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Investigation of cephalosporin-based antimicrobial combination strategies for targeting resistant bacterial infections

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DEDICATION

I dedicate this work to my family, Eliane, Mauro, Rafael, and Neusa, for all their support, care, understanding, and encouragement throughout these years.

EPIGRAPH

"Our greatest weakness lies in giving
up. The most certain way to succeed
is always to try just one more time."
— **Thomas Edison**

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I would like to first thank God, the source of wisdom, who was present at every step of this journey. It was He who gave me strength in moments of exhaustion, hope in times of doubt, and courage to face the challenges this work brought.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AUC	Area under curve
BHI	Brain heart infusion
CC	Clonal complex
CFU	Colony-forming unit
CFX	Cefixime
CLX	Cephalexin
CMX	Cefmenoxime
CPR-Kp	Carbapenem-polymyxin-resistant *Klebsiella pneumoniae*
CTB	Ceftibuten
CTX	Cefotaxime
D-PBS	Dulbecco's phosphate-buffered saline
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter*
ESBL	Extended-spectrum beta-lactamase
FICI	Fractional inhibitory concentration index
HR	Hemolytic rate
IPC	International Patent Classification
KPC	*Klebsiella pneumoniae* carbapenemase
mcr	Mobilized colistin resistance
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
NGM	Nematode growth medium
OD	Optical Density
PBP	Penicillin-binding protein
PCR	Polymyxin-carbapenem-resistant
PK/PD	Pharmaconkinetics/pharmacodynamics

PMB	Polymyxin B
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
ROS	Reactive oxygen species
RSE	Relative standard error
SE	Standard errors
SEM	Scanning electron microscopy
ST	Sequence type
TK	Time-kill
VPC	Visual predictive check
WIPO	Worldwide Intellectual Property Office
ZIP	Zero interaction potency

Investigação de estratégias de combinação antimicrobiana à base de cefalosporinas para o tratamento de infecções bacterianas resistentes

RESUMO

A bactéria Gram-negativa *Klebsiella pneumoniae* representa uma grande ameaça à saúde global devido à sua alta taxa de resistência a antimicrobianos, o que limita as opções de tratamento. Diante disso, o presente estudo teve como objetivo investigar o sinergismo entre combinações de antibióticos como alternativa terapêutica para infecções causadas por *K. pneumoniae*. Para isso, foi realizado o teste de *checkerboard* com combinações das seguintes cefalosporinas - Cefotaxima, Cefmenoxima, Ceftibuteno, Cefalexina, Cefixima - com Polimixina B, e a partir disso, foi calculado o índice de concentração inibitória fracionada (ICIF). Além disso, foram obtidas curvas de sobrevivência e realizadas análises com o software *Synergyfinder* para as combinações estudadas. O potencial de inibição de biofilme bacteriano das combinações também foi explorado. Imagens de microscopia eletrônica de varredura (MEV) de *K. pneumoniae* tratada com as combinações foram obtidas. Ademais, foi realizado o teste de hemólise para investigar o potencial hemolítico das combinações. As combinações Cefotaxima-Polimixina B e Cefmenoxima-Polimixina B foram exploradas em um modelo *in vivo* de *Caenorhabditis elegans*, onde experimentos de toxicidade e eficácia antibacteriana foram realizados. A farmacocinética/farmacodinâmica (PK/PD), baseada em experimentação *in vitro*, foi estudada para a combinação Ceftibuteno-Polimixina B, utilizando um modelo Emax modificado para elucidar o potencial antibacteriano e a eficácia da combinação. Um ensaio *in vivo* com camundongos foi realizado com a combinação Ceftibuteno-Polimixina B. Cefotaxima-Polimixina B, Cefmenoxima-Polimixina B e Ceftibuteno-Polimixina B apresentaram baixos ICIFs, variando de 0,19 a 0,5, de 0,25 a 1,5 e de 0,15 a 0,37, respectivamente, com pontuações de Potência de Interação Zero (ZIP) de 37.484, 15.076 e 45.754, representando sinergismo. Além disso, o estudo de PK/PD demonstrou que a combinação Ceftibuteno-Polimixina B teve maior atividade antibacteriana em comparação com os tratamentos isolados, com base em uma dose de EC50 (concentração do antibiótico necessária para alcançar 50% do efeito máximo) menor e um kmax (constante de morte bacteriana) maior. As três combinações estudadas reduziram significativamente o crescimento e a formação de biofilme de *K. pneumoniae* resistente a carbapenêmicos e polimixinas, sem comprometer a integridade da célula bacteriana, conforme imagens de MEV. As avaliações de segurança demonstraram baixos

percentuais de hemólise para os tratamentos. Ainda, Cefotaxima-Polimixina B e Cefmenoxima-Polimixina B geraram altas taxas de sobrevivência nas avaliações de toxicidade em *C. elegans*. No modelo *in vivo* de infecção por *K. pneumoniae*, Cefotaxima-Polimixina B e Cefmenoxima-Polimixina B aumentaram significativamente a sobrevivência dos nematoides *C. elegans*. Além disso, os estudos *in vivo* revelaram a eficácia de Ceftibuteno-Polimixina B na redução significativa da carga bacteriana no fluido de lavagem peritoneal de camundongos. Esses resultados destacam o potencial da combinação entre as cefalosporinas estudadas e Polimixina B para o tratamento de infecções causadas por *K. pneumoniae* resistente a carbapenêmicos e polimixinas.

Palavras-chave: Resistência, *Klebsiella pneumoniae*, terapia combinada, antibióticos.

Investigation of cephalosporin-based antimicrobial combination strategies for targeting resistant bacterial infections

ABSTRACT

The Gram-negative bacterium *Klebsiella pneumoniae* represents a major threat to global health due to its high rate of resistance to antimicrobials, leading to treatment limitations. Given this, the present study aimed to investigate the synergism between antibiotic combinations as a therapeutic alternative for infections caused by *K. pneumoniae*. For this purpose, a *checkerboard* test was performed with combinations of the following cephalosporins - Cefotaxime, Cefmenoxime, Ceftibuten, Cephalexin, Cefixime - with Polymyxin B and, from this, the fractional inhibitory concentration index (FICI) was calculated. In addition, survival curves were obtained, and analyses were performed with the *Synergyfinder* software for the studied combinations. The bacterial biofilm inhibition potential of the combinations was also explored. Scanning electron microscopy (SEM) images of *K. pneumoniae* treated with the combinations were obtained. In addition, the hemolysis test was performed to investigate the combinations hemolytic potential. The combinations, Cefotaxime-Polymyxin B and Cefmenoxime-Polymyxin B were explored in an *in vivo* model of *Caenorhabditis elegans*, where toxicity and antibacterial efficacy experiments were performed. In vitro pharmacokinetic/pharmacodynamic (PK/PD) was performed for Ceftibuten-Polymyxin B, using a modified E_{max} model to elucidate the combination antibacterial potential and efficacy. *In vivo* assay with Swiss mice was performed with Ceftibuten-Polymyxin B combination. Cefotaxime-Polymyxin B, Cefmenoxime-Polymyxin B and Ceftibuten-Polymyxin B exhibited low FICIs, ranging from 0.19 to 0.5, from 0.25 to 1.5 and from 0.15 to 0.37, respectively, with zero interaction potency (ZIP) scores of 37,484, 15,076 and 45,754, representing synergism. Plus, PK/PD study demonstrated that Ceftibuten-Polymyxin B had enhanced antibacterial activity comparing to treatments alone, based on a lower EC_{50} dose (the antibiotic concentration required to achieve 50% of the maximum effect) and a higher k_{max} (bacterial killing constant). The three studied combinations significantly reduced the growth and biofilm formation of carbapenem-polymyxin resistant *K. pneumoniae*, without compromising the bacterial cell integrity, according to SEM images. Safety evaluations demonstrated low percentages of hemolysis for the treatments. In accordance, Cefotaxime-Polymyxin B and Cefmenoxime-

Polymyxin B generated high survival rates in toxicity evaluations in *C. elegans*. In the *K. pneumoniae* infection *in vivo* model, Cefotaxime-Polymyxin B and Cefmenoxime-Polymyxin B significantly increased the survival of *C. elegans* nematodes. Furthermore, *in vivo* studies revealed the efficacy of Ceftibuten-Polymyxin B in significantly reducing the bacterial load of the peritoneal lavage fluid of mice. These results highlight the potential of the combination between the studied cephalosporins and Polymyxin B for the treatment of infections caused by *K. pneumoniae* resistant to carbapenems and polymyxins.

Keywords: Resistance, *Klebsiella pneumoniae*, combination therapy, antibiotics.

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1. INTRODUCTION

Gram-negative pathogens, such as multidrug-resistant (MDR) *Klebsiella pneumoniae*, directly impact and threaten global population health (KULSHRESTHA; TIWARI; TIWARI, 2024). Infections caused by MDR *K. pneumoniae* raise concerns due to limited therapeutic options, prolonged hospital stays, increased medical costs, and increased mortality and morbidity rates among patients (DE SOUZA et al., 2024). The cited bacteria belongs to a group called ESKAPE (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter*), characterized by the ability to rapidly acquire resistance to most classes of antibiotics through multiple mechanisms, including modifications in the drug target, decreased drug absorption due to mutations in outer membrane proteins, biofilm formation, production of antibiotic-degrading enzymes, overexpression of efflux pumps and adaptation of alternative metabolic pathways (DENISSEN et al., 2022).

A projection suggests that without new effective measures against antimicrobial resistance, around 10 million deaths will occur worldwide due to resistant infections by 2050 (NAGHAVI et al., 2024). However, the process of creating new antibiotics compounds represents a significant challenge. The research and development cycle of an innovative drug requires a considerable investment, both in terms of time (on average 10 to 15 years), as well as financial resources and scientific expertise (HUGHES; KARLÉN, 2014). Therefore, it is imperative to explore therapeutic alternatives capable of overcoming the limitations associated with the development of new drugs, quickly and efficiently.

The combination of existing antibiotics emerges as a promising strategy in this scenario. By exploiting the synergy between two or more drugs, this approach seeks to enhance their therapeutic efficacy. Synergistic interaction can increase antimicrobial activity, overcome resistance, broaden the spectrum of action and reduce treatment toxicity. In addition, to paving the way for innovative solutions against difficult-to-treat infections, this tactic offers a viable and affordable alternative to the development of new drugs, especially in the treatment of infections caused by resistant microorganisms, where therapeutic options are limited (COATES et al., 2020).

Polymyxin B, a cyclic cationic polypeptide antibiotic, had its use restricted in the past due to adverse effects such as nephrotoxicity and neurotoxicity, due to their cationic profile, cellular membrane disrupting potential and cell accumulation (causing oxidative stress and apoptosis pathways activation) (WAGENLEHNER et al., 2021), leading to a significant discontinuation of its application in the 1980s. However, in the current scenario of effective

therapeutic alternatives scarcity, Polymyxin B has re-emerged as a last-line treatment option for serious infections caused by MDR Gram-negative microorganisms (JÚNIOR et al., 2022). Additionally, Polymyxin B is an excellent antibiotic for combined treatment, given its potential to sensitize the bacteria by damaging its cell membrane, amplifying the action of the other drug (HOU; WU; FENG, 2020).

On the other hand, the cephalosporins, a class of beta-lactam antibiotics, exhibit potent antibacterial activity against multiple pathogens and relatively low toxicity, highlighting their potential in combination therapies (LIU et al., 2024; STURARO et al., 2024). Cephalosporins exert their antibacterial activity by binding to penicillin-binding proteins (PBPs), which are essential enzymes involved in the synthesis of the bacterial cell wall. This binding inhibits peptidoglycan cross-linking, ultimately leading to cell lysis and bacterial death (STEWART et al., 2020).

Based on their spectrum of activity and structural characteristics, cephalosporins are classified into five generations (FERNANDEZ; JIMENEZ-RODRIGUEZ; BLANCA-LOPEZ, 2021). First-generation cephalosporins, such as Cephalexin, are primarily effective against Gram-positive bacteria due to the accessibility of PBPs within their thick peptidoglycan layer, which lacks an outer membrane barrier. In contrast, third-generation cephalosporins, including Cefotaxime, Cefmenoxime, Ceftibuten, and Cefixime, exhibit enhanced activity against Gram-negative bacteria. This broader spectrum is attributed to structural modifications in the beta-lactam ring and side chains, such as the introduction of bulky groups and polar substituents, which confer greater resistance to beta-lactamase enzymes and improve penetration through porin channels in the outer membrane of Gram-negative bacteria **(Figure 1)** (BUI; PATEL; PREUSS, 2025). These adaptations enhance their stability, target affinity, and ability to overcome common mechanisms of bacterial resistance (YANKOVA et al., 2023).

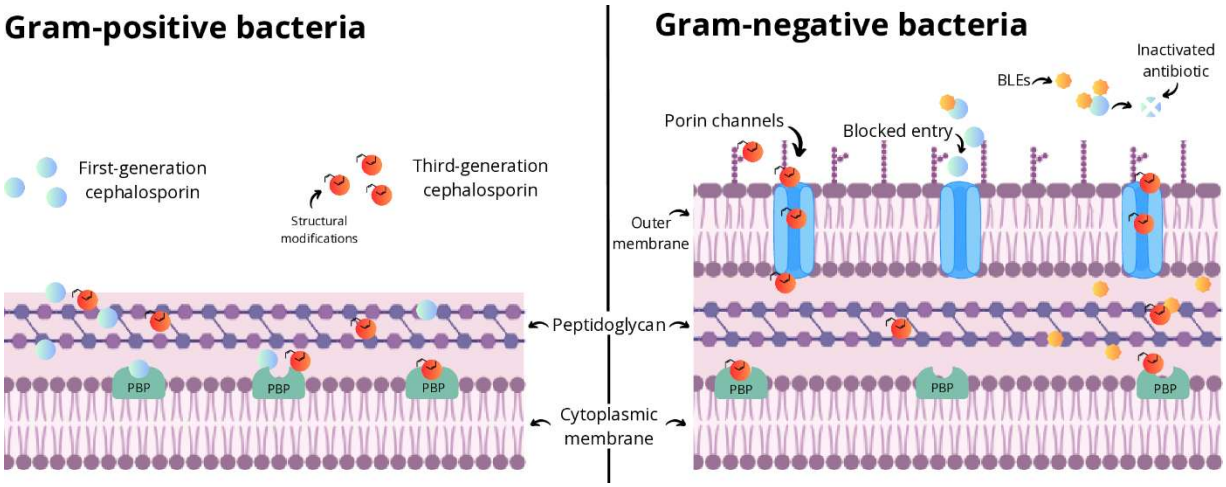

Figure 1. Representative illustration of the interaction between first- and third-generation cephalosporins with Gram-positive and Gram-negative bacterial cell walls. First-generation cephalosporins, characterized by a simpler chemical structure, are primarily effective against Gram-positive bacteria due to the absence of an outer membrane, which allows easy access to their target, the penicillin-binding proteins (PBPs), located within the thick peptidoglycan layer. In contrast, third-generation cephalosporins, with more complex structural modifications, are effective against both Gram-positive and Gram-negative bacteria. This broader activity is attributed to their ability to penetrate Gram-negative outer membrane porin channels and their increased resistance to degradation by beta-lactamase enzymes (BLE).

In clinical settings, Cefotaxime has strong activity against Gram-negative bacteria and some Gram-positive coverage; it is administered intravenously (IV) or intramuscularly (IM) at doses of 1–2 g every 6–12 hours, depending on the severity of the infection (LIU et al., 2024). Cefmenoxime has a similar spectrum to cefotaxime but is less commonly used; it is typically given IV or IM at 1–2 g every 12 hours (CAMPOLI-RICHARDS; TODD, 1987). Ceftibuten, an oral antibiotic, is primarily used for respiratory and urinary tract infections and is dosed at 400 mg once daily (LASKO; ASEMPA; NICOLAU, 2021). Cefixime, another oral third-generation cephalosporin, is used for a variety of infections, including otitis media and gonorrhea, with typical doses of 400 mg once daily or 200 mg every 12 hours (AJMAL et al., 2023). Lastly, Cephalexin, a first-generation cephalosporin, has stronger Gram-positive coverage, commonly used for skin and soft tissue infections, as well as uncomplicated urinary tract infections, with a typical oral dosage of 250–500 mg every 6–12 hours (EVERTS et al., 2021). While all belong to the cephalosporin class, differences in generation, route of administration, and bacterial coverage determine their specific clinical applications.

The cephalosporins extensive use has led to the emergence of resistant bacterial strains, including ESKAPE pathogens, which pose significant therapeutic limitations (MORÁN-DÍAZ et al., 2023). Despite this, cephalosporins remain clinically essential antibacterial agents. To address increasing antibiotic resistance, prudent use of drugs and development of new therapeutic strategies, including innovative combination therapies, are needed.

Although some studies have explored cephalosporin combination therapies targeting ESKAPE pathogens, only a small number have progressed to patent filings, and even fewer have advanced to clinical trials or achieved clinical application. Given the limited progress in this field, reviewing patents within this domain is crucial for addressing the scarcity of available resources, monitoring emerging developments, and gaining insight into the current technological landscape. Patents provide a valuable source of information on novel drug

formulations, mechanisms of action, and potential therapeutic applications (BRAGA et al., 2023; CARNEIRO et al., 2022).

Given the facts presented, this study aims to elucidate the innovative potential of cephalosporin-based combination therapy. Specifically, it seeks to experimentally investigate the interactions between selected cephalosporins—Cefotaxime, Cefmenoxime, Ceftibuten, Cephalexin, and Cefixime— with Polymyxin B against MDR, carbapenem- and polymyxin-resistant *Klebsiella pneumoniae*. The ultimate goal is to develop a new, effective therapeutic alternative for combating difficult-to-treat infections caused by these resistant pathogens.

