. By integrating intracellular drug quantification, membrane permeability assays, and untargeted metabolomics, we aimed to determine whether PMBN-mediated OM permeabilization enhances intracellular accumulation of CLO and thereby restores its antibacterial activity against GNB.

Results

1. Synergistic activity of CLO and PMBN *in vitro*

Checkerboard assays were performed to evaluate the antibacterial activity of CLO in combination with PMBN against a panel of 30 *E. coli* and *Salmonella* isolates, including both CST-susceptible and *mcr-1*-carrying strains. CLO alone showed almost no detectable antibacterial activity against any isolate (MIC > 64 mg/L), whereas PMBN alone exhibited minimal activity (MIC > 256 mg/L). In contrast, combination treatment produced synergistic antibacterial activity against all isolates, with FICI values ranging from 0.012 to 0.250 (**Figure 1a, 1b**). FICI values were significantly lower in *mcr-1*-carrying isolates than in CST-susceptible isolates, whereas no significant difference was observed between *E. coli* and *Salmonella* isolates.

The bactericidal activity of the combination was further assessed by time-kill assays using four representative strains (*E. coli* MG1655, MG1655+pEGN-*mcr-1*, 47 (*mcr-1*-

negative isolate) and 44 (*mcr-1*-positive isolate)), which were subsequently selected for mechanistic investigations. Neither CLO nor PMBN alone substantially reduced bacterial viability during the 24 hours (h) incubation period. In contrast, the combination reduced viable counts by more than 3 lg CFU/mL within 4 h and completely eradicated detectable bacteria after 24 h in all four strains (**Figure 1c**).

2. CLO-PMBN delays resistance evolution

Resistance evolution was evaluated by serial passaging of *E. coli* 44 in the presence of subinhibitory antimicrobial concentrations for 30 consecutive days (**Figure 1d**). Continuous exposure to CST resulted in a progressive increase in MIC, reaching a fourfold increase by the end of the experiment. In contrast, repeated exposure to the CLO-PMBN combination did not increase the MIC throughout the study period.

3. CLO-PMBN retains activity under physiological conditions

The robustness of the CLO-PMBN combination was evaluated under physiologically relevant conditions (**Figure 2a**). Supplementation with Na⁺ or K⁺ had minimal effects on synergy (FICI = 0.094), whereas Mg²⁺ and Ca²⁺ partially reduced the synergistic activity but retained synergy (FICI < 0.5). Ethylenediaminetetraacetic acid (EDTA) enhanced antibacterial activity by further reducing the MIC of CLO in the presence of PMBN. Supplementation with 10% (v/v) Dulbecco's modified Eagle's medium (DMEM) to mimic host cell culture conditions slightly weakened the synergistic effect but maintained synergy (FICI = 0.156). In contrast, 10% (v/v) serum supplementation abolished synergy (FICI = 1), even though serum alone did not affect bacterial growth in the absence of antimicrobial treatment (**Figure S1**).

The haemolytic activity of CLO and PMBN was further evaluated using sheep erythrocytes (**Figure 2b**). Neither CLO nor PMBN, alone or in combination, caused appreciable haemolysis at concentrations up to 32 mg/L, with haemolysis remaining below 5% under all tested conditions.

4. In vivo therapeutic efficacy of CLO-PMBN combination

The therapeutic efficacy of the CLO-PMBN combination was evaluated in a murine peritonitis model using *mcr-1*-carrying *E. coli* 44 (**Figure 2c**). Combination treatment

resulted in a higher survival rate than the untreated group (40% versus 20%), although the difference did not reach statistical significance (**Figure 2d**). Each monotherapy provided limited protection. Consistently, bacterial burdens in the liver and spleen were significantly lower in the combination group than in either monotherapy group (**Figure 2e**).

5. PMBN facilitating uptake of CLO

To determine whether PMBN restores the antibacterial activity of CLO by facilitating intracellular drug entry, outer and inner membrane permeability together with intracellular CLO accumulation were evaluated. PMBN plus CLO markedly increased OM permeability in all four representative strains, as shown by elevated N-phenyl-1-naphthylamine (NPN) fluorescence, whereas CLO alone had little effect (**Figure 3a**). Propidium iodide (PI) fluorescence remained low following treatment with either CLO or PMBN alone but increased significantly after combination treatment, indicating enhanced disruption of the cytoplasmic membrane (**Figure 3b**).

Building on these findings, we further assessed whether PMBN enhances intracellular uptake of CLO. With CLO maintained at a constant sub-MIC concentration (0.0625 mg/L), incremental addition of PMBN (0-2 mg/L) revealed a clear threshold-dependent effect on intracellular CLO accumulation in *E. coli* MG1655. Specifically, PMBN at 0.125 and 0.25 mg/L did not alter the basal accumulation level, whereas a marked enhancement was triggered at 0.5 mg/L. The accumulation further increased at 1 mg/L but plateaued thereafter, with no additional increment observed at 2 mg/L (**Figure 3c**). Consequently, 1 mg/L PMBN was selected for subsequent experiments. When *E. coli* MG1655 was treated with PMBN across a range of CLO concentrations (0.0156-0.25 mg/L), the intracellular CLO levels were consistently and significantly higher than those in the CLO-alone group at every concentration tested (**Figure 3d**). Moreover, the absolute amount of intracellular CLO increased progressively with rising extracellular CLO concentrations in the presence of PMBN, indicating that PMBN not only enhances CLO accumulation but also allows the bacterium to accumulate more CLO as the external concentration increases.

6. CLO-PMBN disrupts bacterial bioenergetics

The physiological consequences of increased intracellular CLO accumulation were investigated by measuring intracellular ATP levels, efflux activity and reactive oxygen species (ROS) production. Neither CLO nor PMBN alone substantially affected intracellular ATP levels in any of the four representative strains. In contrast, combination treatment significantly reduced ATP concentrations in these four strains (**Figure 4a**). Consistent with this finding, ethidium bromide (EtBr) accumulation increased markedly following combination treatment, whereas no significant changes were observed with either monotherapy (**Figure 4b**), indicating reduced efflux activity. Intracellular ROS levels were further assessed using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The combination induced a pronounced increase in ROS production in these four strains compared with either CLO or PMBN alone (**Figure 4c**).

3.7 Metabolomic profiles revealing global metabolic reprogramming

To further characterize the metabolic alterations induced by CLO-PMBN treatment, untargeted metabolomic profiling was performed by comparing the CLO-PMBN combination group with the PMBN-treated group. The combination treatment resulted in 829 significantly upregulated and 709 significantly downregulated metabolites (**Figure 5a**). To identify the metabolites contributing most to group discrimination, the top 30 metabolites ranked by variable importance in projection (VIP) scores were selected for visualization based on their $\log_2$ fold changes ($\log_2\text{FC}$) (**Figure 5b**). Among these metabolites, five were increased, while most of the others displayed reduced abundance in the CLO-PMBN treatment group compared with the control group. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis based on the top 25 pathways ranked by rich factor revealed that differential metabolites were mainly associated with β -alanine metabolism, arginine and proline metabolism, arginine biosynthesis, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, pantothenate and CoA biosynthesis, lysine degradation, and glycerophospholipid metabolism (**Figure 5c**).

Based on their biological functions, the altered metabolic pathways were further

categorized into energy metabolism, redox-associated metabolism, and membrane-associated metabolism modules (**Figure 5d**). Energy-related pathways, including alanine, aspartate and glutamate metabolism, pyruvate metabolism, and pantothenate-CoA biosynthesis, were markedly perturbed. Specifically, pantothenate, a precursor of CoA biosynthesis, was significantly decreased ($\log_2FC = -1.650$), whereas β -alanine accumulated ($\log_2FC = 3.295$), suggesting disruption of CoA-associated metabolic processes. Redox-associated pathways, particularly those involving cysteine/methionine and arginine metabolism, were significantly altered. Notably, this was characterized by cystine accumulation ($\log_2FC = 4.041$), whereas metabolites linked to polyamine biosynthesis, including γ -glutamyl-GABald (γ -glutamyl- γ -aminobutyraldehyde, $\log_2FC = -2.318$) and succinyl-ornithine ($\log_2FC = -4.741$), showed marked decreases. Furthermore, changes in choline- and ethanolamine-related metabolites indicated remodeling of glycerophospholipid metabolism and membrane homeostasis. Collectively, CLO-PMBN treatment induced extensive metabolic reprogramming, primarily affecting energy metabolism, redox-associated pathways, and membrane homeostasis.

Discussion

Previous studies have demonstrated that CLO exhibits broad-spectrum antibacterial activity against GPB, and stereoisomeric analyses suggested that its activity may involve conserved intracellular metabolic targets [9]. In contrast, CLO alone displays limited activity against GNB, whereas combination with colistin produces potent and reproducible synergistic antibacterial effects against diverse Enterobacteriaceae pathogens [11]. Given the high conservation of many intracellular metabolic pathways between GPB and GNB, these observations suggest that restricted intracellular penetration, rather than the absence of susceptible targets, is the possibly primary barrier to CLO activity against GNB [10, 14, 19]. In the present study, we further demonstrated that selective disruption of the OM permeability barrier using PMBN, a non-bactericidal polymyxin derivative, was sufficient to restore CLO activity against

MDR *E. coli* and *Salmonella*, including *mcr-1*-carrying strains.

Notably, no species-specific difference in synergistic activity was observed between *E. coli* and *Salmonella*. Although *mcr-1*-carrying isolates exhibited significantly higher FICI values than CST-susceptible strains, the CLO-PMBN combination-maintained synergy across all tested isolates (FICI < 0.5) (Figure 1A). This indicates that MCR-1-mediated phosphoethanolamine (pEtN) modification of lipid A reduces, but does not abrogate, PMBN-mediated potentiation. Given that lipid A modification decreases the negative charge of LPS and contributes to colistin resistance in GNB, the preserved activity of the CLO-PMBN combination suggests that MCR-1-driven OM remodeling is insufficient to fully prevent PMBN-induced disruption of the LPS permeability barrier [20, 21]. Consistently, supplementation with Mg^{2+} or Ca^{2+} attenuated-but did not abolish-synergy, likely by reinforcing divalent cation-mediated LPS stabilization. In contrast, EDTA enhanced antibacterial activity by chelating divalent cations and destabilizing the OM [17-19]. Most importantly, LC-MS/MS confirmed that PMBN markedly increased intracellular CLO accumulation, providing direct evidence that disruption of the OM barrier is the initial event restoring CLO activity.

Salicylanilides have been increasingly recognized as bioenergetic disruptors rather than classical target-specific antibiotics, with their antibacterial effects associated with perturbation of membrane-associated energy metabolism and proton motive force [12, 22]. The increased intracellular exposure of CLO was accompanied by coordinated physiological alterations, including ATP depletion, reduced efflux activity and increased intracellular ROS accumulation. These findings suggest that CLO exhibits a similar systems-level mode of action once sufficient intracellular concentrations are achieved. Reduced ATP availability may impair energy-dependent cellular processes, including active transport systems such as resistance-nodulation-division (RND) efflux pumps, which rely on electrochemical gradients to drive substrate export [23]. Consequently, impaired efflux activity may further promote intracellular retention of CLO and amplify metabolic stress. In parallel, elevated ROS accumulation indicates disruption of intracellular redox homeostasis, a phenomenon frequently associated with antibiotic-

induced metabolic perturbation and bactericidal activity [24-27]. Although the precise causal relationships among ATP depletion, efflux inhibition and oxidative stress remain to be determined, their coordinated occurrence supports the concept that CLO accumulation initiates progressive disruption of bacterial bioenergetic homeostasis. Untargeted metabolomic profiling revealed extensive metabolic reprogramming induced by CLO-PMBN treatment, encompassing disruptions in energy homeostasis, redox balance, and membrane-associated stress responses. These alterations reflect coordinated perturbation of interconnected metabolic networks rather than isolated metabolic changes, suggesting that CLO-PMBN drives a global metabolic shift that may compromise bacterial growth, stress adaptation, and survival [25, 26, 28]. In particular, disruption of pantothenate and CoA-associated metabolism may compromise central metabolic flux, as CoA serves as an essential cofactor connecting multiple pathways involved in energy generation and biosynthesis [29]. Changes in cysteine-related metabolism and polyamine-associated pathways are consistent with disturbed redox regulation and stress adaptation mechanisms [30, 31], whereas alterations in phospholipid-associated metabolites suggest perturbation of membrane homeostasis [8]. Collectively, these physiological and metabolomic findings support a unified mechanism in which PMBN-mediated intracellular accumulation enables CLO to disrupt multiple interconnected metabolic processes, ultimately resulting in irreversible loss of cellular homeostasis and bacterial death (Figure 6).

Although the CLO-PMBN combination exhibited potent synergistic antibacterial activity *in vitro*, its therapeutic efficacy in the murine infection model remained limited. This discrepancy is likely attributable to pharmacological and formulation-related constraints rather than failure of the proposed antibacterial mechanism. Previous studies have reported unfavorable pharmacokinetic properties of CLO, including extensive serum protein binding [32], and consistent with this limitation, serum supplementation substantially attenuated the synergistic activity of CLO-PMBN in our study. In addition, the poor aqueous solubility of CLO restricted its achievable concentration in culture media, with the maximal soluble concentration in our

experimental conditions reaching only 64 mg/L. The use of dimethyl sulfoxide (DMSO) as a solvent further constrained *in vivo* application, as administration of high CLO doses required relatively high amounts of DMSO, which may introduce potential toxicity concerns and limit the therapeutic window [33]. Future studies should therefore focus on improving CLO exposure and delivery through structural optimization, advanced formulation strategies, or alternative solubilization approaches to enhance its *in vivo* antibacterial efficacy.

Conclusion

In conclusion, this study demonstrates that OM permeabilization restores the antibacterial activity of CLO by promoting intracellular drug accumulation, thereby initiating coordinated disruption of bacterial bioenergetic and metabolic homeostasis. These findings establish intracellular drug exposure as the key determinant underlying CLO activity against GNB. Furthermore, they provide mechanistic support for combining hydrophobic antibacterial agents with OM permeabilizers.

Materials and Methods

1. Bacterial and reagents

30 *E. coli* and *Salmonella* strains were included in this study, comprising 28 MDR clinical isolates and two laboratory strains (MG1655 and MG1655+pEGN-*mcr-1*). Among the clinical isolates, 15 were CST-susceptible and 15 were *mcr-1*-carrying CST-resistant strains. Detailed strain information, antimicrobial susceptibility profiles and genomic characteristics are provided in Tables S1 and S2. CLO (purity $\geq$ 99%) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China), and PMBN (purity $\geq$ 98%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Both compounds were prepared as sterile stock solutions before use.