2. LITERATURE REVIEW

In this section, a patent review will be presented in order to track therapeutic innovations related to cephalosporin-based combination therapy targeting a specific group of critical pathogens, known as the ESKAPE. This analysis will help us assess the market potential and clinical impact of our combinations, as we aim to develop an antimicrobial treatment with real-world applicability.

2.1 Patents methodology search

For this section, a patent review was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Herein, patents filed up to June 2024, were searched on the online database Espacenet provided by the European Patent Office (DE SOUZA et al., 2023).

Key terms were carefully selected after an in-depth analysis of various three-word combinations. The chosen terms were those that yielded the highest number of patents, thereby expanding the scope of our study. The patent search was then performed using the following key terms: “combination” AND “antibiotics” AND “cephalosporin”. The titles, abstracts, and full texts of the resulting patents were examined. The patent-related articles were obtained from PubMed and Google Scholar databases.

The patents obtained from the database search were meticulously screened by three independent reviewers to determine their eligibility for inclusion. The inclusion criteria for this study were as follows: (1) patents featuring at least one antimicrobial combination containing a cephalosporin antibiotic; (2) patents involving cephalosporin combinations tested against ESKAPE pathogens; and (3) patents with relevance to healthcare settings. Conversely, the

exclusion criteria included: (1) patents that did not involve cephalosporin combinations or ESKAPE pathogens, and (2) duplicate entries and inaccessible patents. During the study selection process, challenges such as language barriers and unavailable data were encountered. The Google Patents database was utilized to retrieve content, and patents that remained inaccessible despite efforts were excluded from the analysis.

The database search identified 666 patents, of which 30 were selected for detailed analysis based on inclusion and exclusion criteria, as well as their novelty and relevance to healthcare applications (Figure 2). This review focuses on innovations in cephalosporin combinations targeting ESKAPE pathogens, with the selected patents thoroughly detailed below.

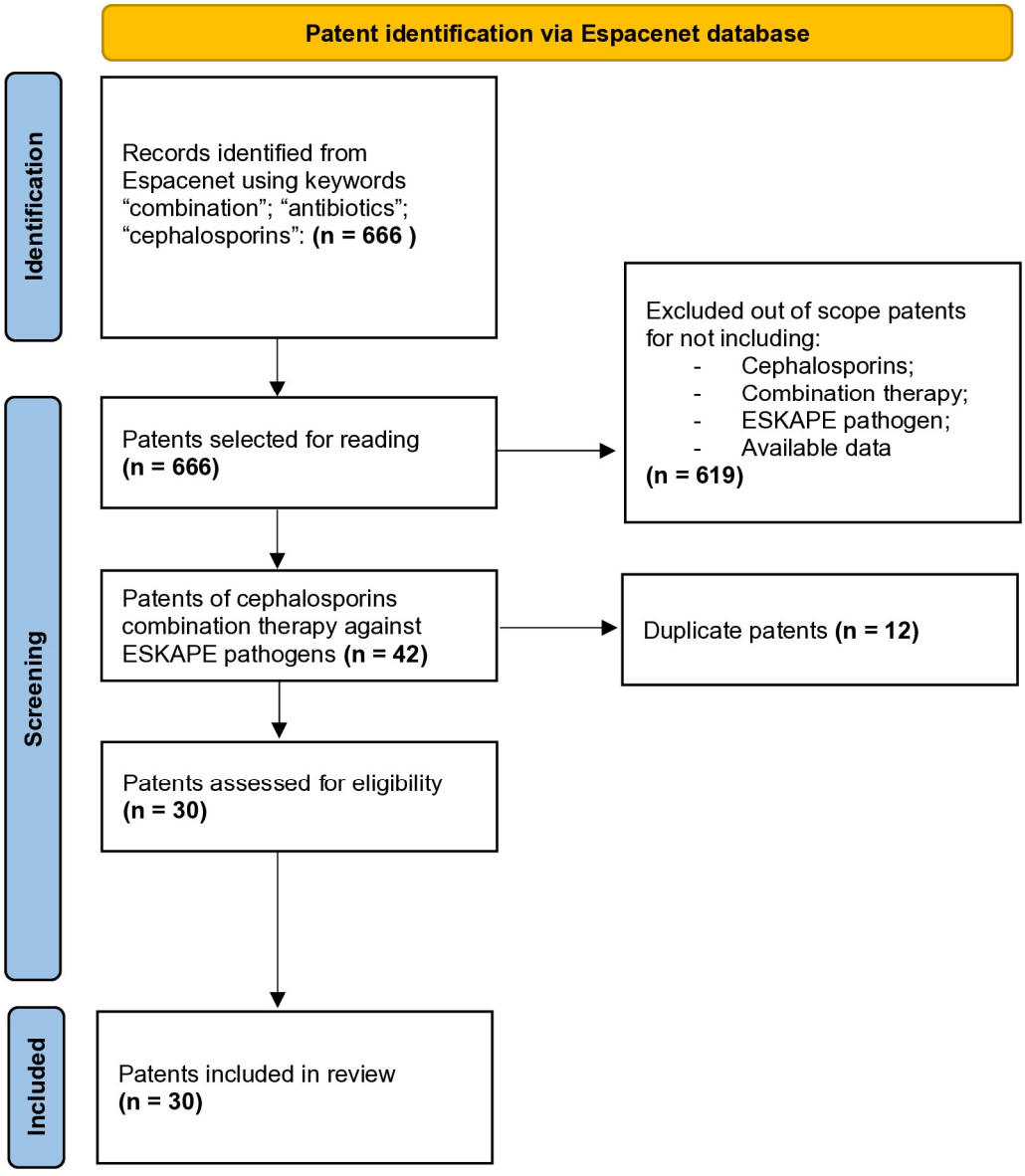

Figure 2. PRISMA flow diagram detailing the selection and screening process for the patent review. The diagram outlines the step-by-step methodology, including the initial identification of patents, screening for relevance and final inclusion of patents for analysis. This systematic approach ensures transparency and reproducibility in the review process.

2.2 Patents Features

The priority country refers to the country where the patent was initially filed or originated. The number of priority filings indicates the research and development level of a specific technology area within a region (PARIHAR; TELANG; OVHAL, 2020). Analysis of patent filings revealed that the priority countries for cephalosporin combination therapy patents were China (30%, 9 patents) and the United States (17%, 5 patents) **(Figure 3A)**. This finding is consistent with the data of the Worldwide Intellectual Property Office (WIPO) (https://www.wipo.int/ipstats/en/statistics/country_profile), which shows China and the United States as the top patent filers, with 1,586,339 and 515,281 applications, respectively, in 2022. These high numbers of patent applications could be attributed to their robust innovation ecosystems, significant investments in research and development, strong intellectual property policies, highly skilled workforces and advanced technological infrastructures, which collectively contribute to their dominance in global patent filings.

In total, 30 cephalosporin combination therapy patents were published between 1974 and 2024 **(Figure 3B).** In 1974, the emergence of antimicrobial resistance prompted initial efforts to explore novel therapeutic approaches utilizing cephalosporin combinations. Over the past 50 years, various combination antimicrobial therapies have been reported worldwide as a time and investment-saving method. The distribution of patent filings revealed both upward and downward trends over the years. Nonetheless, the patent application filings increased during the 21st century, with most filings reported in 2022. This increase may be attributed to increased research investments and technological advancements, which have facilitated scientific exploration and accelerated data generation.

Numerous studies have been conducted on combination therapies for ESKAPE pathogens. Analysis of patent applicants revealed that companies were the primary filers (20 patents), which reflected the economic importance of this strategy in promoting health **(Figure 3C)**. Conversely, universities filed only a few patents (6 patents), which highlights the need for finantial investments in academic research to enhance knowledge and innovation in this field.

Increased academic research funding is critical for driving innovation, advancing scientific knowledge, and addressing pressing global challenges.

The International Patent Classification (IPC) code, established by the WIPO, serves as a global classification system for patents. Notably, cephalosporin combination therapy patents are classified under IPC sections A (Human Necessities) and C (Chemistry) **(Figure 3D).** Herein, most of the patents were categorized under IPC codes A61K (preparations for medical, dental, or toiletry purposes), A61P (specific therapeutic activity of chemical compounds or medicinal preparations), and C07D (heterocyclic compounds), highlighting their importance regarding pharmaceutical applications. These classifications underscore the significant role that drug combinations play in medical and Chemical innovations.

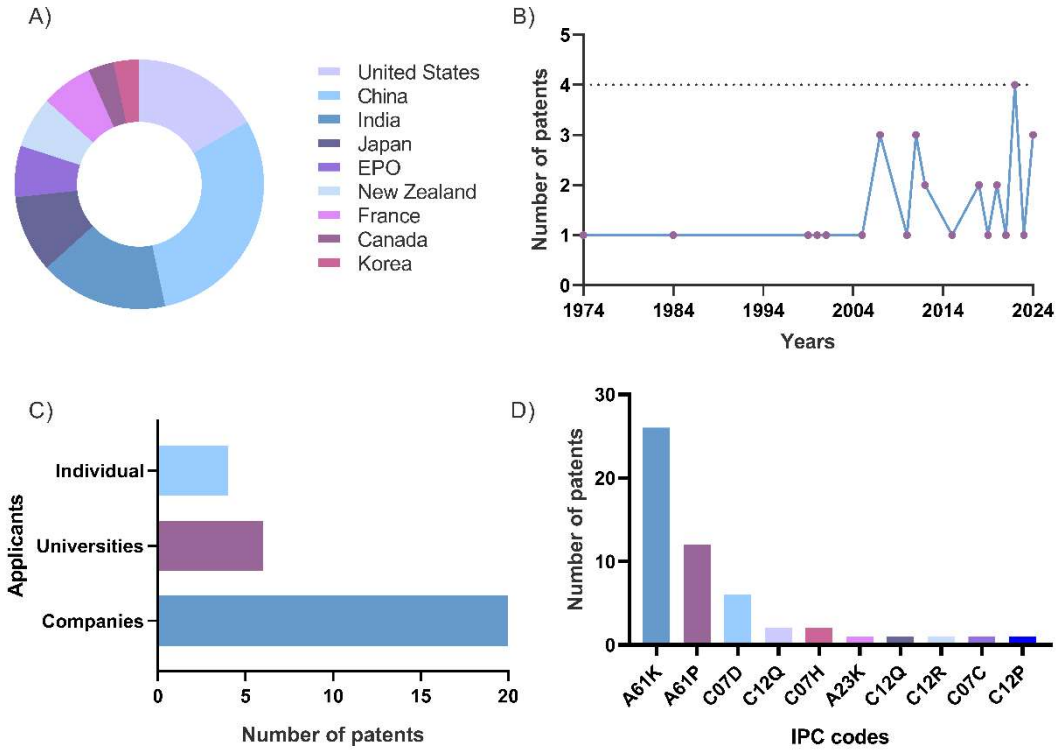

Figure 3. Selected patents features. **A)** Geographical distribution of the selected patents, illustrating global innovation efforts. The United States and China dominate as the top patent applicants, highlighting their leading roles in this field. EPO: European Patent Office. **B)** Publication trends of the selected patents. The data reveal a sharp increase in patent filings, with 2022 marking the highest number of applications, reflecting a surge in research and development. **C)** Patent applicant categories for the selected patents. Companies overwhelmingly lead the rankings, emphasizing the private sector's pivotal contribution to innovation in this domain. **D)** Interrelationships of IPC codes for selected patents. The International Patent Classification (IPC) codes provide insights into the nature of the patented inventions. A significant majority fall under the A61K code, which covers preparations for medical, dental, or toiletry purposes, underscoring the therapeutic focus of these innovations.

2.3 Cephalosporins combination with new developed antimicrobial compounds

Many patents included in this review reported the development of either novel antimicrobial compounds, or cephalosporin derivatives, and described their combinations **(Table 1)**. For example, "EP0911030A2" claims using vinyl-pyrrolidinone cephalosporin derivatives in combination with carbapenems (namely imipenem and meropenem) and beta-lactamase inhibitors (such as clavulanic acid, tazobactam, and sulbactam) to combat a broad spectrum of bacteria, including methicillin-resistant *S. aureus* (MRSA), *K. pneumoniae*, *Escherichia coli*, *Serratia marcescens* and *P. aeruginosa* (ANGEHRN et al., 1999). Moreover, "IN3216MU2013A" also claims the development of nitrogen-containing compounds associated with beta-lactamase inhibitors and other antibiotics, including cephalosporins, to combat bacterial infections (DESHPANDE et al., 2015).

Additionally, "KR101719556B1" describes very innovative products by reporting the development of 68 new cephalosporin derivatives with a siderophore group, a molecule produced by microorganisms to scavenge iron from the environment, to combat MDR Gram-negative bacteria, including *K. pneumoniae*, *A. baumannii*, and notably *P. aeruginosa*. The siderophore group attached to the antibiotic facilitates its internalization through the bacterial outer membrane, and enhance its action. Most of these derivatives presented low minimum inhibitory concentrations (MICs) against the tested strains. Four compounds (namely 4, 8, 11, and 26) were identified with antimicrobial activity, of them, compounds 4 and 8 exhibited notable enhanced antimicrobial activity *in vivo*, showing positive results against *P. aeruginosa* infections model, with high bioavailability and significant concentrations detected in rat blood. Plus, the potential use of these cephalosporin derivatives was explored in combination therapies or as drug carriers, expanding their therapeutic applications (CHO et al., 2012).

Similarly, "WO2020232534A1" reports the development of a broad-spectrum homodimeric tobramycin adjuvant that is notably non-toxic and designed to permeabilize the bacterial outer membrane for enhancing the antimicrobial effectiveness of associated antibiotics (SCHWEIZER; IDOWU, 2020). The homodimeric tobramycin adjuvant did not exhibit hemotoxicity, cytotoxicity, and toxicity in the *Galleria mellonella in vivo* model (at 200 mg/kg). Furthermore, it exhibited a synergic interaction with cephalosporins, such as cefotaxime and ceftazidime, generating fractional inhibitory concentration indices (FICIs) of ≤0.5 (IDOWU et al., 2019).

Additionally, “CA946284A” elucidates the development of rifamycin synthetic derivatives and their combinations with various antibiotics, including cephalosporins, penicillins, streptomycins, kanamycins, gentamycins, tetracyclines, macrolides, polypeptide antibiotics, and chloramphenicol. These combinations were designed to target a wide range of pathogens, including both Gram-negative and -positive bacteria among the ESKAPE pathogens, providing a broad-spectrum approach (KONOPKA; GELZER, 1974).

Comparably, “US2005171035A1” reports the development and use of aminoglycosides for a novel antibiotic strategy. Reportedly, it was used in combination with other antibiotic classes, including cephalosporins, to effectively combat a broad spectrum of bacterial pathogens, enhancing the therapeutic potential and scope of the associated antimicrobial agents (HADDAD; KOTRA; MOBASHERY, 2005). The innovative aminoglycosides were designed based on the neamine mechanism of action to interact and bind the bacterial A-site RNA (HADDAD et al., 2002).

Finally, “SU1075984A3” elucidates the development of novel carbapenem antibiotics C-19393 S_2 and H_2, obtained from *Streptomyces griseus* subsp. *cryophilus*, along with their antimicrobial applications. They act as beta-lactamase inhibitors, sensitizing resistant microorganisms and improving antibiotic action in a combination therapy (IMADA; KHARADA; ASAI, 1984). Reportedly, cephalosporins and penicillin may be great adjuncts for C-19393 S_2 and H_2, considering their beta-lactamase-inhibition ability (IMADA et al., 1980).

This review section highlights advancements in developing new antimicrobial compounds and cephalosporin derivatives, focusing on combating MDR bacteria and expanding therapeutic options. However, many patents in this section emphasize development methods over fully exploring antimicrobial potential through in vitro, in vivo, or clinical testing, for example only two out of seven patents described in vivo assays, and few had detailed combination’s mechanisms of action. Additionally, many patents lack specificity regarding antibiotics used in combinations, often referencing only classes rather than specific drugs, limiting practical application insights. Addressing these issues through increased funding and multidisciplinary collaboration could enhance the described innovations real-world applicability and market potential.

The development of new antimicrobial agents and their transition to real-world applications are essential to combat antimicrobial resistance, and cephalosporins (and their derivatives) have demonstrated significant synergistic potential against ESKAPE pathogens. However, it is notice that further research and well-designed clinical trials are necessary to

comprehensively assess the safety and efficacy of these combinations in healthcare settings. Nevertheless, such experimentation faces substantial regulatory challenges and requires significant financial investment, which is often lacking. Addressing these barriers is vital to advancing the development and application of these promising antimicrobial combinations.

Table 1. Included patents that described the development of new compounds, or cephalosporins derivates, and their respective combination therapy model.

New developed drug	Antibiotic in combination	ESKAPE pathogen	Mechanism of action	*In vivo* assay	Reference
Vinyl-pyrrolidinone Cephalosporin derivatives	Carbapenems and Beta-lactamase inhibitors	MRSA, *K. pneumoniae*, *E. coli*, *S. marcescens* and *P. aeruginosa*	−	−	Angehrn et al., 1999
Nitrogen-containing compounds	Cephalosporins and Beta-lactamase inhibitors	*Acinetobacter, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella*	−	−	Deshpande et al., 2015
Cephalosporin derivatives with a siderophore group	Not specified	*K. pneumoniae*, *A. baumannii* and *P. aeruginosa*	Outer membrane increased permeability	Rat infection model	Cho et al., 2012
Homodimeric Tobramycin adjuvant	Cefotaxime or Ceftazidime	*P. aeruginosa, K. pneumoniae, A. Baumannii* and *E. coli*	Outer membrane increased permeability	*Galleria mellonella* toxicity model	Schweizer; Idowu, 2020
Synthetic derivatives of Rifamycin	Cephalosporins	Gram-negative and -positive bacteria	−	−	Konopka; Gelzer, 1974
Novel Aminoglycosides	Cephalosporins	*Klebsiella, Acinetobacter, S. aureus*, *Pseudomonas*	A-RNA site binding	−	Haddad; kotra; mobashery, 2005
Novel carbapenems (C-19393 S_2 and H_2)	Cephalosporins	*E. coli*	Beta-lactamase inhibition	−	Imada; Kharada; Asai, 1984

− Inaccessible or not found data. MRSA: Methicillin-resistant *S. aureus.*

2.4 Combination of cephalosporins with commercial antibiotics

The majority of the reviewed patents (11 out of 30) highlighted the use of commercial antibiotic combinations as a promising antimicrobial strategy, primarily taking advantage of well-established drugs with known efficacy, safety profiles, and properties. Among these, third-generation cephalosporins emerged as the most frequently utilized class in these combinations

(Figure 4), likely due to their broad spectrum of action and favorable safety profile (STURARO et al., 2024).