2. Antimicrobial susceptibility, drug combination and stability assays, and resistance evolution

Minimum inhibitory concentrations (MICs) were determined by broth microdilution

according to CLSI guidelines [34]. Synergistic activity between CLO and PMBN was evaluated using checkerboard assays, and interactions were interpreted using the fractional inhibitory concentration index (FICI), where $FICI \leq 0.5$ indicated synergy. Time-kill assays were performed to assess bactericidal activity. The stability of the synergistic effect was further evaluated under different physiological conditions by supplementing Mueller-Hinton broth with selected ions, serum or cell culture medium. Resistance evolution was assessed by serial passaging of *E. coli* 44 in subinhibitory concentrations of antimicrobial agents for 30 consecutive days. Experimental details are provided in the Additional File.

3. *In vivo* therapeutic efficacy and haemolytic activity

The *in vivo* antibacterial activity of the CLO-PMBN combination was evaluated using an intraperitoneal infection model in female ICR mice. Mice were infected with *mcr-1*-carrying *E. coli* strain 44 and randomly assigned to treatment groups receiving CLO, PMBN, or the combination. Survival was monitored for 7 days, and bacterial burdens in spleen and kidney tissues were determined in an independent cohort at 24 h post-infection. All animal experiments were approved by the Animal Ethics Committee of South China Agricultural University (approval no. 2026C081) and were conducted in accordance with ARRIVE 2.0 guidelines. Detailed information on animal allocation, randomization, blinding, and humane endpoints is provided in Additional File.

Haemolytic activity was evaluated using sheep erythrocytes. Detailed experimental procedures are described in the Additional File.

4. Determination of intracellular CLO

Intracellular accumulation of CLO was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Mid-logarithmic-phase bacteria were treated with CLO in the presence or absence of PMBN, washed to remove extracellular drug and lysed following addition of the internal standard (CLO-D₆). Intracellular CLO was extracted with acetonitrile and quantified using a previously validated LC-MS/MS method. Chromatographic and mass spectrometric conditions are described in the Additional File.

5. Mechanistic assays for drug combination action

OM permeability, cytoplasmic membrane integrity, intracellular adenosine triphosphate (ATP), efflux activity and reactive oxygen species (ROS) production were assessed using fluorescence-based assays. Untargeted metabolomic profiling was performed using differential isotope labelling coupled with ultra-performance liquid chromatography-quadrupole time-of-flight high-resolution mass spectrometry (UPLC-QTOF-HRMS). Detailed protocols, analytical conditions and data processing procedures are provided in the Additional File.

6. Statistical analysis

Data are presented as mean $\pm$ SD from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 8.0. Data are presented as mean $\pm$ SD from at least three independent biological experiments. Comparisons between two groups were performed using unpaired two-tailed Student's t-tests, whereas comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. A *P* value < 0.05 was considered statistically significant.

Abbreviations

ATP: Adenosine triphosphate

ANOVA : Analysis of variance

CLO: Closantel

CST: Colistin

DCF: 2',7'-Dichlorofluorescein

DCFH-DA: 2',7'-Dichlorodihydrofluorescein diacetate

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediaminetetraacetic acid

EtBr: Ethidium bromide

FICI: Fractional inhibitory concentration index

373 **GNB:** Gram-negative bacteria
374 **GPB:** Gram-positive bacteria
375 **KEGG:** Kyoto Encyclopedia of Genes and Genomes
376 **LC-MS/MS:** Liquid chromatography-tandem mass spectrometry
377 **LPS:** Lipopolysaccharide
378 **MDR:** Multidrug-resistant
379 **MIC:** Minimum inhibitory concentration
380 ***mcr-1*:** Mobilized colistin resistance gene 1
381 **NPN:** N-Phenyl-1-naphthylamine
382 **OM:** Outer membrane
383 **pEtN:** Phosphoethanolamine
384 **PI:** Propidium iodide
385 **PMBN:** Polymyxin B nonapeptide
386 **ROS:** Reactive oxygen species
387 **RND:** Resistance-nodulation-division
388 **UPLC-QTOF-HRMS:** Ultra-performance liquid chromatography-quadrupole time-of-
389 flight high-resolution mass spectrometry
390 **VIP:** Variable importance in projection
391 **Log₂FC:** Log₂ fold change

392

393 **Ethics approval and consent to participate**

394 Ethical approval for all animal experiments was obtained from the Institutional Animal
395 Care and Use Committee of [Institution name] (approval no. 2026C081). All animal
396 procedures were conducted in accordance with the relevant institutional guidelines and
397 regulations for the care and use of laboratory animals. The murine peritonitis infection
398 model was performed following the approved experimental protocols, and all efforts
399 were made to minimize animal suffering throughout the study.

400

401 **Consent for publication**

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Author contribution

WG: Investigation, Data curation, Formal analysis, Writing-original draft. ZS: Investigation, Data analysis. QL: Investigation, Data analysis. JW: Investigation. WM: Investigation, Data curation. MZ: Investigation. YL: Data analysis. LH: Supervision, Conceptualization, Review & editing, Funding acquisition. LF: Supervision, Conceptualization, Review & editing.

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Figure Legend

Figure 1. Synergistic antibacterial activity of CLO and PMBN against *E. coli* and *Salmonella*

(a) Fractional inhibitory concentration index (FICI) values of the CLO-PMBN combination against 30 Gram-negative isolates, including 22 *Escherichia coli* (*E. coli*) and 8 *Salmonella* strains. Isolates were categorized according to the presence (orange) or absence (blue) of *mcr-1*. The CLO-PMBN combination showed synergistic activity across all tested isolates (FICI < 0.5).

(b) Representative checkerboard assay heat maps showing the interaction between CLO and PMBN against four representative *E. coli* strains, including CST-susceptible strains (*E. coli* MG1655 and 47) and *mcr-1*-carrying strains (*E. coli* MG1655+pGEN-*mcr-1* and 44). Red dots indicate the combination concentrations used for FICI calculation.

(c) Time-kill kinetics of CLO and PMBN, alone or in combination, against the four representative *E. coli* strains.

(d) Effect of CLO-PMBN combination treatment on the development of CST resistance during a 30-day serial passage experiment in *E. coli* 44. Sub-MIC CST exposure was included as a positive control for resistance selection. Data are presented as mean $\pm$ SD. Statistical significance was determined using an unpaired two-tailed Student's t-test. (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

Abbreviations: CLO, closantel; PMBN, polymyxin B nonapeptide; FICI, fractional inhibitory concentration index; MIC, minimum inhibitory concentration; CST, colistin; *mcr-1*, mobilized colistin resistance gene 1; h, hours.

Figure 2. Stability, biosafety, and *in vivo* therapeutic efficacy of the CLO-PMBN combination against *E. coli* 44

(a) Effects of different physiological conditions on the synergistic antibacterial activity of CLO and PMBN. FICI values were determined by checkerboard assays in the presence of different metal ions or environmental supplements, including Na^+ , K^+ , Ca^{2+} ,

Mg²⁺, ethylenediaminetetraacetic acid (EDTA), 10% Dulbecco's modified Eagle's medium (DMEM), and 10% serum. The dashed line indicates the threshold for synergy (FICI = 0.5).

(b) Schematic illustration of experimental protocol for the animal trial.

(c) Hemolytic activity of CLO and PMBN against sheep erythrocytes. Triton X-100 (0.2%) was used as the positive control representing 100% hemolysis.

(d) Therapeutic efficacy of CLO and PMBN in a murine peritonitis-sepsis infection model caused by *E. coli* 44.

(e) Bacterial burdens in the liver and spleen of infected mice following treatment.