Within this group of patents, "CN1305375A" proposed using penicillin, amoxicillin or amikacin in combination with a cephalosporin, notably cefixime or cefdinir, to treat mixed respiratory infections caused by *Streptococcus*, *Moraxella*, *Haemophilus*, and/or *Klebsiella* species (YOSHIMI; SHUICHI; HITOSHI, 2001). The cefixime/amikacin combination presented a synergic profile, with a mean FICI of 0.6 and remarkable antibacterial activity in a mouse respiratory tract infection model (MATSUMOTO, 1998). The claimed combinations could enhance the treatment efficacy against various pathogens.

Moreover, "WO0057882A1" claims various effective combinations of antibiotics against MRSA, particularly emphasizing the combination of cefdinir with oxytetracycline hydrochloride, ofloxacin, gentamicin sulfate, clarithromycin, or erythromycin. These combinations presented a low FICI in checkerboard assays, indicating a potent synergistic effect in combating MRSA strains (YOKOTA, 2000).

Additionally, "WO2007086013A1" elucidates a pharmacological composition combining ceftazidime with tazobactam, a beta-lactamase inhibitor, which was further enhanced using linezolid. This advanced formulation could target a broad spectrum of bacteria, including resistant Gram-negative and -positive bacteria. Moreover, administration routes—oral, topical, and parenteral—were recommended to maximize its utility and effectiveness across different clinical settings (SRINIVAS, 2007a). Similarly, "WO2007086012A1" reports combining cefpodoxime, as the cephalosporin component and clavulanic acid, as the beta-lactamase inhibitor to diversify the potential applications of their antimicrobial strategies (SRINIVAS, 2007b). Furthermore, "CN113194943A" established a pharmaceutical composition containing beta-lactamase inhibitors combined with cephalosporins to sensitize resistant bacteria (SUN; GAO; JIANG, 2021).

In addition, "CN102292079A" claims using ceftaroline, a third-generation cephalosporin, in combination with various antibiotics, including beta-lactams, aminoglycosides, tetracycline, sulfonamide, trimethoprim, fluoroquinolone, vancomycin, macrolide, polymyxin, glycylcycline, chloramphenicol, and lincosamide. The combinations were designed to treat skin infections and community-acquired pneumonia, offering a broad-spectrum approach to managing these serious infections effectively (BIEK, 2010).

The patent “US2020289610A1” also discusses the use of cephalosporins, such as cefazolin, cefuroxime, ceftazidime, cephalexin, cephaloridine, cefamandole, cefsulodin, cefonicid, cefoperazone, cefprozil, and ceftriaxone, individually or in any combination, with at least one polymyxin. Additionally, it claims the use of fluoroquinolones, aminoglycosides, and corticosteroids in the combination for treating ESKAPE pathogens in ocular, skin, and internal organ infections, where the drugs can be used simultaneously or sequentially. The combination of Cefuroxime, Polymyxin B, and Amikacin has demonstrated effectiveness against MRSA. Furthermore, the combination of Polymyxin B with Cefuroxime, Ceftazidime, and Levofloxacin has shown bactericidal properties while maintaining low toxicity (GARDNER, 2020).

Following the same concept, “NZ535648A” claims using an oxazolidinone antibiotic, commonly used to treat diabetic foot infections in mammals, in combination with cephalosporins, via multiple administration routes, to enhance antimicrobial activity against infection-causing resistant Gram-positive bacteria, including *S. aureus*, *Staphylococcus epidermidis*, and *Staphylococcus hemolyticus* (NORDEN, 2007).

Finally, “EP3560489A1” reports the use of various antibiotic combinations for developing targeted therapies and pharmaceutical compositions against bacterial infections, highlighting cephalosporins, a class of beta-lactam antibiotics, among these combinations. Cephalosporins are considered for their synergistic effects when used with other drugs, such as aminoglycosides, macrolides, and fluoroquinolones. This approach is aimed at enhancing the treatment efficacy against both Gram-positive and -negative bacteria, including challenging MDR strains (GONTAO; TYPAS; GÖTTIG, 2019).

This review section once again highlights a recurring pattern within patents for antibiotic combinations, revealing significant barriers to their real-world application. A notable issue is the lack of specificity in the patented combinations, particularly regarding the antibiotics used. Most patents focused exclusively on the in vitro antimicrobial activity of the combinations, with little to no investment in in vivo assays. Furthermore, most of the patents didn’t provide detailed insights into the antibacterial mechanisms of action of the included combinations. This lack of specific and comprehensive data poses challenges for the practical application of these innovations, particularly when considering ethical principles in their development and use.

Together, these patents highlight the promising potential of combining cephalosporins with commercial antibiotics to strengthen antibacterial therapy. Although these innovative strategies have real-world applicability and are frequently administrated in clinical settings, there remains a gap in evaluating their efficacy and safety for patient use. This gap should be addressed through scientific studies, which could, in turn, lead to further patent filings.

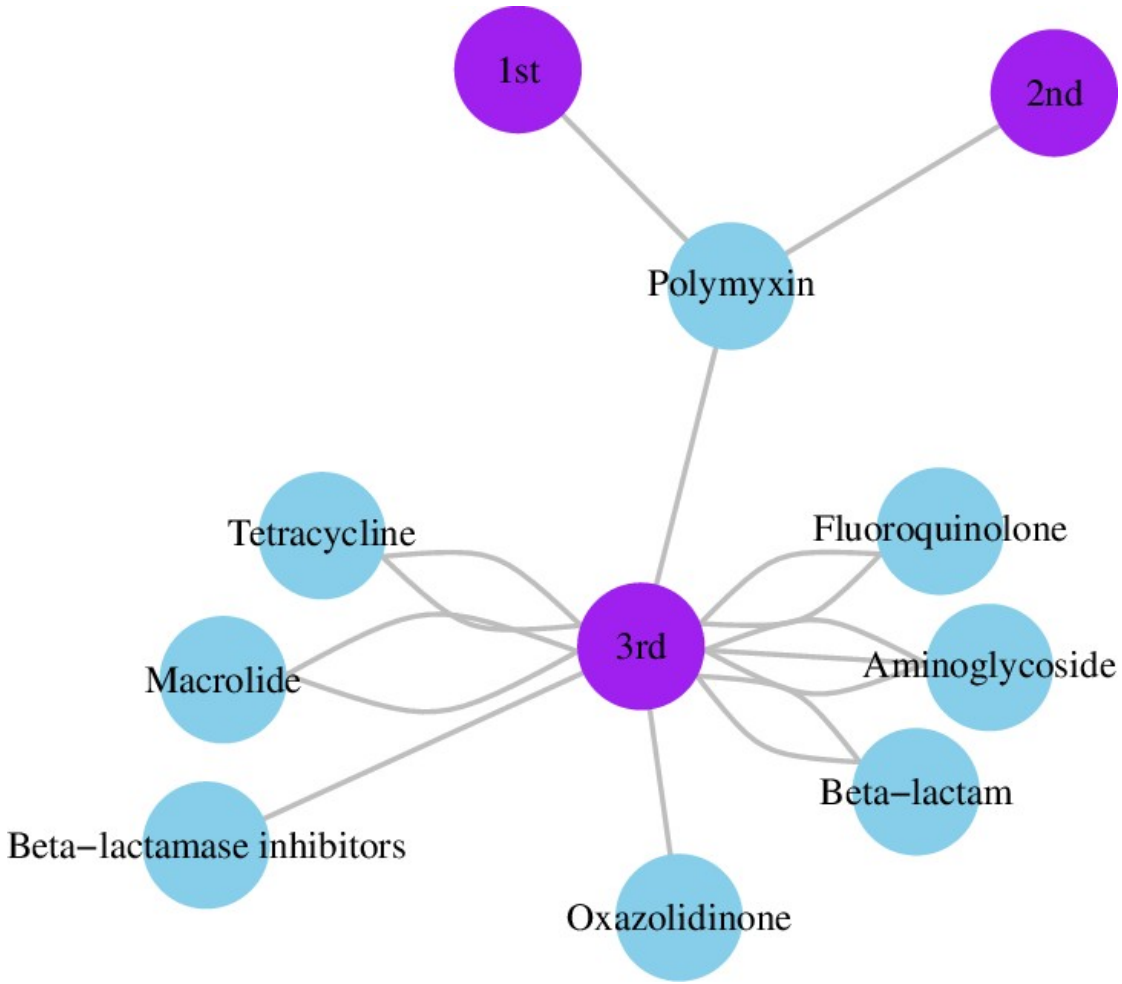

Figure 4. Commercial antibiotic combination network graphic illustrating the relationships between antibiotic classes and cephalosporin generations in patented combinations. Blue nodes represent antibiotic classes (including beta-lactamase inhibitors), while purple nodes indicate the generation of the associated cephalosporins (first – 1st, second – 2nd, or third – 3rd generation). The connections between nodes show which antibiotic classes are combined with specific cephalosporin generations in the patents and the number of connections reflects its frequency in the dataset mentions. Third-generation cephalosporins were the most frequently cited in combination patents, particularly alongside aminoglycosides.

2.5 Cephalosporins combinations with repositioned compounds

Many patents describe the combination of cephalosporins with non-antibiotic compounds **(Table 2)**, aiming to explore novel applications for them, thereby characterizing a

repurposing strategy. As an example, “CN108125954A” proposed the use of amlodipine, an antihypertensive medicine, in combination with cephalosporins to combat *A. baumannii* and MRSA infections (ZIYUE, 2018). Amlodipine demonstrated potent beta-lactamase inhibition activity, effectively covering a broader range of beta-lactamases compared to clavulanic acid and sulbactam. This capability helps counteract antimicrobial resistance mediated by lytic enzymes. Additionally, amlodipine exhibited synergistic interaction with cefuroxime against an MRSA strain, achieving a remarkably low FICI of 0.125 within 22 hours of exposure (YI; PEI; XIAOYAN, 2019).

Additionally, “CN117442734A” claims a pharmaceutical composition containing α-pinene and beta-lactam antibiotics for combating MDR *A. baumannii*. α-Pinene, a monoterpene, is obtained from essential oils and exhibits antimicrobial activity against Gram-positive bacteria. Notably, α-pinene and meropenem exhibited synergistic interaction, showing good results in checkerboard assays, biofilm inhibition, and in a mice model of infection. Although meropenem was the main studied associated antibiotic, the combination of α-pinene with cephalosporins such as cefoperazone/sulbactam or ceftazidime was also proposed by inventors (ZENG et al., 2024). Similarly, “WO2023047421A1” proposed using plant-based compositions with cephalosporins against resistant Gram-negative and -positive pathogens (LAKSHMISUBRAMANIAN, 2023).

Innovatively, “CN114652716A” claims the application of dimetridazole (an antiprotozoal agent) with cephalosporins, especially cefotaxime (a third-generation cephalosporin), for combating drug-resistant *E. coli* in veterinary environments. Although *E. coli* is not officially included in the ESKAPE pathogens, it is considered an active member of the group because of its increased pathogenicity and ability to acquire antimicrobial resistance (AYOBAMI et al., 2022; CRAVEN et al., 2024). The combination FICI ranged from 0.3125 to 0.375, indicating synergism between dimetridazole and cefotaxime. The combination potential for bacterial sensibilization and treatment dosage reduction were highlighted by the authors (WEI et al., 2022a). Similarly, the “CN114831994A” discusses the efficacy of cefotaxime–dimenidazole combination for combating drug-resistant *K. pneumoniae*. The combination had FICIs ranging from of 0.25 to 0.375, demonstrating a synergistic antimicrobial interaction (WEI et al., 2022b).

Some innovative patents, such as “CN117771379A” claim the combination of a fully human antibody or antigen-binding fragment with an antibiotic, such as cephalosporin, for combating resistant *S. aureus* (LIAO et al., 2024). Additionally, “CN114432428A” proposed

the combination of the polypeptide PGLa with cephalosporins to increase the sensibility of bacteria (HUPING et al., 2022), further, "CN117343131A" proposed the innovative combination of snake venom peptides (SVP) with cephalosporins antibiotics to treat *A. baumannii* and *E. coli* infections, in special, the combination of cefotaxime with SVP yielded a FICI of 0.31 against an ESBL-positive *E. coli* strain, indicating a strong synergistic interaction (ZHILIANG et al., 2024), which demonstrates the potential antimicrobial activity of peptides, especially when associated to a cephalosporin.

Interestingly, "WO2018141063A1" elucidates an innovative use for bicarbonate as an enhancer for various antimicrobial agents, including cephalosporins, fluoroquinolones, macrolides, tetracyclines, and various other agents that inhibit the growth of viruses, bacteria, fungi, and parasites (BROWN et al., 2018). Bicarbonate can dissipate the pH gradient of the proton motive force in bacterial cytoplasmic membranes, thereby enhancing the action of conjugated antibiotics (FARHA et al., 2018).

These patents underscore the promising potential of combining cephalosporins with repurposed compounds to address the growing challenge of antibiotic resistance. By enhancing the efficacy of cephalosporins, these innovative strategies offer a pathway to developing more effective antimicrobial treatments. However, the transition of repositioned compounds into clinical practice faces significant challenges, including stringent regulatory requirements, limited funding, scalability to industrial production, skepticism from healthcare providers and patients, and the need to optimize dosing regimens. Moreover, the reviewed patents once again revealed a notable lack of information regarding the toxicity profile and the combinations progression to clinical experimentation. Considering the barriers to developing new antimicrobial therapies, addressing these gaps is essential to fully realize the potential of drug repurposing within combination treatments, ultimately advancing the fight against resistant infections.

Table 2. Reviewed patents of cephalosporins in combination with repurposed compounds.

Combination				
Cephalosporin	**Repurposed compound**	**Nature of the repurposed compound**	**Microorganism**	**Reference**

Ceftazidime, Ceftriaxone or Cefuroxime	Amlodipine	Antihypertensive medicine	*A. baumannii* and MRSA	Ziyue 2018
Cefoperazone/sulbactam or Ceftazidime	A-pinene	Essential oils component	*A. baumannii*	Zeng et al., 2024
Ceftriaxone	Plant-based MDRi	Plants extracts (from a variety of specimens)	Gram-negative and –positive bacteria	Lakshmisubramanian, 2023
Cefotaxime	Dimetridazole	Antiprotozoal	*E. coli*	Wei et al., 2022a
Cefotaxime	Dimenidazole	Antiprotozoal	*K. pneumoniae*	Wei et al., 2022b
Cefazolin	TRN1029, TRN1030, TRN1031, TRN1032 or TRN1033	Fully human antibody or antigen-binding fragment	MSSA	Liao et al., 2024
Cefazolin, Cephalexin, Cefoxitin, Cefotaxime, Cefuroxime or Ceftazidime	PGLa	Polypeptide	Gram-negative and –positive bacteria	Huping et al. 2022
Cefotaxime	Peptide	Snake venom	*A. baumannii* or *E. coli*	Zhiliang et al. 2024
−	Bicarbonate	Weak base	Gram-negative and –positive bacteria	Brown et al., 2018

− Not specified, authors have cited a wide range of cephalosporins. MRSA: Methicillin-resistant *S. aureus*. MSSA: Methicillin-sensitive *S. aureus*.

2.6 Identification methodologies using cephalosporins' combinations

Some studies included in this review aimed to develop detection methods for resistant ESKAPE bacteria utilizing cephalosporin combinations. While these combinations do not directly target the pathogens, they aim to support the selection of the most appropriate treatment, making this process more assertive **(Figure 5)**. For instance, "US2011165604A1" presents a reaction medium that utilizes antibiotic combinations at sub-inhibitory concentrations for identifying MRSA. Reportedly, these antibiotic combinations enhanced both the specificity and sensitivity of the medium in detecting and isolating MRSA. Notably, various combinations of cephalosporins, from all generations, and carbapenems were used, with the combinations of cefoxitin with ceftriaxone, cefotaxime, ertapenem, cefoperazone, or cefpodoxime being particularly significant (ORENGA; ROBICHON; ZAMBARDI, 2011).

Additionally, "CN102586390A" claims the development of a specialized detection culture medium that incorporates antibiotic combinations, including cephalosporins,

specifically designed to differentiate resistant Gram-negative microorganisms. This innovative medium could distinguish three different microorganisms from a single biological sample, offering a notable advancement in diagnostic microbiology (ORENGA et al., 2012).

Finally, "CA3175879A1" elucidates an advanced system for identifying strain in polymicrobial infections caused by ESKAPE and other pathogens to optimize antibiotic therapy selection. This system integrates genetic identification, through polymerase chain reaction and genomic sequencing, and analyzes of the antimicrobial resistance profile using genomic markers and phenotypic studies. Thereafter, antibiotics are determined based on the resistibility or susceptibility of the polymicrobial sample, thereby guiding a precise selection of a cephalosporin combination therapy (BAUNOCH et al., 2022).

The reviewed patents show the notable advancements in using cephalosporin combinations for identifying resistant ESKAPE pathogens. These methodologies leverage the enhanced specificity and sensitivity provided by antibiotic combinations to improve diagnostic accuracy and guide effective treatment strategies. However, extensive research to validate these methodologies in diverse healthcare settings is warranted.

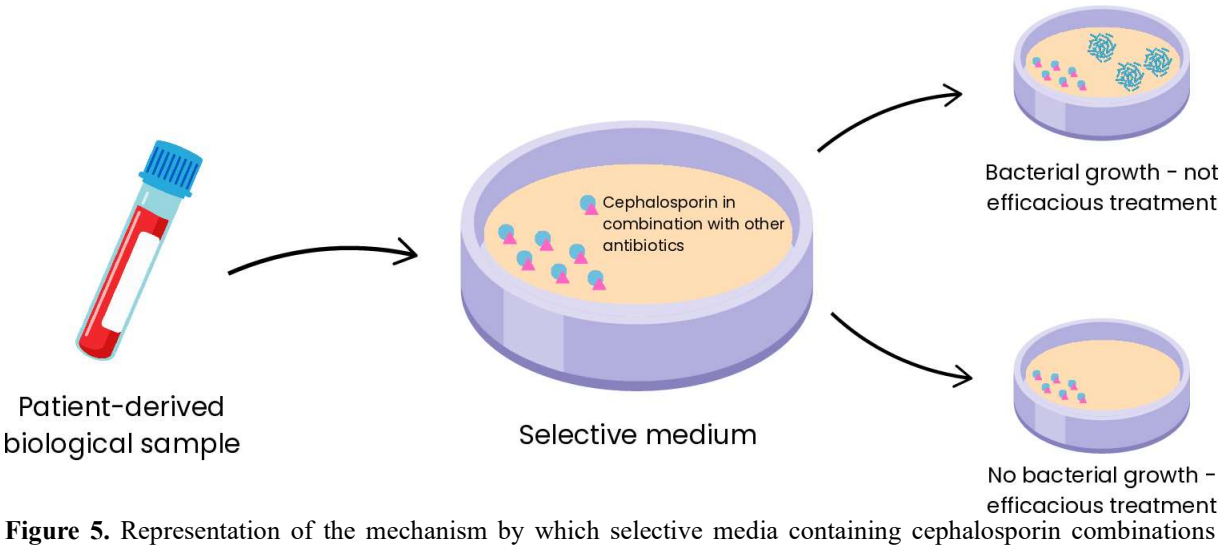

Figure 5. Representation of the mechanism by which selective media containing cephalosporin combinations enable precise selection of antibiotic regimens for treating infections caused by ESKAPE pathogens. The diagram illustrates how the selective media function to isolate and identify resistant bacterial strains. By leveraging the activity of specific cephalosporin combinations, the media inhibit non-target organisms while promoting the growth of resistant strains, allowing clinicians to determine the most effective antibiotic therapies for combating these high-priority pathogens.

2.7 Cephalosporins combination therapy with clinical trials

Interestingly, only two patent-associated studies progressed to clinical trials (Figure 5). "NZ555076A" is one of them and claims the amikacin–cefepime combination for treating infections caused by pathogens such as E. coli, K. pneumoniae, Streptococcus pneumoniae, Enterococcus faecalis, P. aeruginosa, and S. aureus. Associated study showed that this combination could reduce hospitalization time for patients and decrease the treatment nephrotoxicity and overall healthcare costs (CHAUDHARY, 2010). The combination therapy efficacy was evaluated in a clinical trial where patients with nosocomial pneumonia were divided into two groups (n = 100 each). One group was treated with the amikacin–cefepime combination, whereas the other group received an intravenous injection of cefepime alone for 7–10 days.

The study indicated that the Cefepime-Amikacin combination was significantly more effective than Cefepime alone. In patients receiving the combination therapy, 89% showed a clinically successful outcome, compared to 71% in the Cefepime-only group. Bacteriological success was also higher, with 90% of patients in the combination group showing pathogen eradication, against 66% in the Cefepime-only group. This trend was particularly pronounced in patients infected with *P. aeruginosa*, where the combination led to a 92% clinical success rate compared to 46% in the Cefepime-only group ($p<0.05$). Further, the combination was well tolerated, with no major adverse events reported. Laboratory parameters remained stable across both treatment groups (CHAUDHARY et al., 2008).

Similarly, "US2011257079A1" reports a pharmacological composition combining glycopeptides and cephalosporins for treating drug-resistant Staphylococci bacteria, particularly highlighting the vancomycin–ceftriaxone combination. This combination exhibited potential for treating non-ocular infections via parenteral administration (CHAUDHARY, 2011).

In the clinical study, the vancomycin–ceftriaxone combination was efficacious, with 70% of patients achieving clinical cure within seven days. Significant reductions in total leukocyte count and erythrocyte sedimentation rate indicated recovery from infections. The combination was particularly effective due to its broad antibacterial spectrum, targeting both Gram-positive and Gram-negative pathogens. The treatment was well-tolerated with no major adverse effects. Key safety parameters, including liver (serum glutamyl oxaloacetic transaminase and glutamyl pyruvic transaminase levels) and kidney (serum creatinine and urea

levels) function tests, showed no significant changes post-treatment, suggesting low toxicity. Common mild side effects included pain at the injection site, nausea, and dizziness, affecting less than 5% of patients (CHAUDHARY; SHRIVASTAVA; SEHGAL, 2008).

The amikacin/cefepime and vancomycin/ceftriaxone combination presents the potential of cephalosporin-based combination therapies to improve the treatment outcomes for severe and resistant infections. Despite promising results, both studies are limited by a lack of long-term post-treatment outcomes, insufficient data on potential resistance development, and the absence of pathogen-specific regimens, which together challenge the real-world applicability of these treatments. Further research and clinical trials are necessary to explore other potential cephalosporin-related combinations.

Additionally, it is crucial to ensure ongoing monitoring of resistance patterns and adverse effects in larger and more diverse patient populations to ensure efficacy and enhance the developed strategies. Innovations in drug delivery systems and formulations may also contribute to enhancing the efficacy and safety of these combination therapies. However, the complexity and resource-intensive nature of such experimentation can pose significant challenges and act as a barrier for scientists in advancing their research to the clinical trial stage. This is evident in the fact that only two patents included clinical data. Such limitations not only hinder progress but also affect the real-world applicability of cephalosporin combinations, as clinical trials are a critical step in validating the safety, efficacy, and practicality of these therapies for widespread use.

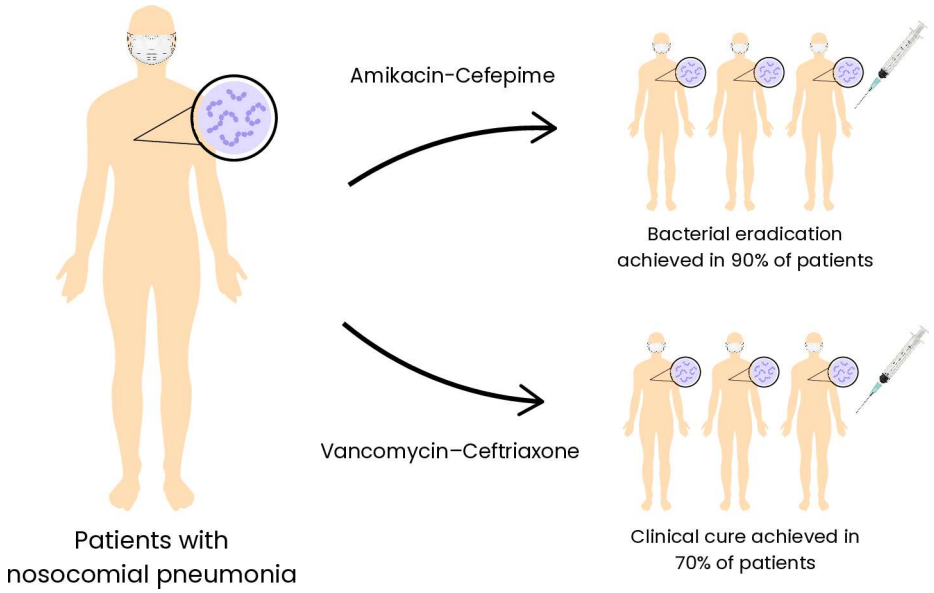

Figure 6. Representation of the two patented antibiotic combinations undergoing clinical trials and their therapeutic outcomes. The amikacin-cefepime combination demonstrated remarkable efficacy, eradicating bacterial pathogens in 90% of the patients included in the study. Similarly, the vancomycin-ceftriaxone combination achieved a clinical cure in 70% of the patients. The figure highlights the potential of these patented combinations in addressing infections caused by bacterial pathogens and their promising results in clinical applications.

2.8 Resistance profile of ESKAPE pathogens

Some of the patents included in this review focused on combating antimicrobial-resistant bacteria, particularly due to the broad-spectrum activity of antibiotic combinations. MRSA was a primary target for cephalosporin combination therapies. According to "US2011165604A1" MRSA is characterized by its resistance to methicillin and oxacillin, mediated by the *mecA* gene, which encodes the modified protein PBP2a. MRSA accounts for a significant proportion of nosocomial infections and is often associated with severe and potentially fatal health issues. MRSA infections, which are frequently cross-transmitted between patients via healthcare staff, are highly contagious and responsible for endemic outbreaks that are challenging to control (ORENGA; ROBICHON; ZAMBARDI, 2011).

Several patents specifically aimed to address MRSA and other gram-positive pathogens. For instance, "EP0911030A2" targeted beta-lactamase-producing MRSA strains (ANGEHRN et al., 1999). Additionally, "NZ555076A" focused on treating hospital-acquired pneumonia caused by beta-lactam-resistant MRSA (CHAUDHARY, 2010). "WO0057882A1" also claimed combination therapies targeting gram-positive pathogens, including MRSA and Methicillin-resistant *Staphylococcus epidermidis* (MRSE), both of which cause severe infections in immunocompromised or elderly patients (YOKOTA, 2000). Other patents, such as "CN108125954A", "CN102292079A", and "US2020289610A1" addressed the challenge of combating MRSA through various combinations (BIEK, 2010; GARDNER, 2020; ZIYUE, 2018).

Resistant Gram-negative bacteria were also a significant focus of cephalosporin combination therapies due to their severe impact on public health. The patent "KR101719556B1" targeted resistant *P. aeruginosa* strains with mutations in outer membrane and porin channels, which hinder antibiotic efficacy (CHO et al., 2012). "WO2020232534A1" described a combination with potent bactericidal activity against Gram-negative carbapenem-resistant and colistin-resistant strains harboring plasmid-borne *mcr-1* genes (SCHWEIZER;

IDOWU, 2020). Additionally, "WO2007086013A1" and "WO2007086012A1" addressed penicillin-resistant organisms with penicillin-binding-protein-mediated resistance mechanisms, including *P. aeruginosa* and *E. coli* (SRINIVAS, 2007b, 2007a).

The patent "US2016257684A1" introduced novel approaches to combat Extended-Spectrum Beta-Lactamase (ESBL) producing strains of *K. pneumoniae*, *E. coli*, and *P. aeruginosa* (DESHPANDE et al., 2015). "CN117442734A" claimed a new combination for treating beta-lactamase-producing *A. baumannii*, particularly focusing on the carbapenemase oxacillinase (ZENG et al., 2024). Furthermore, "CN114831994A" aimed to combat *K. pneumoniae* resistant to multiple antibiotics, including gentamicin, tetracycline, chloramphenicol, ceftriaxone, cefazolin, cefuroxime, cefepime, and lomefloxacin (WEI et al., 2022b) . "CN114652716A" targeted beta-lactam-resistant *E. coli* (WEI et al., 2022a), while "CN113194943A" addressed beta-lactamase-producing Gram-negative bacteria (SUN; GAO; JIANG, 2021). Finally, "WO2023047421A1" aimed to combat multi-drug-resistant bacteria such as *A. baumannii*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, focusing on efflux pumps and the production of lytic enzymes (LAKSHMISUBRAMANIAN, 2023).

2.9 Conclusion

Overall, patent reviews play a critical role in tracking innovations and investments, offering valuable insights into future advancements and guiding research and development efforts toward new and effective antimicrobial therapies to combat the growing threat of antimicrobial resistance. The analysis of patents on cephalosporin combination therapies highlights their significant potential in addressing ESKAPE pathogens. Our findings reveal that cephalosporins exhibit synergistic interactions with a wide range of compounds, including new developed ones, repositioned drugs and established antibiotics. Notably, these combinations often expand the antimicrobial spectrum, reduce treatment toxicity, and demonstrate potent antibacterial efficacy, thereby improving treatment success rates.

Our review identified significant barriers to the market implementation of these innovative therapies. We encountered a notable limitation concerning the variability in data comprehensiveness across the patents analyzed. While some patents provided extensive details, including animal experimentation and complex experimental data, others offered only preliminary findings, merely indicating synergism and proposing combinations. The

inconsistency in experimental standards may contribute to the limited real-world application and technology transfer of patents related to healthcare settings.

In addition, many patents lacked specificity regarding the antibiotics used, relied primarily on in vitro experimentation without sufficient in vivo studies, and failed to progress to clinical trials. This stagnation is largely attributed to the extensive financial resources required for clinical trial validation, which has hindered the advancement of these combinations. To unlock the full potential of cephalosporins-based therapies, it is crucial to prioritize future research efforts encompassing comprehensive preclinical and clinical validation, alongside strategic implementation initiatives. Such efforts are essential to effectively address infectious diseases and realize the promise of the proposed technologies in real-world applications.

3. OBJECTIVES

3.1 GENERAL

- Investigate the effectiveness of cephalosporin-based combinations with Polymyxin B against polymyxin-carbapenem-resistant *K. pneumoniae*, aiming to identify promising and innovative therapeutic possibilities.

3.2 SPECIFICS

- Review patent fillings for cephalosporin-based combination therapies, to increase the comprehension regarding the innovative potential of these antimicrobial approaches in combating resistant pathogens.
- Evaluate the antibacterial activity and interaction of cephalosporins-based combination with Polymyxin B on bacterial survival.
- Assess the ability of the combinations to prevent or reduce biofilm formation, a key factor in bacterial persistence and resistance.
- Evaluate the toxicity of the antibiotic combinations to ensure minimal toxicity to host cells.
- Assess the pharmacokinetic/pharmacodynamic (PK/PD) parameters of the Ceftibuten-Polymyxin B combination against polymyxin-carbapenem-resistant *K. pneumoniae*.

- Investigate the *in vivo* potential of the combinations as candidates for innovative therapeutic strategies to address antimicrobial resistance.

4. REFERENCES

AJMAL, M. et al. Clinical pharmacokinetics of cefixime: a systematic review. **Xenobiotica; the Fate of Foreign Compounds in Biological Systems**, v. 53, n. 3, p. 149–162, mar. 2023

ANGEHRN, P. et al. Antimicrobial composition comprising a vinyyl pyrrolidinon derivative and a carbapenem antibiotic or a beta-lactamase inhibitor., 28 abr. 1999.

AYOBAMI, O. et al. Antibiotic resistance in hospital-acquired ESKAPE-E infections in low- and lower-middle-income countries: a systematic review and meta-analysis. Emerging Microbes & Infections, v. 11, n. 1, p. 443–451, dez. 2022.

BAUNOCH, D. A et al. Methods and Systems for Determining Suitability of Compositions for Inhibiting Growth of Polymicrobial Samples., 25 ago. 2022.

BIEK, D. Compositions and Methods of Treatment Comprising Ceftaroline. , 4 mar. 2010.

BRAGA, L. et al. The global patent landscape of artificial intelligence applications for cancer. Nature Biotechnology, v. 41, n. 12, p. 1679–1687, dez. 2023.

BROWN, E. et al. Bicarbonate as a Potentiator for Antimicrobial Agents. , 9 ago. 2018.

BUI, T.; PATEL, P.; PREUSS, C. V. Cephalosporins. Em: StatPearls. Treasure Island (FL): StatPearls Publishing, 2025.

CAMPOLI-RICHARDS, D. M.; TODD, P. A. Cefmenoxime. A review of its antibacterial activity, pharmacokinetic properties and therapeutic use. **Drugs**, v. 34, n. 2, p. 188–221, ago. 1987.

CARNEIRO, D. C. et al. A patent review of the antimicrobial applications of lectins: Perspectives on therapy of infectious diseases. Journal of Applied Microbiology, v. 132, n. 2, p. 841–854, fev. 2022.

CHAUDHARY, M. et al. Efficacy and Safety Evaluation of Fixed Dose Combination of Cefepime and Amikacin in Comparison with Cefepime Alone in Treatment of Nosocomial Pneumonia Patients. Current Clinical Pharmacology, v. 3, n. 2, p. 118–122, 1 maio 2008.

CHAUDHARY, M. Antibiotic combinations for providing total solution to the treatment of infections. , 29 jan. 2010.

CHAUDHARY, M. Parenteral Combination Therapy for Infective Conditions With Drug Resistant Bacterium., 20 out. 2011. Disponível em: <https://patents.google.com/patent/US20110257079A1/en?oq=US2011257079A1>. Acesso em: 29 abr. 2024

CHAUDHARY, M.; SHRIVASTAVA, S. M.; SEHGAL, R. Efficacy and Safety Study of Fixed-Dose Combination of Ceftriaxone-Vancomycin Injection in Patients with Various Infections. Current Drug Safety, v. 3, n. 1, p. 82–85, 1 jan. 2008.

CHO, Y. L. et al. Novel Cephalosporin Derivatives and Pharmaceutical Compositions Thereof. , 18 out. 2012.

COATES, A. R. M. et al. Antibiotic combination therapy against resistant bacterial infections: synergy, rejuvenation and resistance reduction. Expert Review of Anti-Infective Therapy, v. 18, n. 1, p. 5–15, jan. 2020.