Data are presented as mean $\pm$ SD. Statistical significance was determined using an unpaired two-tailed Student's t-test. (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

Figure 3. PMBN-mediated membrane permeabilization enhances intracellular accumulation of CLO in *E. coli*

(a) Outer membrane (OM) permeability of four representative *E. coli* strains, including CST-susceptible strains (*E. coli* MG1655 and 47) and *mcr-1*-carrying strains (MG1655+pEGN-*mcr-1* and 44), following treatment with CLO, PMBN, or their combination. OM permeability was evaluated by measuring N-phenyl-1-naphthylamine (NPN) fluorescence after 1 h exposure to the indicated treatments. Increased NPN fluorescence reflects enhanced OM permeabilization.

(b) Membrane permeability of *E. coli* MG1655, 47, MG1655+pEGN-*mcr-1*, and 44 following treatments with CLO, PMBN, or their combination. Membrane integrity was assessed using propidium iodide (PI) staining after 1 h drug exposure. Increased PI fluorescence intensity indicates disruption of membrane integrity and enhanced membrane permeability.

(c) Effect of PMBN concentration on intracellular accumulation of CLO in *E. coli* MG1655. Bacteria were incubated with a fixed concentration of CLO (0.0625 mg/L) in the presence of increasing concentrations of PMBN (0-2 mg/L). Intracellular CLO

concentrations were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

(d) Concentration-dependent intracellular accumulation of CLO in the presence of PMBN. *E. coli* MG1655 were treated with a fixed concentration of PMBN (1 mg/L) and increasing concentrations of CLO (0.0156-0.25 mg/L). Data are presented as mean $\pm$ SD from three independent experiments.

Statistical significance was determined using unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, as appropriate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Different lowercase letters indicate significant differences among treatments ($P < 0.05$).

Figure 4. CLO-PMBN effects on ATP, efflux, and ROS in *E. coli*

(a) Intracellular ATP levels in *E. coli* MG1655, 47, MG1655+pEGN-*mcr-1*, and 44 following treatments with CLO, PMBN, or their combination. ATP production was quantified using a bioluminescence-based ATP assay kit after 1 h treatment.

(b) Effects of CLO and PMBN on bacterial efflux pump activity determined by ethidium bromide (EtBr) accumulation assay. Efflux activity was assessed by measuring intracellular EtBr fluorescence after 1 h drug exposure. Increased fluorescence intensity indicates enhanced intracellular accumulation of EtBr resulting from impaired efflux activity.

(c) ROS accumulation induced by CLO and PMBN treatment. ROS levels were measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) after 1 h exposure to the indicated treatments. Oxidation of intracellular DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) was quantified by fluorescence intensity measurement.

Data are presented as mean $\pm$ SD from three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences among treatments within the same strain ($P < 0.05$).

Figure 5. Metabolic remodeling induced by CLO-PMBN combination treatment in *E. coli* 44 (*mcr-1*)

(a) Volcano plot showing differential metabolites between the CLO-PMBN combination group and the PMBN-treated group. Red dots represent significantly upregulated metabolites, blue dots represent significantly downregulated metabolites, and gray dots represent metabolites without significant differences.

(b) Log₂ fold changes (log₂FC) of the top 30 differential metabolites ranked by variable importance in projection (VIP).

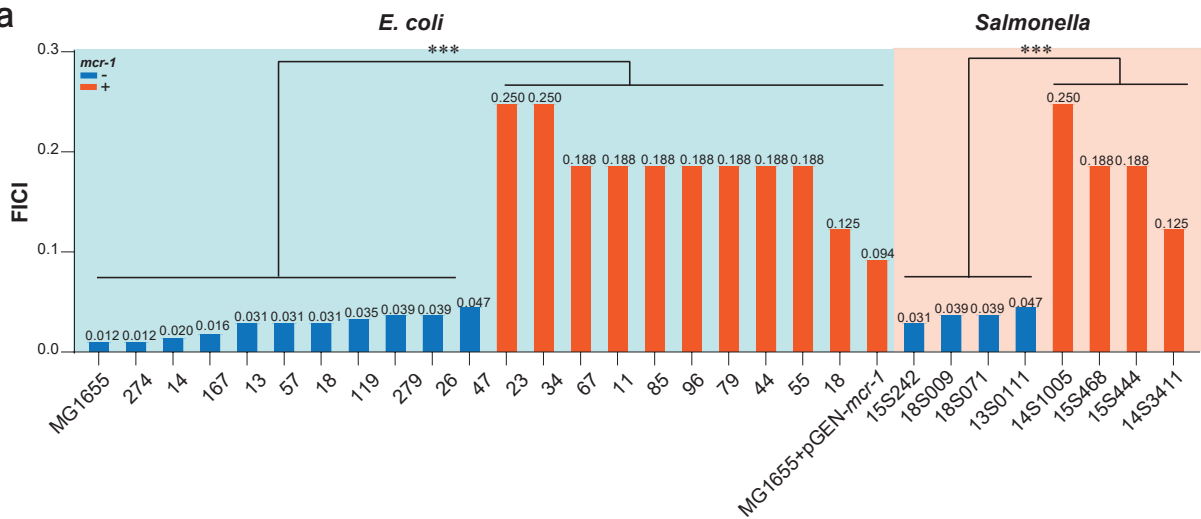
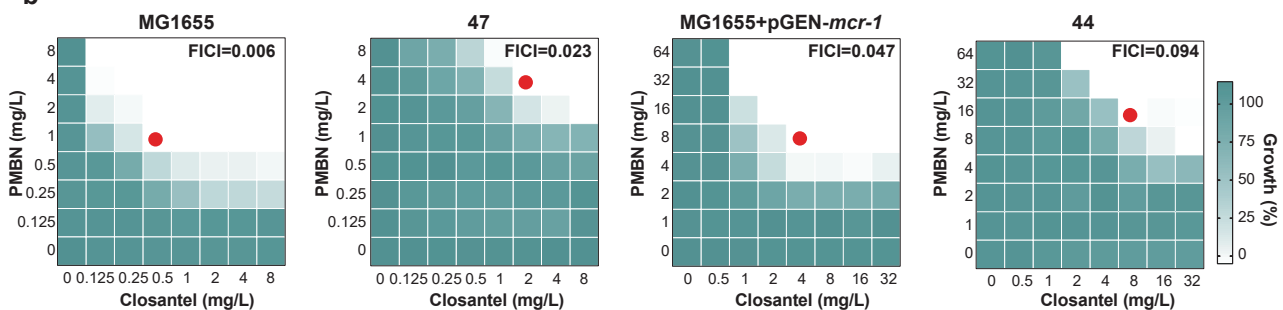
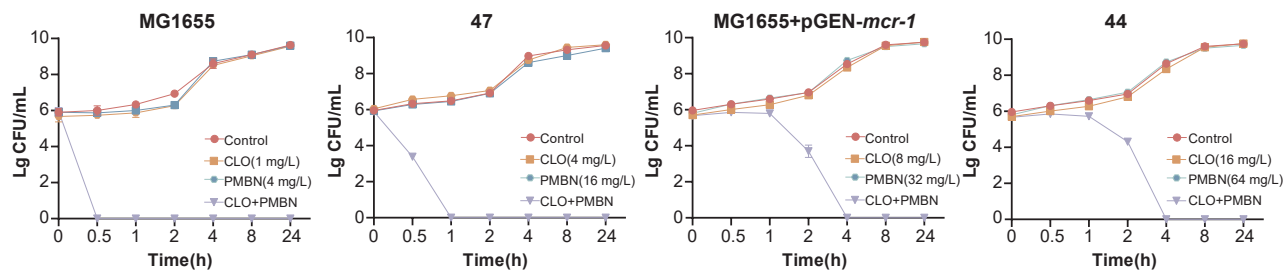
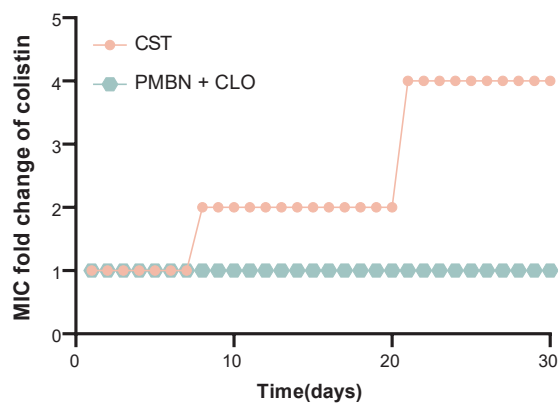
(c) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differential metabolites. The top 25 enriched pathways are shown. Bubble size represents the number of metabolites annotated to each KEGG pathway, while color intensity indicates the corresponding significance values expressed as -lg (*P*-value).