CRAVEN, T. W. et al. Computational Design of Cyclic Peptide Inhibitors of a Bacterial Membrane Lipoprotein Peptidase. ACS chemical biology, v. 19, n. 5, p. 1125–1130, 17 maio 2024.

DE SOUZA, G. H. et al. Synergistic interaction of polymyxin B with carvacrol: antimicrobial strategy against polymyxin-resistant Klebsiella pneumoniae. Future Microbiology, 8 fev. 2024.

DE SOUZA, G. H. DE A. et al. Antimicrobial peptides against polymyxin-resistant Klebsiella pneumoniae: a patent review. World Journal of Microbiology & Biotechnology, v. 39, n. 3, p. 86, 1 fev. 2023.

DENISSEN, J. et al. Prevalence of ESKAPE pathogens in the environment: Antibiotic resistance status, community-acquired infection and risk to human health. International Journal of Hygiene and Environmental Health, v. 244, p. 114006, 1 jul. 2022.

DESHPANDE, P. K. et al. Nitrogen Containing Compounds and Their Use. , 3 jul. 2015.

EVERTS, R. J. et al. Probenecid effects on cephalexin pharmacokinetics and pharmacodynamics in healthy volunteers. **The Journal of Infection**, v. 83, n. 2, p. 182–189, ago. 2021

FARHA, M. A. et al. Bicarbonate Alters Bacterial Susceptibility to Antibiotics by Targeting the Proton Motive Force. ACS Infectious Diseases, v. 4, n. 3, p. 382–390, 9 mar. 2018.

FERNANDEZ, J.; JIMENEZ-RODRIGUEZ, T. W.; BLANCA-LOPEZ, N. Classifying cephalosporins: from generation to cross-reactivity. Current Opinion in Allergy and Clinical Immunology, v. 21, n. 4, p. 346–354, 1 ago. 2021.

GARDNER, S. Antimicrobial Formulations and Applications Thereof. , 17 set. 2020.

GONTAO, B. A. R.; TYPAS, A.; GÖTTIG, S. Pharmaceutical Compositions for Prevention and/or Treatment of Infections and Antibacterial-Induced Dysfunctions. , 30 out. 2019.

HADDAD, J. et al. Design of Novel Antibiotics that Bind to the Ribosomal Acyltransfer Site. Journal of the American Chemical Society, v. 124, n. 13, p. 3229–3237, 1 abr. 2002.

HADDAD, J.; KOTRA, L.; MOBASHERY, S. Aminoglycosides as antibiotics. , 4 ago. 2005.

HOU, S.-Y.; WU, D.; FENG, X.-H. Polymyxin monotherapy versus polymyxin-based combination therapy against carbapenem-resistant Klebsiella pneumoniae: A systematic review and meta-analysis. Journal of Global Antimicrobial Resistance, v. 23, p. 197–202, dez. 2020.

HUGHES, D.; KARLÉN, A. Discovery and preclinical development of new antibiotics. Upsala Journal of Medical Sciences, v. 119, n. 2, p. 162–169, maio 2014.

HUPING, X et al. Application of PGLa in improving sensitivity of bacteria to antibiotics and delaying generation of bacterial drug resistance. , 6 maio 2022. Disponível em: <https://patents.google.com/patent/CN114432428A/en?oq=CN114432428A>. Acesso em: 10 jun. 2024

IDOWU, T. et al. Homodimeric Tobramycin Adjuvant Repurposes Novobiocin as an Effective Antibacterial Agent against Gram-Negative Bacteria. Journal of Medicinal Chemistry, v. 62, n. 20, p. 9103–9115, 24 out. 2019.

IMADA, A. et al. C-19393 S2 and H2, new carbapenem antibiotics. I. Taxonomy of the producing strain, fermentation and antibacterial properties. The Journal of Antibiotics, v. 33, n. 12, p. 1417–1424, dez. 1980.

IMADA, A.; KHARADA, S.; ASAI, M. Process for Preparing Antibiotic C-19393 S2 and/or C-19393 H2. , 23 fev. 1984.

JÚNIOR, S. D. DA C. et al. Dissemination and prevalence of polymyxin-resistant bacteria of clinical and environmental origin. Research, Society and Development, v. 11, n. 13, p. e94111334996–e94111334996, 29 set. 2022.

KONOPKA, E. A; GELZER, J. M. Synergistic Antibiotics. , 30 abr. 1974.

KULSHRESTHA, M.; TIWARI, M.; TIWARI, V. Bacteriophage therapy against ESKAPE bacterial pathogens: Current status, strategies, challenges, and future scope. Microbial Pathogenesis, v. 186, p. 106467, jan. 2024.

LAKSHMISUBRAMANIAN, S. Plant-Based Compositions for Inhibiting Multi-Drug Resistance (mdr) and Potentiating Antibiotics in Bacterial Pathogens. , 30 mar. 2023.

LASKO, M. J.; ASEMPA, T. E.; NICOLAU, D. P. Pharmacodynamics of Ceftibuten: An Assessment of an Oral Cephalosporin against Enterobacterales in a Neutropenic Murine Thigh Model. **Antibiotics**, v. 10, n. 2, p. 201, 19 fev. 2021

LIAO, H. et al. Combination of fully human antibodies and antibiotics that specifically bind to staphylococcus aureus Hla toxin. , 29 mar. 2024.

LIU, G. et al. Effectiveness and safety of cefotaxime combined with avibactam for treating multidrug-resistant E coli infections: A systematic review and meta-analysis. **Medicine**, v. 103, n. 3, p. e36938, 19 jan. 2024.

MATSUMOTO, Y. Combination Cefixime/Amoxicillin against Penicillin-Resistant Streptococcus pneumoniae Infection. Chemotherapy, v. 44, n. Suppl. 1, p. 6–9, 15 out. 1998.

MORÁN-DÍAZ, J. R. et al. QSAR analysis of five generations of cephalosporins to establish the structural basis of activity against methicillin-resistant and methicillin-sensitive Staphylococcus aureus. Molecular Diversity, 21 set. 2023.

NAGHAVI, M. et al. Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. **The Lancet**, v. 404, n. 10459, p. 1199–1226, 28 set. 2024.

NORDEN, C. Parenteral, intravenous, and oral administration of oxazolidinones for treating diabetic foot infections. , 31 maio 2007.

ORENGA, S. et al. Culture medium for the specific detection of resistant organisms. , 18 jul. 2012.

ORENGA, S.; ROBICHON, D.; ZAMBARDI, G. Reaction Medium for Methicillin-Resistant Staphylococcus Aureus (mrsa) Bacteria. , 7 jul. 2011.

PARIHAR, K.; TELANG, M.; OVHAL, A. A patent review on strategies for biological control of mosquito vector. World Journal of Microbiology and Biotechnology, v. 36, n. 12, p. 187, 24 nov. 2020.

SCHWEIZER, F.; IDOWU, T. Homodimeric tobramycin adjuvant repurposes novobiocin as an effective antibacterial agent against gram-negative bacteria. , 26 nov. 2020. Disponível em:

<https://patents.google.com/patent/WO2020232534A1/en?oq=WO2020232534A1>. Acesso em: 25 abr. 2024

SRINIVAS, J. Formulation Comprising of Ceftazidime, Tazobactam and Linezolid. , 2 ago. 2007a.

SRINIVAS, J. Formulation of Cefpodoxime, Clavulanic Acid and Linezolid. , 2 ago. 2007b.

STEWART, A. G. et al. Oral cephalosporin and β-lactamase inhibitor combinations for ESBL-producing Enterobacteriaceae urinary tract infections. Journal of Antimicrobial Chemotherapy, v. 75, n. 9, p. 2384–2393, 1 set. 2020.

STURARO, M. C. et al. Synergistic antimicrobial combination of third-generation cephalosporins and polymyxin B against carbapenem-polymyxin-resistant Klebsiella pneumoniae: an in vitro and in vivo analysis. Antimicrobial Agents and Chemotherapy, v. 68, n. 10, p. e0093024, 8 out. 2024.

SUN, T.; GAO, T.; JIANG, J. Beta-lactamase inhibitor composition with stable quality as well as application and method of beta-lactamase inhibitor composition. , 30 jul. 2021.

WAGENLEHNER, F. et al. Systematic review on estimated rates of nephrotoxicity and neurotoxicity in patients treated with polymyxins. Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, p. S1198- 743X(20)30764–3, 6 jan. 2021.

WEI, X. et al. Application of dimetridazole in preparation of anti-beta-lactam drug-resistant escherichia coli medicine. , 24 jun. 2022a.

WEI, X. et al. Pharmaceutical composition for resisting drug-resistant klebsiella pneumoniae and application of pharmaceutical composition. , 2 ago. 2022b.

YANKOVA, N. et al. CEPHALOSPORIN ANTIBIOTIC RESISTANCE. Archiv Euromedica, v. 13, n. 1, 27 fev. 2023.

YI, Z.; PEI, Z.; XIAOYAN, M. Evaluation of Amlodipine Inhibition and Antimicrobial Effects. International Journal of Pharmacy and Chemistry, v. 5, n. 1, p. 12–14, abr. 2019.

YOKOTA, Y. Medicaments concomitants pour la prevention ou le traitement de l'infection par des bacteries gram positives. , 5 out. 2000. Disponível em: <https://patents.google.com/patent/WO2000057882A1/en?oq=WO0057882A1>. Acesso em: 25 abr. 2024

YOSHIMI, M.; SHUICHI, T.; HITOSHI, N. Antibacterial agents. , 25 jul. 2001.

ZENG, H. et al. Composition for resisting multi-drug-resistant acinetobacter baumannii and application thereof. , 26 jan. 2024.

ZHILIANG, S et al. Snake venom polypeptide and application thereof. , 5 jan. 2024. Disponível em: <https://patents.google.com/patent/CN117343131A/en?oq=CN117343131A>. Acesso em: 10 jun. 2024

ZIYUE, Y. Application of amlodipine and cephalosporin medicines to preparation of medicine-resistant combined antibiotics. , 8 jun. 2018. Disponível em: <https://patents.google.com/patent/CN108125954A/en?oq=CN108125954A>. Acesso em: 10 jun. 2024

5. ATTACHMENTS

Optimizing Ceftibuten/Polymyxin B combination regimens for carbapenem-polymyxin-resistant *Klebsiella pneumoniae*: insights from an *in vitro* PK/PD model

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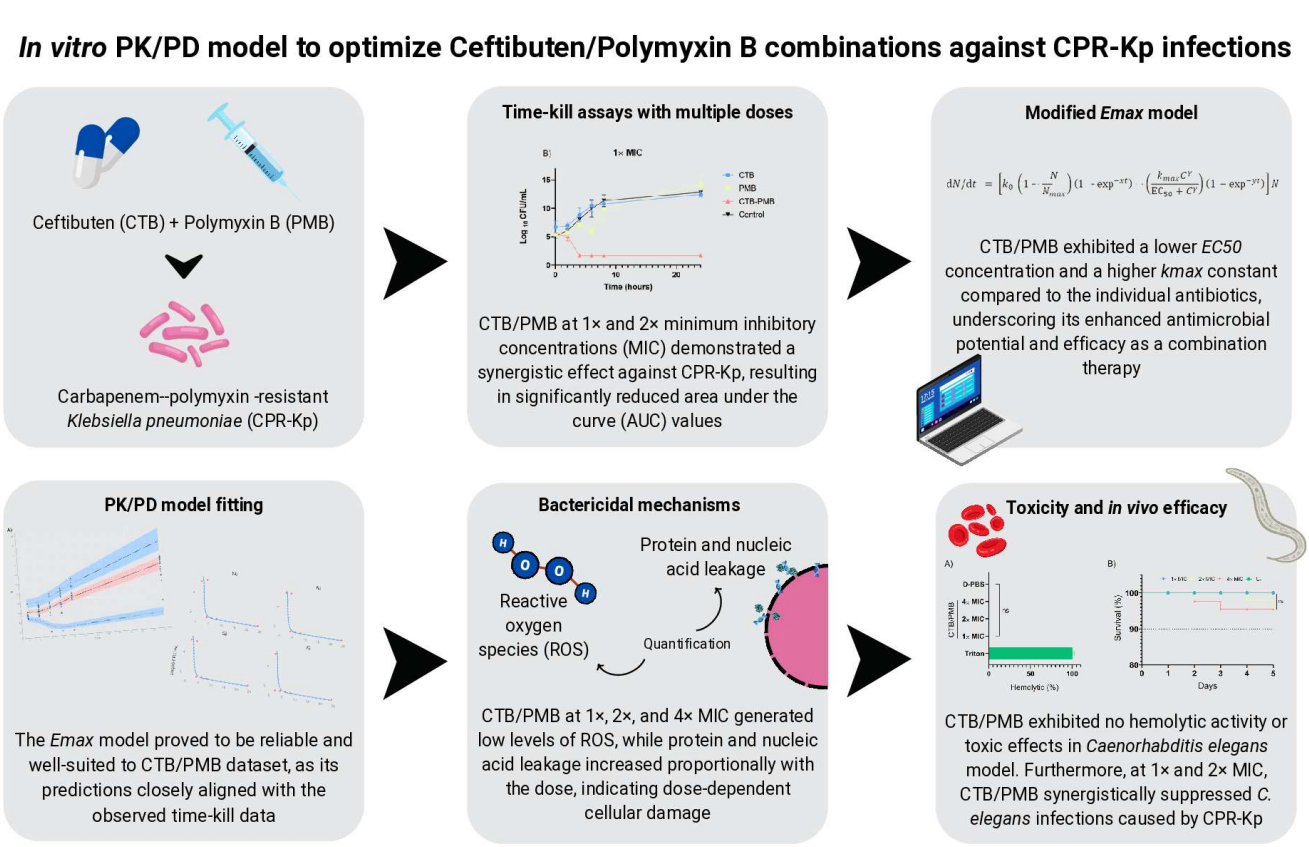
GRAPHICAL ABSTRACT



ABSTRACT

This study aimed to evaluate the synergistic effects of ceftibuten (CTB) combined with polymyxin B (PMB) and to optimize dosing using a modified E_{max} *in vitro* PK/PD model, offering a potential new treatment approach for Carbapenem-polymyxin-resistant *Klebsiella pneumoniae* (CPR-Kp).

Time-kill experiments were conducted with seven doses of CTB/PMB, the resulting data was fitted to a modified E_{max} model. Area under curve (AUC) values were calculated. Reactive oxygen species (ROS) generated by CTB/PMB upon exposure to CPR-Kp were evaluated, and the CPR-Kp intracellular components were quantified to assess the effect of the treatment on bacterial cells. Treatments toxicity was accessed through hemolysis assay and by determining the influence on the lifespan of *Caenorhabditis elegans*. An *in vivo C. elegans* model was used to examine the ability of CTB/PMB to control CPR-Kp infection.

The results revealed a synergistic effect for CTB/PMB at 1× and 2× minimum inhibitory concentration (MIC), with reduced AUC values compared to those of the antibiotics used individually. The combination also resulted in a lower EC_{50} concentration and a higher k_{max} constant than the individual antibiotics. CTB/PMB produced low levels of ROS, which probably enhanced its antibacterial properties. Protein and nucleic acid externalization was minimal at lower doses but increased at 4× MIC, indicating a dose-dependent damaging effect. CTB/PMB showed no hemolytic effect, no toxic effects on the *C. elegans* model, and effectively controlled infection at the 1× and 2× MIC doses.

To summarize, CTB/PMB at 1× and 2× MIC represent a promising alternative for treating CPR-Kp infections.

KEYWORDS: *K. pneumoniae*; Ceftibuten; Polymyxin B; Combination Therapy; PK/PD; *C. elegans*.

INTRODUCTION

The carbapenem-polymyxin resistance in bacteria, such as *Klebsiella pneumoniae*, poses a significant public health threat, as it severely limits infection's treatment options, leading to high mortality and morbidity rates for patients[1]. Infections caused by carbapenem-polymyxin-resistant *Klebsiella pneumoniae* (CPR-Kp) are frequently associated with higher treatment failure occurrence and longer hospital stays[2]. The economic burden of managing these infections is also significantly higher owing to the need for more intensive and prolonged therapies, as well as the potential risk for clinical complications[3].

To address antimicrobial resistance, the ongoing development of alternative therapies and optimized antibiotic regimens are essential. In this context, antibiotic combinations offer a promising solution, as it can be evaluated more quickly and with less investment than new antibiotics[4]. These combinations can broaden the antibacterial spectrum and lower required dosages, consequently reducing associated toxicity[5]. Polymyxin B (PMB), a last-resort antibiotic used in Brazil[6], is an excellent adjuvant for combined treatments, given its potential to sensitize the bacteria by damaging its cell membrane, amplifying the action of the other drug[7]. Further, cephalosporins, such as Ceftibuten (CTB), exhibit potent antibacterial activity against multiple pathogens and relatively low toxicity, which highlights their potential in combination therapies[8,9].

Given the advantages of antibiotic combinations, their use has surged over the past few decades[10], however it is extremely important to explore and validate suitable dosing regimens considering the antibiotics interaction on the time-course of bacterial

growth and killing to optimize the proposed treatment[11]. In this scenario, semi-mechanistic pharmacokinetic/pharmacodynamic (PK/PD) methods are valuable tools considering that they can be performed *in vitro* and in a short period, providing deeper insights[12]. Therefore, the aim of this study was to assess the potential synergy between the Ceftibuten/Polymyxin B (CTB/PMB) combination against CPR-Kp using time-kill analysis, followed by an evaluation through an *in vitro* PK/PD modified E_{max} based model with multiple drugs concentrations to determine the optimal one.

RESULTS

Bacterial strain

According to a previous study, the CPR-Kp strain used within the experiments was identified as having the sequence type 11, belonging to clonal complex 258, a major lineage associated with KPC production. The strain carried the *bla*$_{KPC-2}$ gene, which encodes the KPC-2 enzyme responsible for carbapenem resistance, as well as several extended-spectrum beta-lactamase (ESBL) genes, including *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, and *bla*$_{SHV-11}$, which impart resistance to β-lactam antibiotics such as cephalosporins. Whole-genome sequencing revealed an alteration in the *mgrB* gene (a frameshift mutation at nucleotide 89 +2), which is linked to chromosomal resistance to polymyxins. The strain remained susceptible only to tigecycline and amikacin, as determined by the minimum inhibitory concentration (MIC)[13].

Additionally, a previous study, performed by our research group, reported that the MIC for the CPR-Kp strain was 64 mg/L for PMB and 32 mg/L for CTB. However, when used in combination, the MICs of both antibiotics significantly decreased, with PMB decreasing to 2 mg/L and CTB decreasing to 4 mg/L, indicating a synergistic effect

between the drugs, confirmed by a low fractional inhibitory concentration index (FICI) of 0.15[14].

Time-kill

The time-kill (TK) experiments revealed no interaction of CTB/PMB at lower concentrations (0.125, 0.25 and 0.5× MIC) (Figure S1A-B, 1A), exhibiting no influence under the bacterial survival. The combination had synergistic activity at 1× MIC (Figure 1B), decreasing > $10\log_{10}$ CFU/mL compared to the activity of the antibiotics alone. Moreover, the time to kill of the combination was 4 h.

The CTB/PMB combination maintain its killing activity and synergistic profile at 2× MIC (Figure 1C). Meanwhile, at 4× MIC concentration, CTB begun to demonstrate killing potential when used in isolation, with a time to kill of 6h, mitigating the synergistic potential of CTB/PMB (Figure 1D). Further, the same occurs with 8× MIC concentration, CTB and PMB presented relatively high doses capable of killing in isolation (Figure S1C).

The area under curve (AUC) results (Figure 1D) were consistent with the TK findings. At lower concentrations (0.125, 0.25, and 0.25× MIC), no killing activity was detected, resulting in higher AUC values (> 200 CFU/mL.h) for both antibiotics, whether in combination or in isolation. At 1× and 2× MIC, the AUC values for CTB/PMB were significantly lower (51.25 and 49.43 CFU/mL.h, respectively) than those for the isolated antibiotics ($p < 0.001$), indicating a synergistic effect. At higher concentrations (4 and 8× MIC), CTB/PMB had lower AUC values than the antibiotics alone ($p < 0.01$), but the difference was less pronounced than the results observed at 1 and 2× MIC.

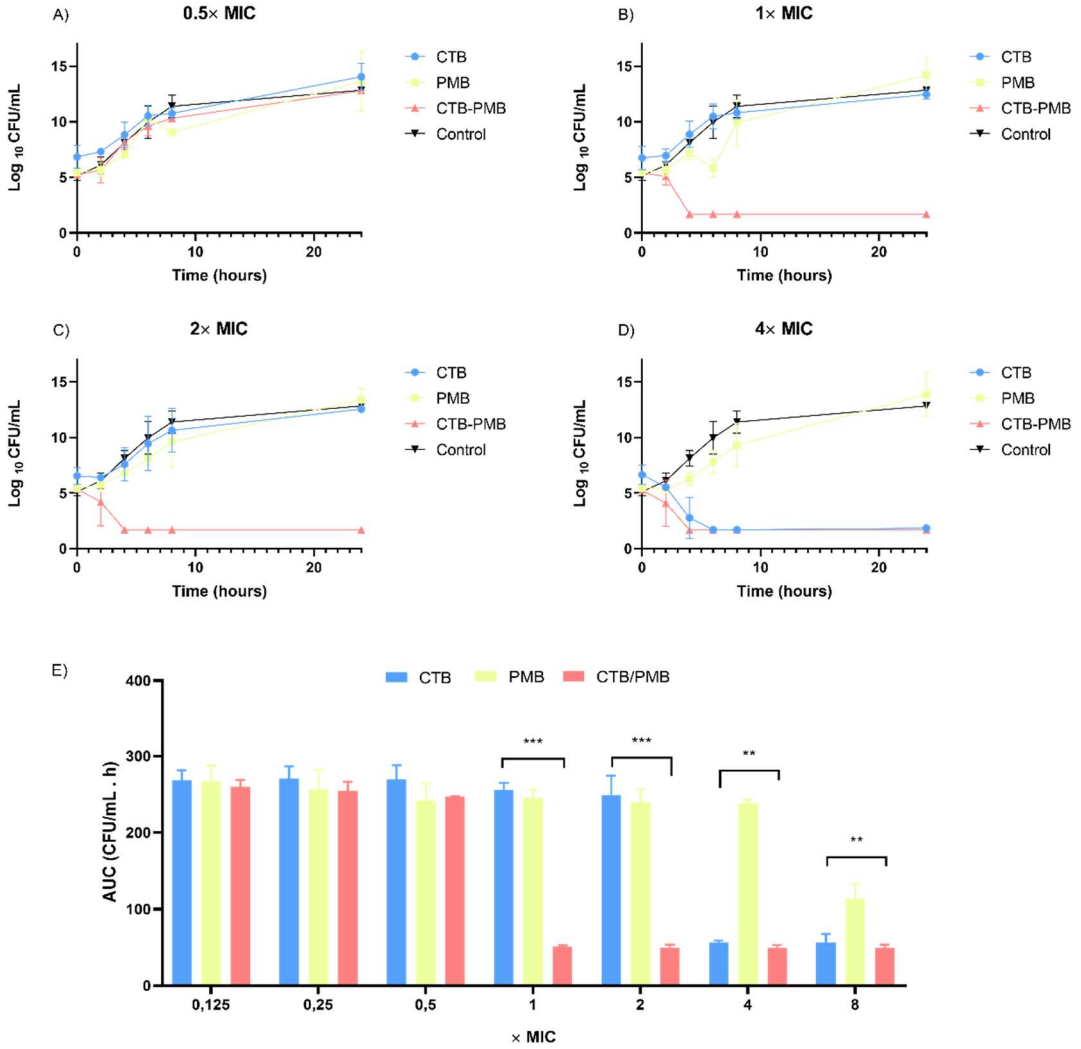


Figure 1. Time-kill curves for CTB/PMB treatment, and individual antibiotics, against CPR-Kp, at the doses of A) 0.5× MIC; B) 1× MIC; C) 2× MIC and D) 4× MIC. A bacterial control was used to attest CPR-Kp viability. E) Area under the curve (AUC) values for CTB/PMB, and individual antibiotics, at multiple doses, calculated from the TK curves for CPR-Kp. AUC is directly proportional to bacterial survival. One-way ANOVA was performed, and differences were considered statistically significant for $p<0.01$ (**) and $p<0.001$ (***).

Pharmacodynamics parameters

Table 1. Pharmacodynamic parameters obtained using an E_{max} model fit for CTB and PMB, both in combination and individually, against CPR-Kp.

Pharmacodynamics Parameters	PMB			CTB			CTB/PMB		
	Value	S.E.	R.S.E.	Value	S.E.	R.S.E.	Value	S.E.	R.S.E.
k_0 (h^{-1})	2			1.45			2		
k_{max} (h^{-1})	0.64	5.48	8.54	0.8	0.05	6.25	1.08	0.052	4.76
EC_{50} (× MIC)	7.45	5.11	28.5	3.04	0.17	5.45	0.76	0.074	9.73
γ	6.52	5.46	33.7	7.79	1.03	13.2	11.21	3.42	30.5

The determined PD parameters of CTB and PMB, both in combination and individually, against CPR-Kp are presented in Table 1. The results indicate that the selected model effectively fits the data, with all parameters estimated accurately, showing low standard errors (SE) and relative standard errors (RSE), which reflects the reproducibility achieved across triplicates. A more precise fit was obtained by incorporating an additional Hill (γ) factor and fixing the k_0 value for each group.

The k_{max} represents the bacterial killing rate, and notably, CTB/PMB exhibited a higher k_{max}, of 1.08 h^{-1}, compared to the antibiotics used in isolation (PMB 0.64 and CTB 0.8 h^{-1}), indicating an enhanced bactericidal potential. Moreover, according to the model, CTB/PMB demonstrated greater efficacy against the CPR-KP strain, as the EC_{50} dose was significantly lower (0.76× MIC) than the EC_{50} values for the antibiotics used individually (PMB 7.45 and CTB 3.04× MIC).

TK curve fitting

The observed data, along with the predicted fitted data based on a modified E_{max} modeling approach, are shown in Figure 2. The predicted values closely aligned with the observed data from TK experiments, in each triplicate, for CTB/PMB against CPR-Kp. This alignment was also observed for the antibiotics in the isolated treatment groups, where the predicted data matched the observed values (Figures S2, S3).

The model indicates enhanced antibacterial activity for CTB/PMB, with concentrations 1–8× MIC (Figure 2J–U) demonstrating significant killing activity. In contrast, PMB alone only exhibits predictive bacterial killing at the sigmoidal curve of the dose 8× MIC (Figure S2S). CTB alone presents a moderate antibacterial effect, with killing activity observed within 4× MIC (Figure S3P), which was consistent with the results obtained from TK and AUC data.

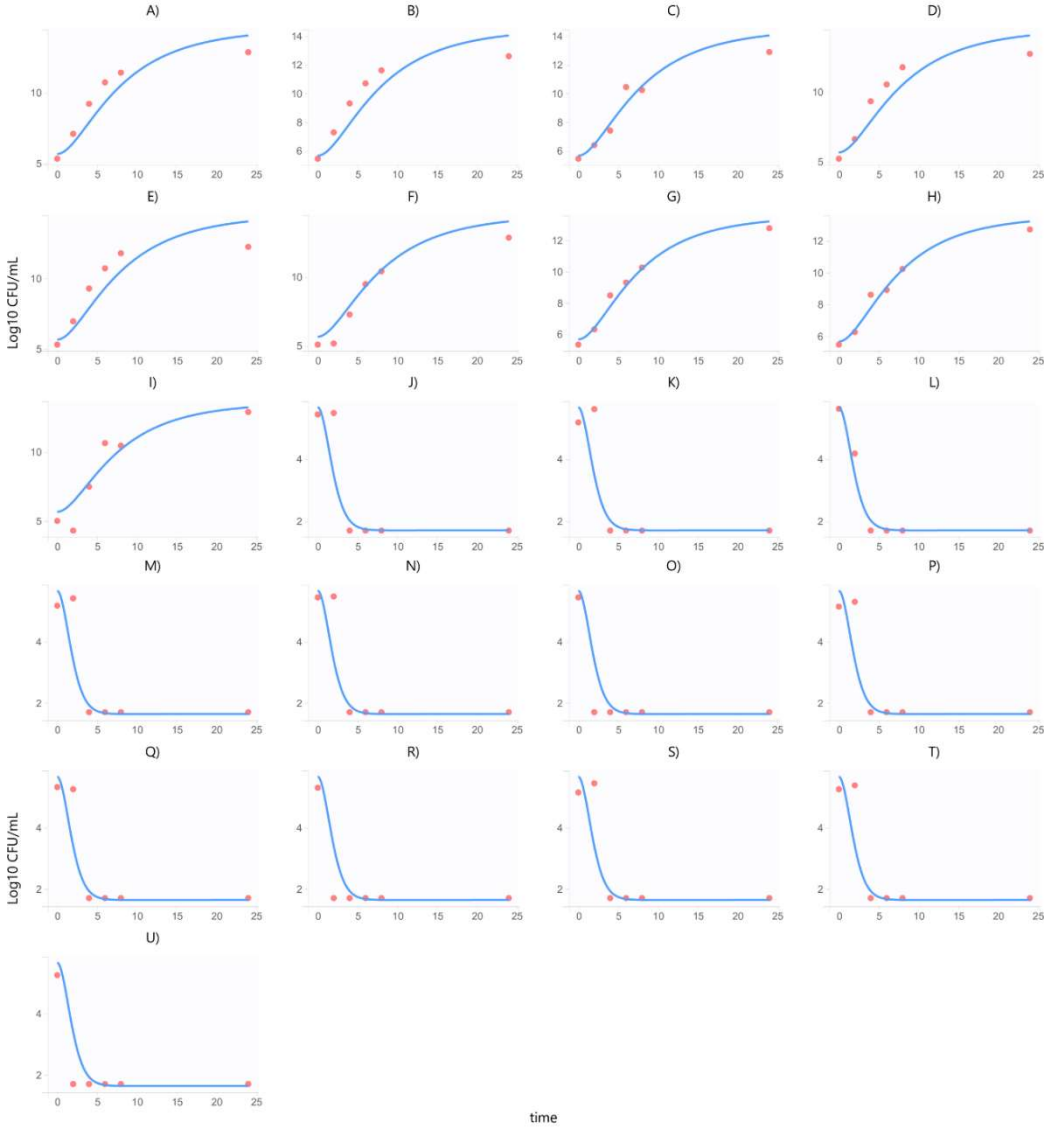


Figure 2. Predicted and observed CTB/PMB TK fitted data profiles using a modified E-max modelling approach, in triplicates, for each concentration. CTB/PMB at 0.125× MIC (A – C), at 0.25× MIC (D – F), at 0.5× MIC (G – I), at 1× MIC (J – L), at 2× MIC (M –

O), at 4× MIC (P – R), at 8× MIC (S – U). Red dots: observations; solid line: individual model predictions. The y-axis represents the bacterial load in $\log_{10}$ CFU/mL, while the x-axis shows the time duration of treatment exposure (hours) in CPR-Kp.

Residual error plots (Figure 3A–C) displayed a symmetric distribution centered around zero, indicating minimal bias in the model's predictions across the CTB and PMB datasets, in combination or individually. The lack of systematic trends in the residuals further supports the model's accuracy, as no significant over- or under-prediction (> or < 4) patterns were evident.

Additionally, the observed versus predicted data plot (Figure 3D–E) demonstrated a strong correlation between the actual and predicted values, reinforcing that the predictive model reliably mirrors the observed outcomes. Together, these findings suggested that the robustly performed model can be considered a good fit for predicting the target variable within the data range used.

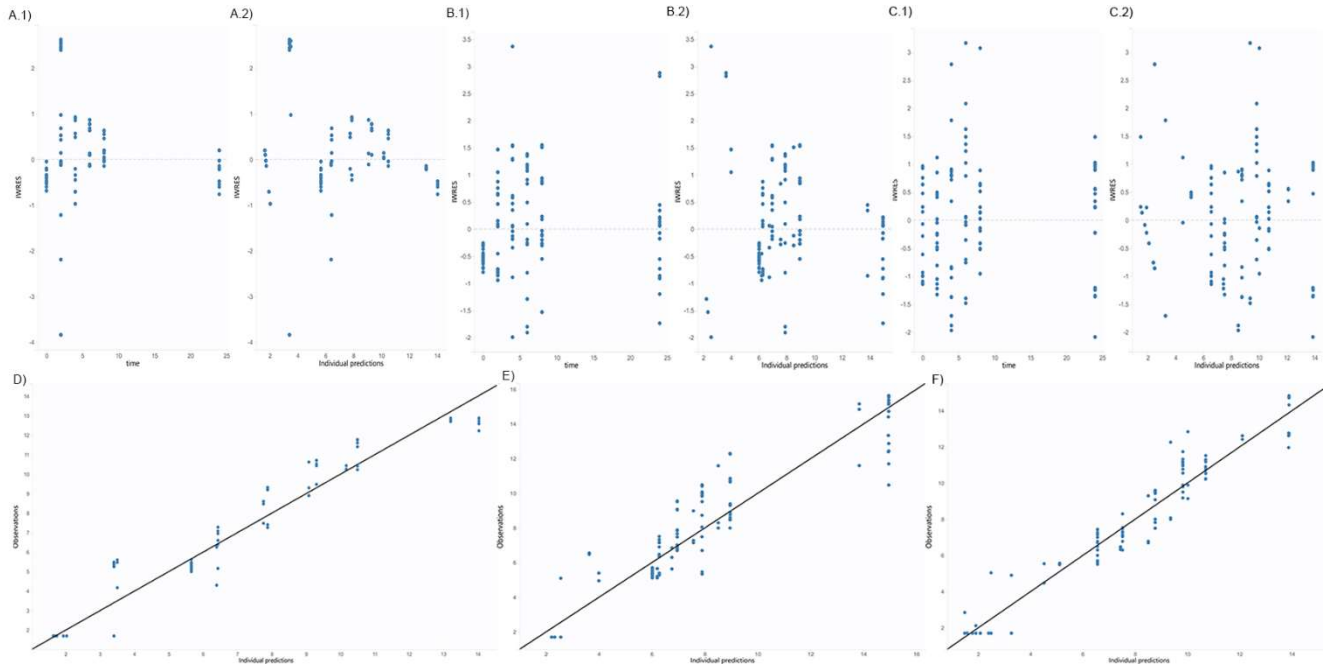


Figure 3. Goodness-of-fit plots for the modified E_{max} model, applied to TK data, in the residual plots. The line is the Locally Estimated Scatterplot Smoothing (LOESS) smooth fit representing the trend in the data for A1-2) CTB/PMB, B1-2) PMB and C1-2) CTB.

The observations vs. individual model predictions plot for D) CTB/PMB, E) PMB and F) CTB.

Finally, the visual predictive check (VPC) plot (Figure 4) for the modified E_{max} model suggests that it effectively predicts CPR-Kp bacterial killing over time with CTB and PMB treatments, irrespective of whether used individually or in combination. The close alignment between observed data and prediction intervals demonstrates that the chosen model captures both the central tendency and variability of the data accurately, implying that the approach is reliable for forecasting bacterial killing dynamics under similar conditions, offering valuable insights into CTB/PMB treatment efficacy over time.