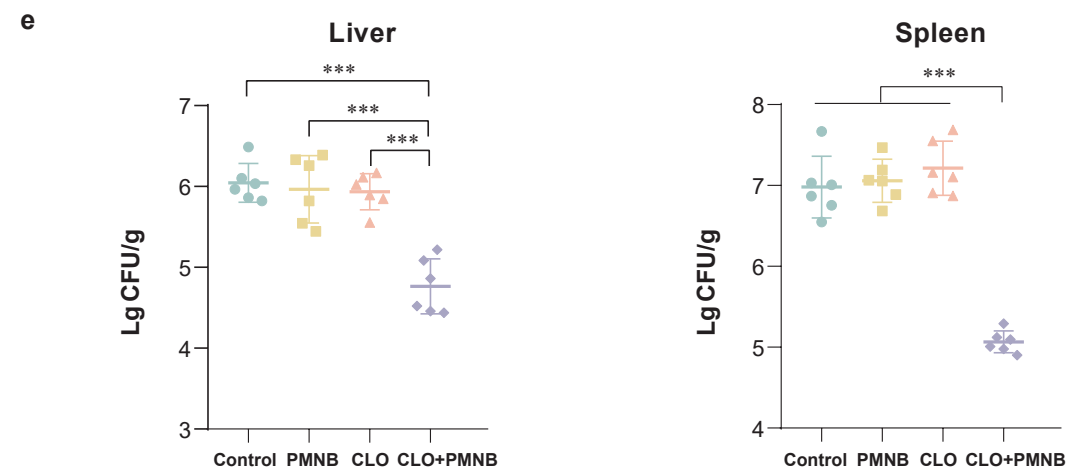
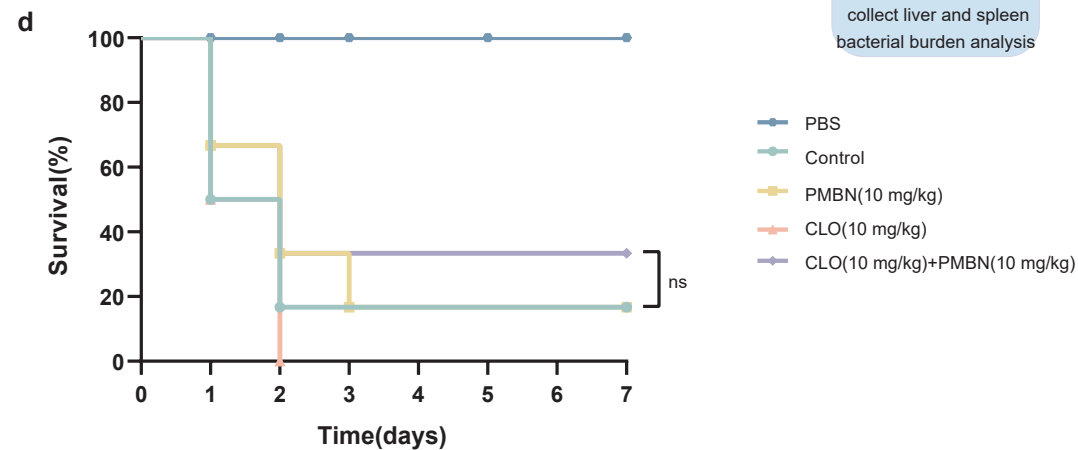
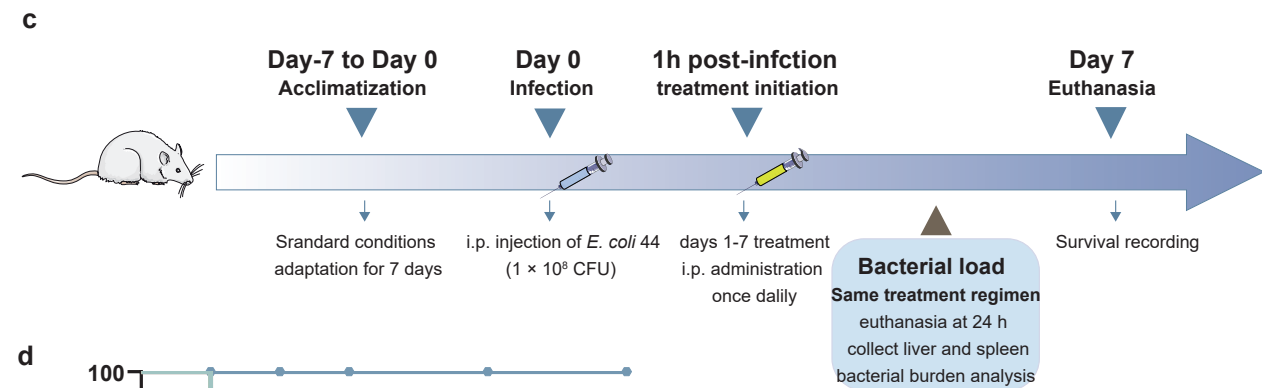
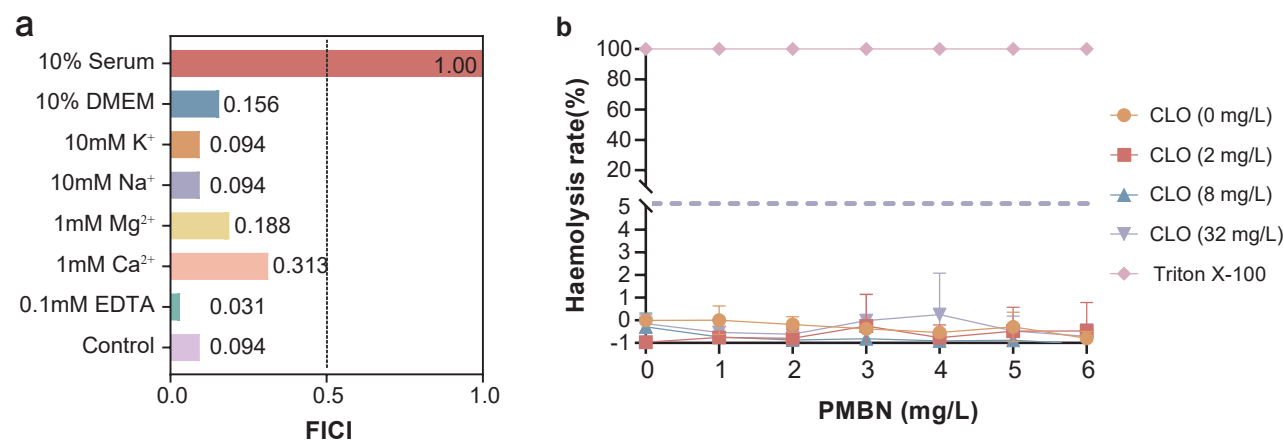
(d) Mapping of differential metabolites to key physiological modules. Differential metabolites identified between the CLO-PMBN combination and PMBN-treated groups were classified into 3 major functional modules: energy metabolism, redox balance, and membrane stability. Each module is connected to its associated metabolites, with the corresponding log₂FC values indicated. Positive values (red) represent upregulated metabolites, and negative values (blue) represent downregulated metabolites in the combination treatment.

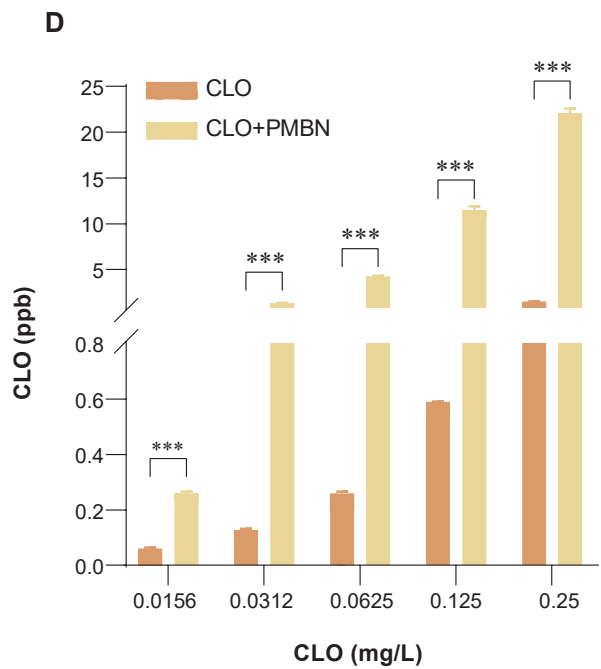
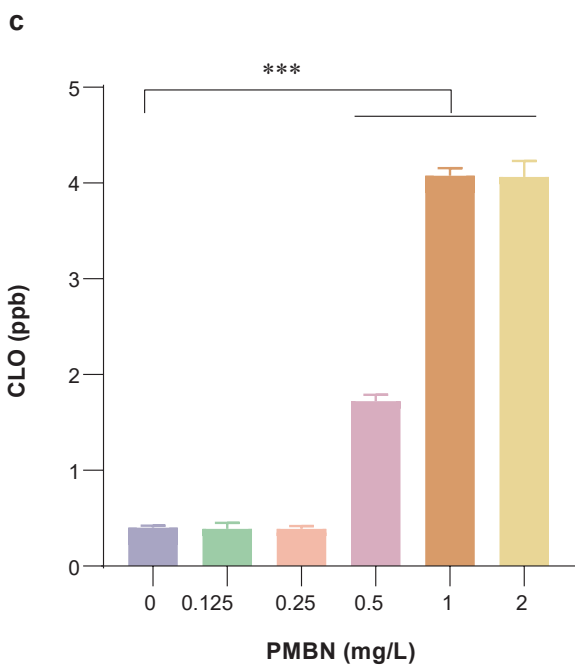
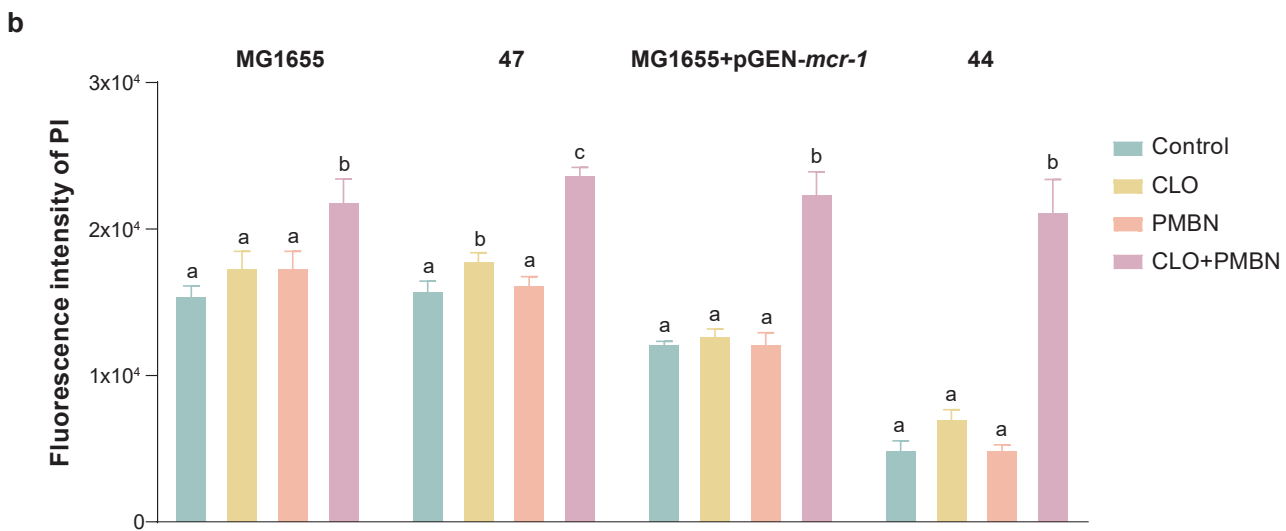
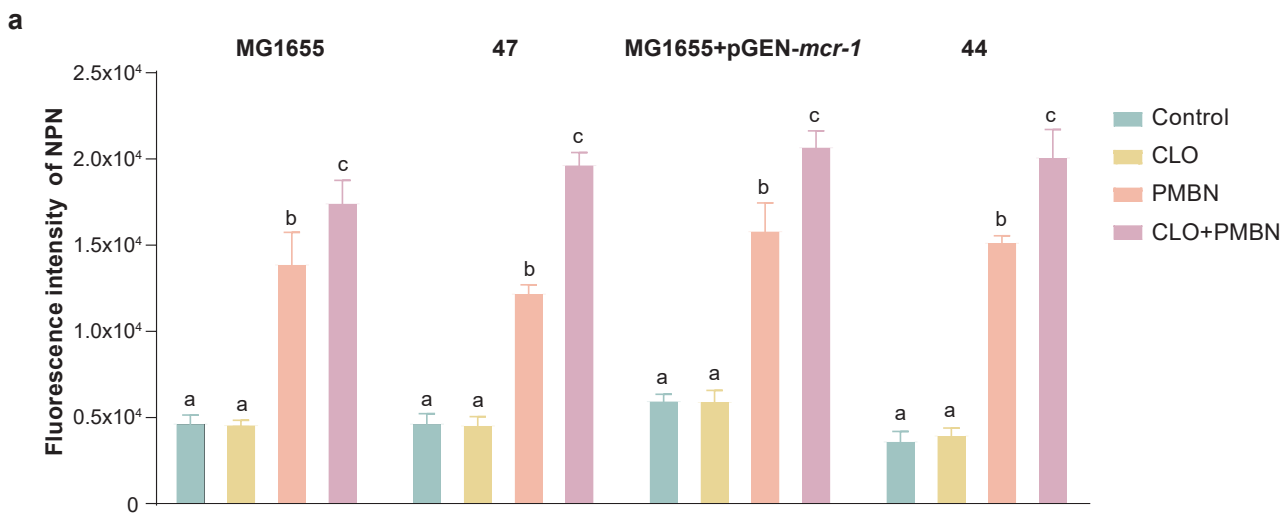
Figure 6. Proposed mechanistic model underlying the synergistic antibacterial activity of CLO-PMBN against Gram-negative bacteria.