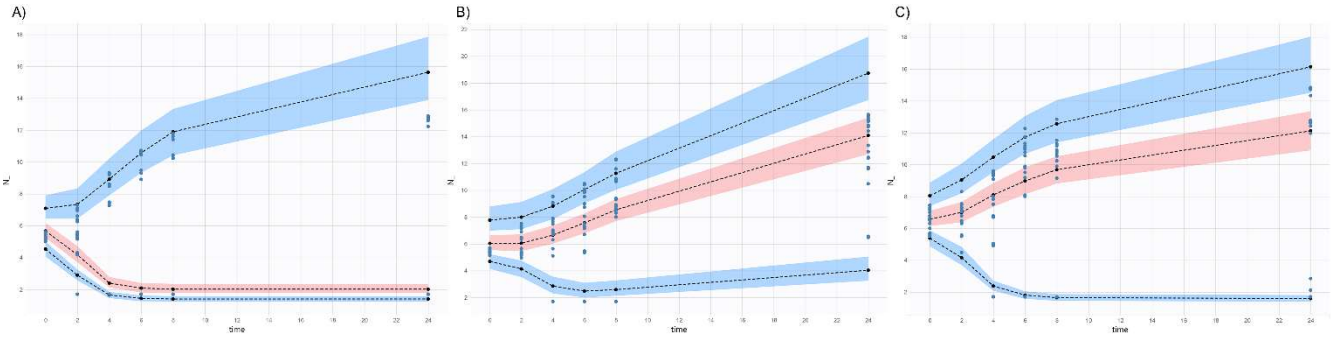


Figure 4. Visual predictive check for A) CTB/PMB, B) PMB and C) CTB. Blue dots represent the observed bacterial growth (log_{10} CFU/mL) over the time course, and solid lines represent the 10th, 50th, and 90th percentiles of simulated data. Red and blue shaded bands represent the 95% confidence intervals for the corresponding model predicted percentiles.

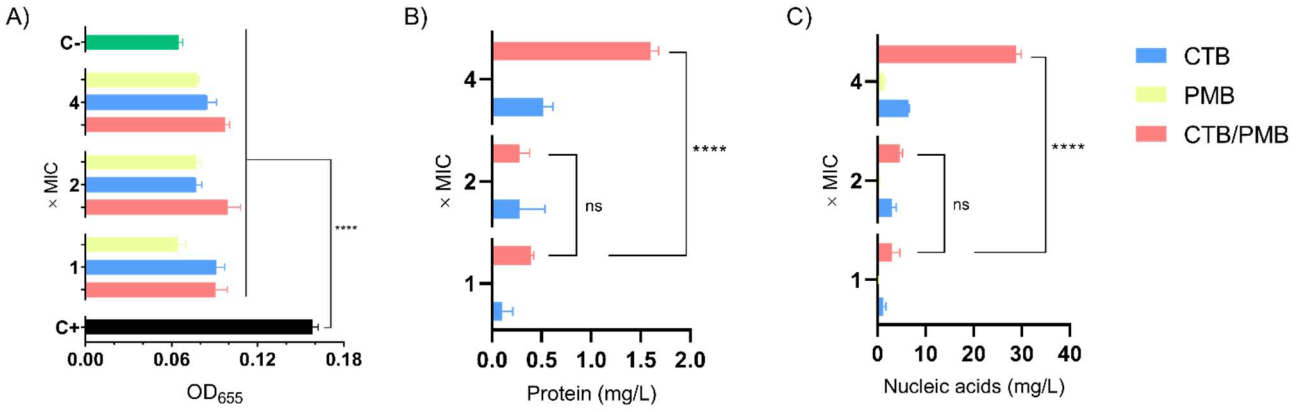
Reactive oxygen species (ROS) quantification

Reactive oxygen species (ROS) generation was quantified to evaluate the oxidative potential of the CTB/PMB treatment regimens (Figure 5A). Although CTB/PMB at 1, 2, and 4× MIC resulted in significant differences compared to the positive

control (Triton), a slight increase in ROS generation was observed within the combination groups. This outcome indicates that the treatment might induce minimal oxidative stress in CPR-Kp, corroborating to its death. Furthermore, no significant differences were observed between the tested doses.

Cell membrane permeability

To evaluate whether the combination treatment strongly influenced the integrity of the bacterial cell membrane, experiments were performed involving protein and nucleic acid quantification. After 4 h of treatment exposure (combination time for bacterial killing defined on TK experiment), at the lower doses of 1 and 2× MIC, no considerable leakage was observed. However, at 4× MIC concentration, CTB/PMB caused higher damaging on the CPR-Kp cell membrane, making it possible to detect both



protein and nucleic acids externalization via the applied methodologies (Figure 5B-C).

Figure 5. Results of antibacterial mechanisms assays. A) ROS quantification for CTB/PMB, or individual antibiotics, at 1, 2 and 4× MIC. Untreated CPR-Kp cells and Triton 0.1% were used as negative (C-) and positive (C+) controls, respectively. B) Intracellular protein leakage for CTB/PMB, or individual antibiotics, at 1, 2 and 4× MIC; C) Nucleic acids leakage for CTB/PMB, or individual antibiotics, at 1, 2 and 4× MIC.

One-way ANOVA was performed, and differences were considered statistically significant for $p<0.0001$ (****).

Toxicity evaluation

The hemolytic profile of the combination was also evaluated (Figure 6A). Although the safety profiles of CTB and PMB are established, their toxicity remains undefined when used in combination. CTB/PMB exhibited no hemolytic activity, even at higher concentrations (4× MIC); the hemolytic activity was not significantly different from that recorded in the negative control, D-PBS.

According to the *Caenorhabditis elegans* toxicity evaluation model, CTB/PMB had no significant effect on nematode survival (Figure 6B). At 1×, 2×, and 4× MIC, survival rates were comparable to those of the control group (nematodes exposed only to M9 buffer). The survival percentile decreased slightly for the higher doses of 2× and 4× MIC (95% survival) compared to the lower doses of 1× MIC and the control (100% survival), but this difference was not significant.

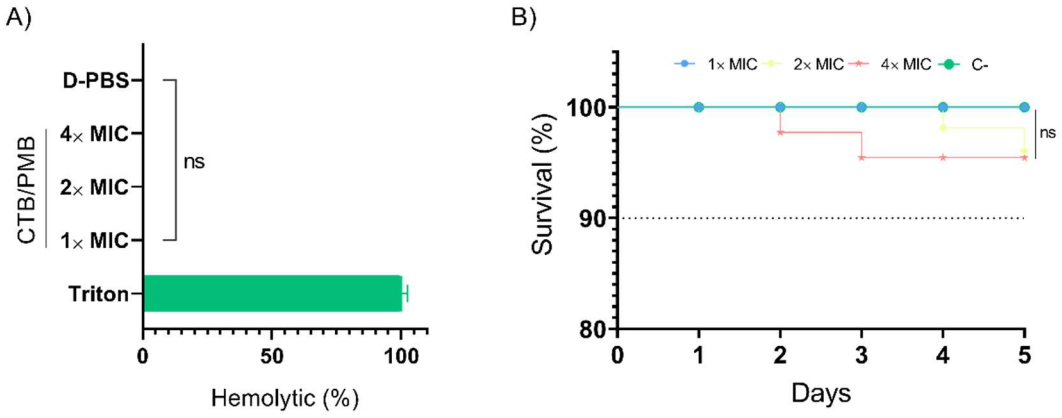


Figure 6. Toxicity evaluations results. A) Hemolytic rate (%) of CTB/PMB at 1, 2 and 4× MIC concentrations. Triton 0.1% was used as positive control and D-PBS as negative control. One-way ANOVA was performed, and differences were considered statistically

significant when $p<0.05$. B) *C. elegans* toxicity assay results represented as a Kaplan–Meier survival curve for CTB/PMB at 1, 2 and 4× MIC. Nematodes exposed only to M9 buffer were used for viability control (C-). Log-rank test was performed for statistical analyses and difference was considered significant when $p<0.05$.

C. elegans infection model

The *C. elegans* model was also employed to investigate the efficacy of CTB/PMB in controlling CPR-Kp infections (Figure 7A-C). Remarkably, at doses of 1×, 2×, and 4× MIC, the combination treatment significantly improved the survival of infected *C. elegans*, yielding results comparable to both the negative control (uninfected nematodes) and the standard treatment with tigecycline.

When the antibiotics were individually examined, neither CTB nor PMB showed antibacterial activity at the 1× MIC dose, resulting in a significantly lower survival rate than that of the negative control. At 2× MIC, CTB alone extended the lifespan of infected *C. elegans*, although not to the same degree as the combination treatment. However, at 4× MIC, each antibiotic alone exhibited strong antibacterial effects, with survival rates closely matching those recorded in the negative and tigecycline-treated controls.

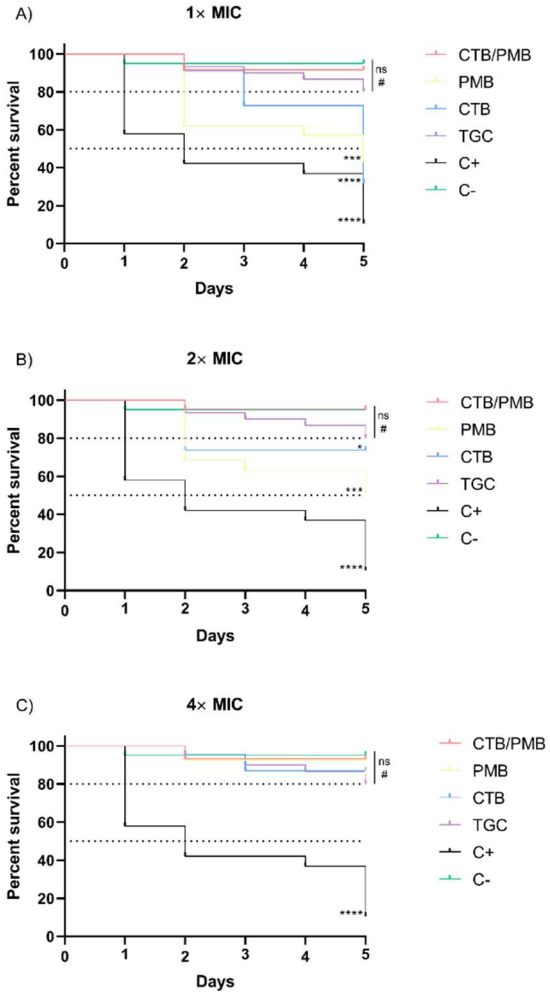


Figure 7. *C. elegans* Kaplan–Meier survival curve for CTB/PMB, or individual antibiotics, against CPR-Kp infection at the doses of A) 1× MIC; B) 2× MIC; and C) 4× MIC. Tigecycline (TGC) 16mg/L was used as a standard treatment for comparison. Uninfected nematodes were considered the negative control (C-) and infected but untreated nematodes were considered the positive control (C+). Log-rank test was performed for statistical analyses and difference was considered significant when $p<0.05$ (*), $p<0.001$ (***) and $p<0.0001$ (****).

DISCUSSION

The ongoing development of alternative treatment options for *K. pneumoniae* infection control has become critically important due to its facility in acquiring

antimicrobial resistance mechanism and its strong impact on global health[15]. Thus, older antimicrobial agents, such as polymyxins and fosfomycin, continue to serve as alternative treatment options for infections caused by resistant-bacteria[16]. PMB, despite its associated toxicity and rising resistance levels, remains a viable option for treating *K. pneumoniae* infections in combination therapies[7], largely due to its dose reduction and widespread availability in Brazilian hospitals[17].

While the MIC value is widely used to assess the antibacterial potential of new compounds, it may lack comprehensiveness as a standalone approach[18]. The present study employed a more advanced method, based on TK data and PK/PD analyses of CTB/PMB at multiple doses, as no previous studies have, to our knowledge, investigated this approach. Unlike the MIC methodology, kill curve analysis provides a deeper understanding of PD effects through two key parameters, k_{max}, and EC_{50}, derived from an E_{max} model, rather than relying solely on a single threshold value. This allows for the identification of optimal dosing regimens[19,20].

Initially, CTB/PMB interaction was investigated, and it was notice that it demonstrated enhancing antibacterial therapeutic activity at 1× and 2× MIC doses against CPR-Kp on the TK curves, achieving a reduction of more than 2 $\log_{10}$ CFU compared to the activity of each antibiotic in isolation, which characterizes the combination as synergic[21]. These results were consistent with our previous findings[14]. Additionally, AUC values supported these synergistic evidences[22], as CTB/PMB significantly decreased the AUC at 1× and 2× MIC doses compared to the antibiotics in isolation. Higher concentrations also demonstrated bactericidal activity; however, they did not exhibit a synergistic effect, as each antibiotic alone also showed potential to combat CPR-Kp.

Applying the modified E_{max} model to the TK data was essential for confirming the synergistic effects of CTB/PMB against CPR-Kp, as indicated by the PD parameters established in this study. The combination decreased the EC_{50} dose for the antibiotics, underscoring the enhanced potency of the treatment compared to each antibiotic used in isolation. Additionally, CTB/PMB exhibited a higher k_{max} constant, demonstrating greater bactericidal efficacy. Similar findings were observed with the combination of colistin and fusidic acid against *Acinetobacter baumannii*, where colistin's EC_{50} decreased by 83% and k_{max} increased by 58% when paired with fusidic acid[23]. These findings underscore the superior effectiveness of the combination therapy.

The selected E_{max} model accurately captured the trend of the CTB/PMB TK data, as evidenced by the predicted TK graphs, goodness-of-fit plots, and VPC analyses. The observed data showed minimal variation compared to the predicted data, underscoring its consistency[24]. Interestingly, isolated PMB at the higher dose demonstrated initial antibacterial activity, followed by bacterial regrowth, as predicted by the model. This pattern has also been observed for PMB against *A. baumannii*[25], suggesting that such regrowth may be characteristic of polymyxin-resistant bacteria. Consequently, these results imply that the model is a reliable tool for predicting bacterial killing dynamics when applying CTB/PMB treatments in similar scenarios, providing valuable insights into their efficacy over time.

Interestingly, the performed experiments suggest that ROS generation may contribute to the antibacterial potential of CTB/PMB. While only minimal levels of ROS were detected, their presence can induce oxidative stress, leading to damage of critical bacterial components such as nucleic acids, lipids, and proteins. This oxidative damage can result in irreversible cellular dysfunction and ultimately bacterial death[26]. Plus, protein and nucleic acid leakage quantification was conducted to assess whether the

CTB/PMB affected CPR-Kp cellular membrane integrity. Although the combination demonstrated bactericidal activity, doses at 1× and 2× MIC did not release sufficient protein/nucleic acids to be detected by our methods. This suggests that, at lower doses, the combination may not cause full cell rupture but only minor cellular damage[14]. However, the 4× MIC CTB/PMB dose significantly increased the externalization of cellular components, indicating a dose-dependent damaging effect.

Third-generation cephalosporins, such as CTB, act by inhibiting microbial cell wall synthesis, binding to penicillin-binding proteins, thereby disrupting cell division[27]. PMB may support this process by increasing bacterial cell wall rigidity and penetrating the cell membrane to interfere directly with cell division machinery[28]. While the treatment influences cellular division, it may not lead to extensive externalization of cellular components. Still, further experimentation might be performed to consolidate this hypothesis.

The CTB/PMB did not induce hemolysis, suggesting a favorable safety profile. Also, it showed no significant toxicity in the alternative *C. elegans* model at any tested dose, indicating the absence of lethal effects[29]. While *C. elegans* is a valuable and promising *in vivo* model[30], additional studies are necessary to confirm these findings in mammalian models and clinical settings. Moreover, CTB/PMB effectively controlled CPR-Kp infections in the nematodes at 1×, 2×, and 4× MIC doses, significantly extending the lifespan of *C. elegans* in a manner comparable to the standard antibiotic treatment, tigecycline. However, at the 4× MIC dose, each antibiotic alone also demonstrated antibacterial activity, suggesting that the combination's lower doses may be more promising for an effective regimen. These findings hold the potential for advancing new therapeutic approaches against CPR-Kp.

In conclusion, our study provided promising insights into the synergistic effects and enhanced activity of CTB/PMB against CPR-Kp, according to the applied modified E_{max} model for static TK experiments data, offering valuable guidance for optimizing dosing regimens based on treatment exposure time. Still, further *in vivo* studies are necessary to deepen our understanding of the antibiotics' combination *in vivo* interaction, half-life, bioavailability, and organ-specific effects. Information on these aspects can help advance our findings toward clinical assessment, making them more applicable in real-world settings.

MATERIAL AND METHODS

Antibiotics

CTB hydrate (Lot 0000124698), and PMB solution (Lot BCCG2613) were purchased from Sigma (St. Louis, USA) and prepared according to the manufacturer's instructions.

Bacterial strain

The CPR-Kp strain used in the experiments was isolated from a blood sample of a patient admitted to a tertiary hospital in Dourados, Mato Grosso do Sul, Midwest, Brazil, in a previous study[13]. Specie identification was performed using the automated Vitek2 system (bioMerieux, Hazelwood, MO), followed by validation of the identified species through matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF) using a Microflex LT spectrometer (Bruker Daltonics, Massachusetts, USA). The MIC of the strain was determined through serial dilution across multiple antibiotics to evaluate its resistance profile[13]. The MIC for CTB/PMB

was established using a checkerboard assay, following the FICI calculation performed in another study[14].