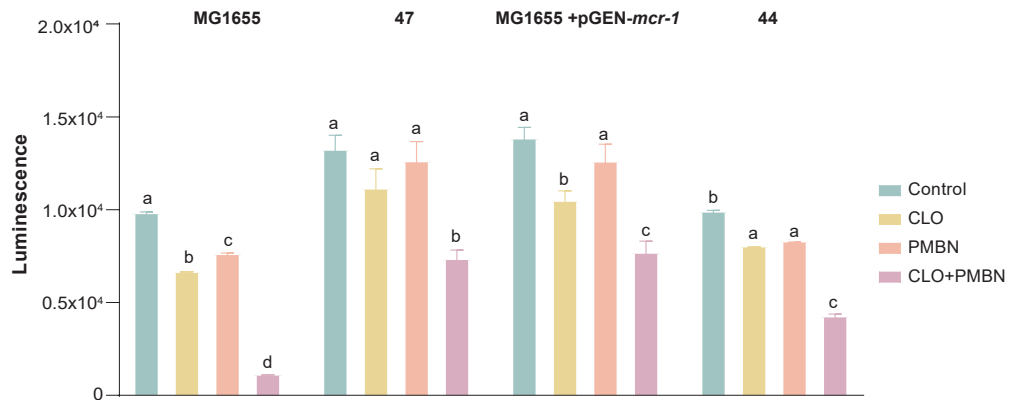
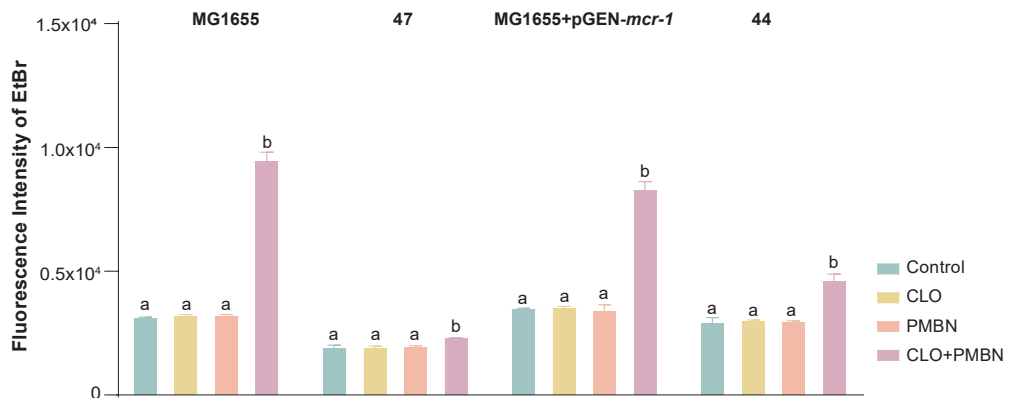
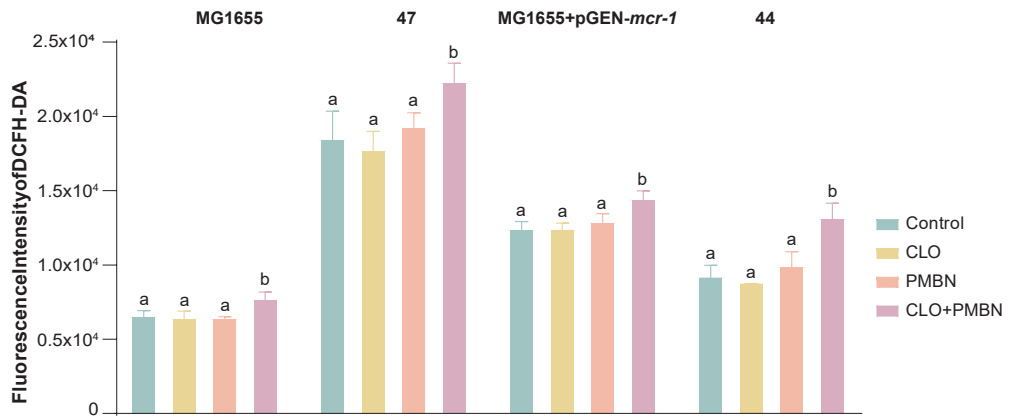
PMBN compromises the outer membrane barrier by interacting with lipopolysaccharide, allowing increased intracellular entry and accumulation of CLO. Elevated intracellular CLO disrupts bioenergetic homeostasis by reducing ATP production, which subsequently impairs ATP-dependent efflux activity and promotes further CLO accumulation, creating a reinforcing intracellular accumulation loop. CLO accumulation also induces excessive ROS generation, resulting in oxidative stress. The combined

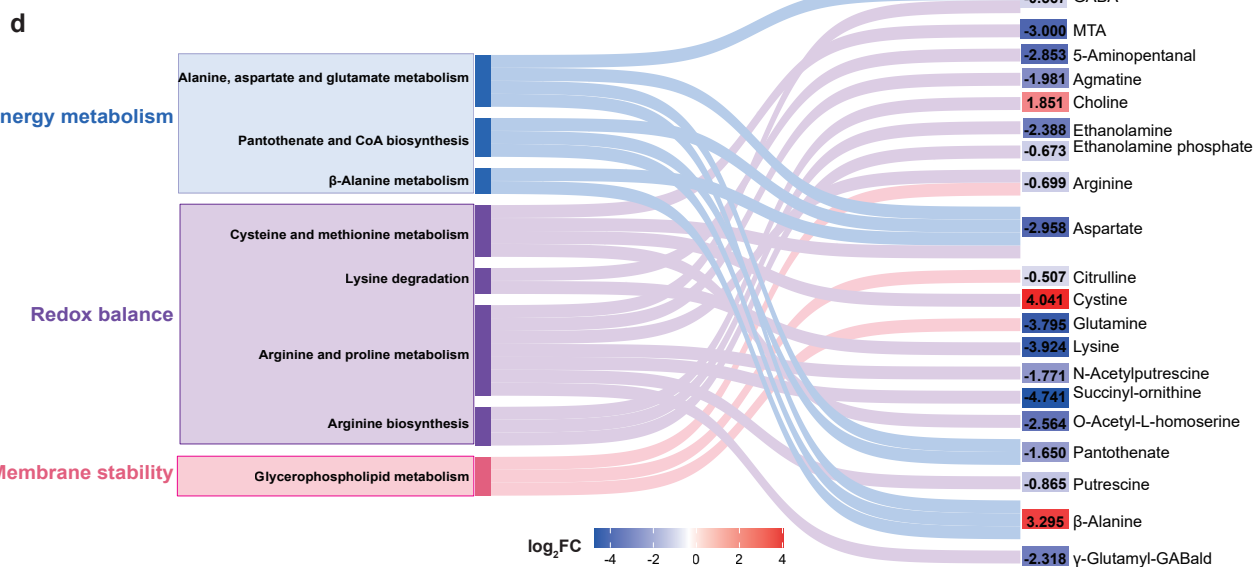
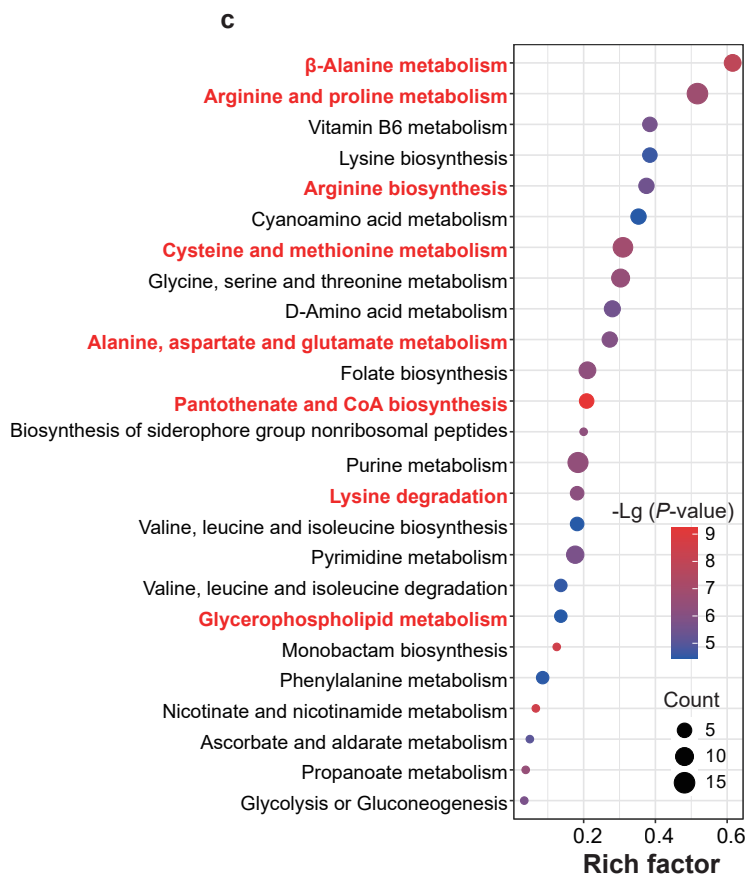
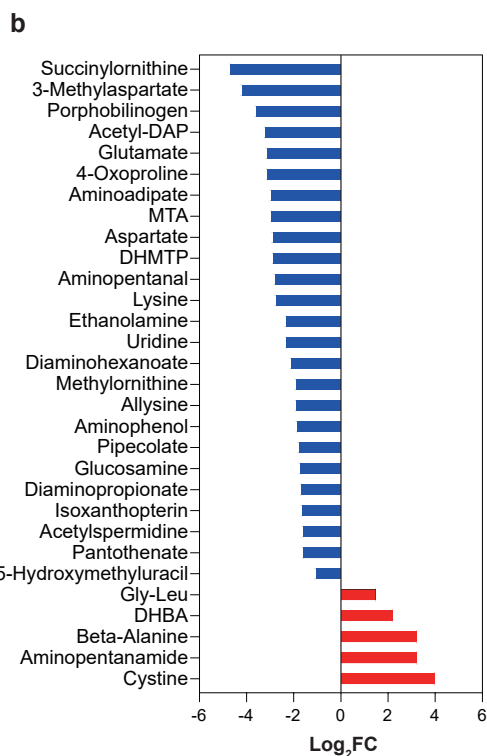
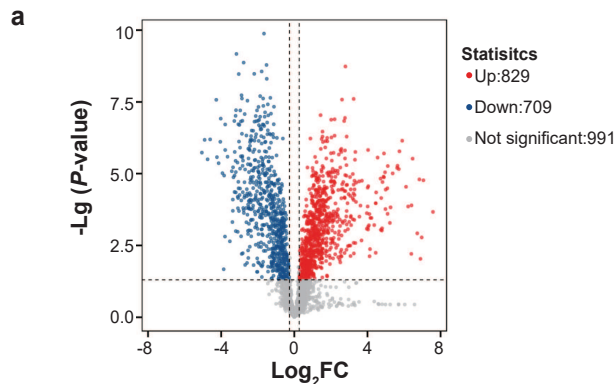
637 disruption of energy metabolism, efflux capacity, redox homeostasis, and global
638 metabolic processes ultimately drives bacterial death.

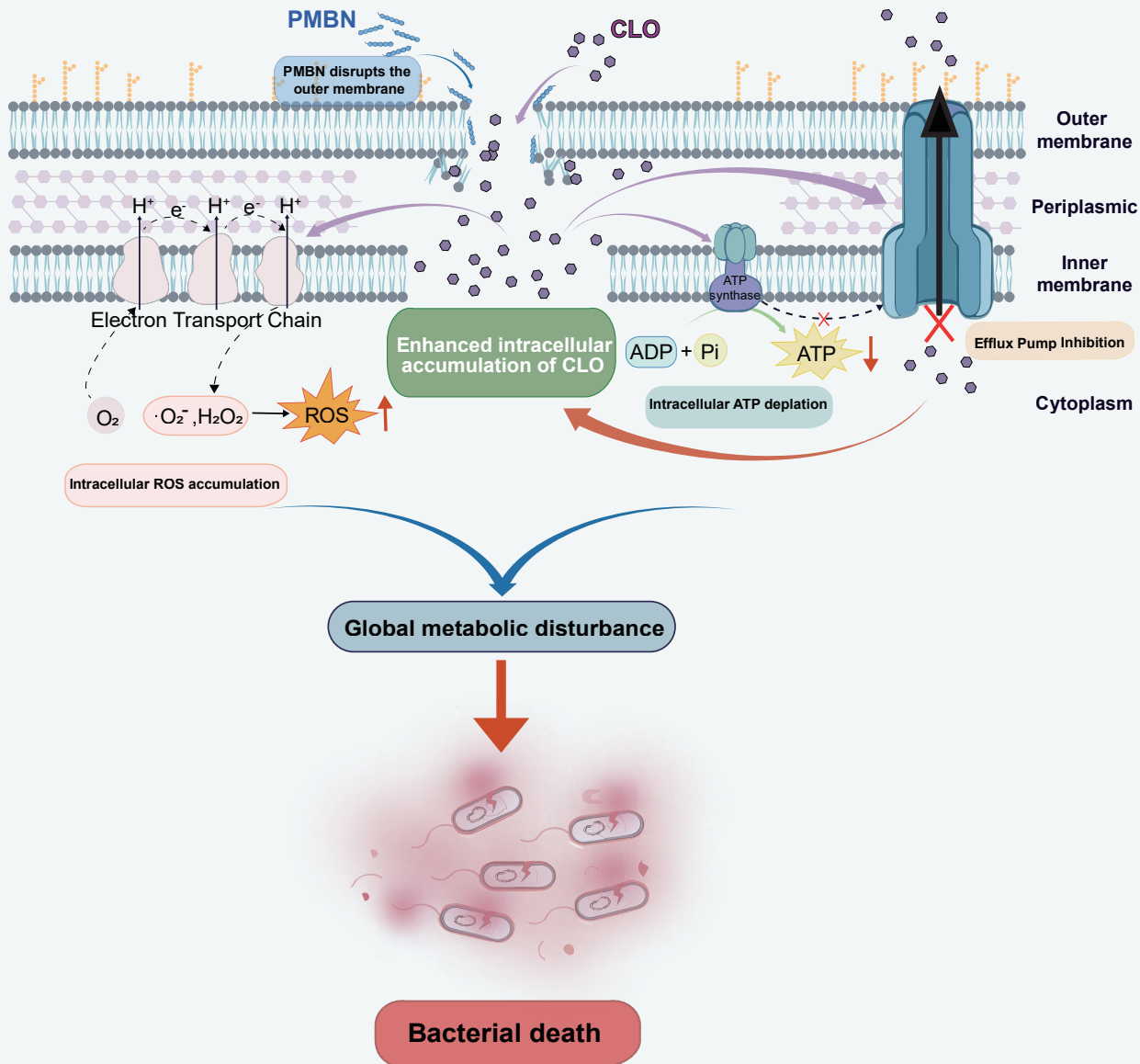
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