Time-kill

TK assays were conducted for the CTB/PMB, as previously described[31], at varying doses of 0.125, 0.25, 0.5, 1, 2, 4, and 8 × MIC **(Table 2)** to encompass a wide range of possible dosages and identify the synergistic optimal ones. Briefly, a CPR-Kp solution was prepared based on the 0.5 McFarland's scale and then diluted to achieve a final concentration of 1.5×10^6 CFU/mL. The CPR-Kp inoculum was then added to the antibiotics, either individually or in combination, at different concentrations. At 0, 2, 4, 6, 8, and 24 h after inoculation, 10 µL aliquots were collected, serially diluted in BHI broth, and plated on BHI agar. The plates were incubated at 37 °C for 24 hours, after which the colonies were counted. A positive control without antibiotics was used to assess bacterial growth. The lower detection limit was set at 1.69 $\log_{10}$ CFU/mL. Synergy was defined as a reduction in bacterial growth of ≥ 2 $\log_{10}$ CFU/mL at 24 h when using the combination therapy, compared to the effect of the individual antibiotics[32]. The treatments area under the curve (AUC) were determined through a trapezoidal rule as previously described[11].

Table 2. Multiple doses of CTB/PMB, in combination or individually, used in the experiments.

	Combination/Individually	
× MIC	**Ceftibuten (mg/L)**	**Polymyxin B (mg/L)**
0.125	0.5	0.25
0.25	1	0.5
0.5	2	1
1	4	2
2	8	4

4	16	8
8	32	16

PK/PD modeling

The TK curves analysis and their corresponding mathematical modeling were performed within the Monolix Software 2024R1 (LIXOFT, Paris, France). The PD data were fitted into the following modified E_{max} model.

$$\mathrm{d}N/\mathrm{d}t = \left[k_0 \left(1 - \frac{N}{N_{max}}\right)(1 - \exp^{-xt}) - \left(\frac{k_{max}C^{\gamma}}{\mathrm{EC}_{50} + C^{\gamma}}\right)(1 - \exp^{-yt})\right]N$$

Here, d*N*/d*t* represents the change in bacterial count over time, k_0 (h^{-1}) represents the bacterial growth rate constant in the absence of antibiotics (growth control), k_{max} (h^{-1}) represents the maximum killing rate constant, EC_{50} (× MIC) represents the antibiotic concentration required to achieve 50% of the maximum effect, *C* (× MIC) represents the antibiotic concentration at any given time (t), *N* (CFU/mL) represents the number of viable bacteria and γ represents the Hill coefficient for determining the curve's steepness[33]. Considering the static *in vitro* system's limitations, including nutrient availability and space constraints, growth saturation was addressed in the model by N_{max}, which represents the maximum bacterial count. As CPR-Kp had not reached the logarithmic growth phase at time zero, exponential correction factors were incorporated for delayed growth ($1 - \exp^{-xt}$) and delayed killing ($1 - \exp^{-yt}$)[18].

To assess the proper fit of the TK data to the selected E_{max} model, the precision of parameter estimates and goodness-of-fit plots were evaluated. Model stability was further confirmed through a visual predictive check (VPC) based on 1,000 simulated scenarios[34].

ROS quantification

To assess the oxidative potential of CTB/PMB in CPR-Kp cells, ROS were quantified using a Nitro Blue Tetrazolium (NBT) assay, as previously described[35]. Briefly, 100 mL bacterial culture was exposed to 500 μL of CTB/PMB, or individual antibiotics at 1, 2 or 4 × MIC, and incubated at 37 °C for 6 h. Next, bacterial pellets were obtained by centrifuging at 10000×*g* for 10 min at 4 °C and resuspended in 2% NBT solution. After 1h, the mixture was centrifuged at 8000×*g* for 2 min. The generated pellet was then washed twice, once with PBS and once with methanol, and then treated with 2 M KOH for cell membrane disruption. A 50% DMSO solution was added, and the mixture was incubated at room temperature for 10 min to dissolve the formazan crystals. The sample was centrifuged at 8000×*g* for 2 min, and 100 μL of the resulting supernatant was transferred to a 96-well plate. Absorbance was recorded at 655 nm using the iMark™ Microplate Absorbance Reader (Bio-Rad, São Paulo, Brazil). Untreated bacterial cultures and Triton 0.1% served as the negative and positive controls, respectively.

Cell membrane permeability

Bacterial cell membrane damage was evaluated by measuring the release of intracellular proteins. The CTB/PMB at concentrations of 1, 2, and 4 × MIC, along with the individual antibiotics, were added to a 96-well microplate, followed by the introduction of the CPR-Kp strain at 1.5×10^8 CFU/mL. A control, with only bacteria and no antibiotic, was used. The microplate was incubated at 37 °C for 4 h. After incubation, each well was centrifuged at 2500 rpm for 5 min at 4 °C. The resulting supernatant was then analyzed for cytoplasmic protein release using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, MA, USA), and optical density (OD) was measured at 595 nm with the iMark™ Microplate Absorbance Reader (Bio-Rad, São Paulo, Brazil)[36].

Nucleic acids leakage

Modulation of cell membrane permeability was also assessed by monitoring the release of nucleic acids. The CTB/PMB, at concentrations of 1, 2, and 4 × MIC, as well as the individual antibiotics, were added to a 96-well microplate, followed by the addition of the CPR-Kp strain at 1.5×10^8 CFU/mL. A setup containing only bacteria without antibiotics was included as a control. Distilled water was used as a negative control. The microplate was incubated at 37 °C for 4 h, after which each well was centrifuged at 6000 rpm for 10 min at 4 °C. The resulting supernatant was collected, and its OD was measured at 260 nm using a Nanodrop spectrophotometer (Bio Drop, Cambridge, England)[37].

Hemolysis evaluation

Hemolysis assays were conducted to assess the hemolytic potential and toxicity profiles of the antibiotics when used in combination. Briefly, 100 µL of mouse red blood cells (the study was approved by the Research Ethics Committee on Animal Use of the Universidade Federal da Grande Dourados (no. 23018)) were exposed to 100 µL of CTB/PMB at 2 and 4 × MIC concentration for 4 h. Next, the samples were centrifuged for 5 min at 2500 rpm, supernatant aliquots were removed, and hemolysis was quantified by measuring the OD at 595 nm using the iMark™ Microplate Absorbance Reader. Triton 0.1% and D-PBS were used as positive and negative controls, respectively. Hemolysis rate of CTB/PMB at 1 × MIC was determined in a previous study[14].

Toxicity assay in *C.elegans* model

The safety profile of CTB/PMB was evaluated using wild-type *C. elegans* nematodes (N2). The *C. elegans* strain was propagated on nematode growth medium (NGM) supplemented with *Escherichia coli* OP50 as a food source. Age synchronization was achieved by bleaching with alkaline hypochlorite and sodium hydroxide. The obtained embryos were placed on NGM plates at 16 °C and grown until they reached the

young adult stage (L4 phase). At this stage, 15–30 worms were transferred to 24-well plates containing M9 buffer and CTB/PMB at 1, 2, and 4 × MIC. The number of viable and dead nematodes was recorded every 24 h over the five-day incubation period. Worms were considered dead if no spontaneous movement or response to stimulation with a platinum loop was observed[38].

In vivo infection model

An *in vivo* infection model was established using the *C. elegans* AU37 (glp-4; sek-1) strain, which is immunocompromised due to the *sek-1* mutation, making it susceptible and increasing the sensitivity of the assay[39]. The worms were propagated, age-synchronized to the L4 stage, and exposed to the CPR-Kp strain (1.5×10^8 CFU/mL) for 3 h. After removing excess bacteria by washing in M9 buffer, 15–30 worms were transferred to 24-well plates containing M9 along with CTB/PMB or individual antibiotics at 1, 2, and 4 × MIC. Uninfected worms served as the negative control, and infected untreated worms as the positive control. Tigecycline (16 mg/L) was used as the reference antibiotic. Viability was assessed every 24 h during a five-day incubation at 16 °C. Worms were classified as dead when no movement or response to a platinum loop was observed[40].

Statistical analysis

Experiments were all conducted in triplicate to ensure reproducibility. Statistical analysis, including AUC, was performed via One-way ANOVA. *C. elegans* survival was assessed through Kaplan–Meier survival curves, with significance determined by a log-rank test. All differences were considered to be statistically significant at $p < 0.05$. All statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

ASSOCIATED CONTENT

Supporting Information. Additional results details, including extra figures of time-kill experiments (Figure S1), and prediction analyses for Polymyxin B (Figure S2) and Ceftibuten (Figure S3) (PDF) are described in the supplementary material.

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Author Contributions

Mariana C Sturaro, Nathalia d S Damaceno, Luccas P Pires, Ediane B Cornelius and Izabel A Alves were responsible for the methodology execution and data accuracy. The manuscript was written by Mariana C Sturaro. Luana Rossato, Gleyce H d A d Souza, Izabel A Alves and Simone Simionatto contributed with editing and reviewing the manuscript. Izabel A Alves and Simone Simionatto were responsible for the project conceptualization, supervision and administration, these authors have contributed equally for this work (‡). All authors have given approval to the final version of the manuscript.

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Data Availability Statement

All data are available from the corresponding authors upon reasonable request.

Conflicts of Interest

The authors have no conflict of interest to declare.

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REFERENCES

(1) Pu, D.; Zhao, J.; Lu, B.; Zhang, Y.; Wu, Y.; Li, Z.; Zhuo, X.; Cao, B. Within-Host Resistance Evolution of a Fatal ST11 Hypervirulent Carbapenem-Resistant *Klebsiella Pneumoniae*. *International Journal of Antimicrobial Agents* **2023**, *61* (4), 106747. https://doi.org/10.1016/j.ijantimicag.2023.106747.

(2) de Souza, G. H.; Vaz, M. S.; Dos Santos Radai, J. A.; Fraga, T. L.; Rossato, L.; Simionatto, S. Synergistic Interaction of Polymyxin B with Carvacrol: Antimicrobial Strategy against Polymyxin-Resistant Klebsiella Pneumoniae. *Future Microbiol* **2024**. https://doi.org/10.2217/fmb-2023-0070.

(3) Gomes, M. Z. R.; de Lima, E. M.; Martins Aires, C. A.; Pereira, P. S.; Yim, J.; Silva, F. H.; Rodrigues, C. A. S.; Oliveira, T. R. T. e; da Silva, P. P.; Eller, C. M.; de Souza, C. M. R.; Rybak, M. J.; Albano, R. M.; de Miranda, A. B.; Machado, E.; Catanho, M. Outbreak Report of Polymyxin-Carbapenem-Resistant Klebsiella Pneumoniae Causing Untreatable Infections Evidenced by Synergy Tests and Bacterial Genomes. *Sci Rep* **2023**, *13* (1), 6238. https://doi.org/10.1038/s41598-023-31901-4.

(4) Hughes, D.; Karlén, A. Discovery and Preclinical Development of New Antibiotics. *Ups J Med Sci* **2014**, *119* (2), 162–169. https://doi.org/10.3109/03009734.2014.896437.

(5) Coates, A. R. M.; Hu, Y.; Holt, J.; Yeh, P. Antibiotic Combination Therapy against Resistant Bacterial Infections: Synergy, Rejuvenation and Resistance Reduction. *Expert Rev Anti Infect Ther* **2020**, *18* (1), 5–15. https://doi.org/10.1080/14787210.2020.1705155.

(6) Mattos, K. P. H.; Gouvêa, I. R.; Quintanilha, J. C. F.; Cursino, M. A.; Vasconcelos, P. E. N. S.; Moriel, P. Polymyxin B Clinical Outcomes: A Prospective Study of Patients Undergoing

Intravenous Treatment. *Journal of Clinical Pharmacy and Therapeutics* **2019**, *44* (3), 415–419. https://doi.org/10.1111/jcpt.12801.

(7) Hou, S.-Y.; Wu, D.; Feng, X.-H. Polymyxin Monotherapy versus Polymyxin-Based Combination Therapy against Carbapenem-Resistant Klebsiella Pneumoniae: A Systematic Review and Meta-Analysis. *J Glob Antimicrob Resist* **2020**, *23*, 197–202. https://doi.org/10.1016/j.jgar.2020.08.024.

(8) Marshall, W. F.; Blair, J. E. The Cephalosporins. *Mayo Clin Proc* **1999**, *74* (2), 187–195. https://doi.org/10.4065/74.2.187.

(9) Stewart, A. G.; Harris, P. N. A.; Henderson, A.; Schembri, M. A.; Paterson, D. L. Oral Cephalosporin and β-Lactamase Inhibitor Combinations for ESBL-Producing Enterobacteriaceae Urinary Tract Infections. *Journal of Antimicrobial Chemotherapy* **2020**, *75* (9), 2384–2393. https://doi.org/10.1093/jac/dkaa183.

(10) Kmeid, J. G.; Youssef, M. M.; Kanafani, Z. A.; Kanj, S. S. Combination Therapy for Gram-Negative Bacteria: What Is the Evidence? *Expert Review of Anti-infective Therapy* **2013**, *11* (12), 1355–1362. https://doi.org/10.1586/14787210.2013.846215.

(11) Leonard, S. N. Synergy between Vancomycin and Nafcillin against Staphylococcus Aureus in an In Vitro Pharmacokinetic/Pharmacodynamic Model. *PLoS One* **2012**, *7* (7), e42103. https://doi.org/10.1371/journal.pone.0042103.

(12) Brill, M. J. E.; Kristoffersson, A. N.; Zhao, C.; Nielsen, E. I.; Friberg, L. E. Semi-Mechanistic Pharmacokinetic–Pharmacodynamic Modelling of Antibiotic Drug Combinations. *Clinical Microbiology and Infection* **2018**, *24* (7), 697–706. https://doi.org/10.1016/j.cmi.2017.11.023.

(13) da Silva, K. E.; Thi Nguyen, T. N.; Boinett, C. J.; Baker, S.; Simionatto, S. Molecular and Epidemiological Surveillance of Polymyxin-Resistant Klebsiella Pneumoniae Strains Isolated from Brazil with Multiple mgrB Gene Mutations. *International Journal of Medical Microbiology* **2020**, *310* (7), 151448. https://doi.org/10.1016/j.ijmm.2020.151448.

(14) Sturaro, M. C.; de Souza, G. H. de A.; Damaceno, N. da S.; Silva, O. N.; de Aquino, T. M.; Freire, N. M. L.; Alcântara, M. G. dos S.; Monteiro, K. L. C.; Martins, A. A.; Rossato, L.; Fraga, T. L.; Borsuk, S.; Dellagostin, O. A.; Simionatto, S. Antimicrobial Activity of Ceftibuten/Polymyxin B Combination against Polymyxin/Carbapenem-Resistant Klebsiella Pneumoniae. *Journal of Antimicrobial Chemotherapy* **2024**, dkae382. https://doi.org/10.1093/jac/dkae382.

(15) Miller, W. R.; Arias, C. A. ESKAPE Pathogens: Antimicrobial Resistance, Epidemiology, Clinical Impact and Therapeutics. *Nat Rev Microbiol* **2024**, 1–19. https://doi.org/10.1038/s41579-024-01054-w.

(16) Ribeiro, A. C. da S.; Chikhani, Y. C. dos S. A.; Valiatti, T. B.; Valêncio, A.; Kurihara, M. N. L.; Santos, F. F.; Minarini, L. A. da R.; Gales, A. C. In Vitro and In Vivo Synergism of Fosfomycin in Combination with Meropenem or Polymyxin B against KPC-2-Producing Klebsiella Pneumoniae Clinical Isolates. *Antibiotics* **2023**, *12* (2), 237. https://doi.org/10.3390/antibiotics12020237.

(17) Sturaro, M. C.; Damaceno, N. da S.; Faccin, I. D.; Silva, O. N.; de Aquino, T. M.; Freire, N. M. L.; Alcântara, M. G. D. S.; Monteiro, K. L. C.; Rossato, L.; de Souza, G. H. de A.; Simionatto, S. Synergistic Antimicrobial Combination of Third-Generation Cephalosporins and Polymyxin B against Carbapenem-Polymyxin-Resistant Klebsiella Pneumoniae: An in Vitro and in Vivo

Analysis. *Antimicrob Agents Chemother* **2024**, *68* (10), e0093024. https://doi.org/10.1128/aac.00930-24.

(18) Treyaprasert, W.; Schmidt, S.; Rand, K. H.; Suvanakoot, U.; Derendorf, H. Pharmacokinetic/Pharmacodynamic Modeling of in Vitro Activity of Azithromycin against Four Different Bacterial Strains. *International Journal of Antimicrobial Agents* **2007**, *29* (3), 263–270. https://doi.org/10.1016/j.ijantimicag.2006.08.049.

(19) Hu, J.; Zhang, J.; Chen, Y.; Liang, W.; Wu, S. In Vitro Anaerobic Pharmacokinetic/Pharmacodynamic Model to Simulate the Bactericidal Activity of Levornidazole Against Bacteroides Fragilis. *Clinical Therapeutics* **2017**, *39* (4), 828–836. https://doi.org/10.1016/j.clinthera.2017.03.003.

(20) O'Donnell, J.; Tanudra, A.; Chen, A.; Hines, D.; Tommasi, R.; Mueller, J. Pharmacokinetic/Pharmacodynamic Determination and Preclinical Pharmacokinetics of the β-Lactamase Inhibitor ETX1317 and Its Orally Available Prodrug ETX0282. *ACS Infect. Dis.* **2020**, *6* (6), 1378–1388. https://doi.org/10.1021/acsinfecdis.0c00019.

(21) Dousa, K. M.; Shin, E.; Kurz, S. G.; Plummer, M.; Nantongo, M.; Bethel, C. R.; Taracila, M. A.; Nguyen, D. C.; Kreiswith, B. N.; Daley, C. L.; Remy, K. E.; Holland, S. M.; Bonomo, R. A. Synergistic Effects of Sulopenem in Combination with Cefuroxime or Durlobactam against Mycobacterium Abscessus. *mBio* **2024**, *15* (6), e00609. https://doi.org/10.1128/mbio.00609-24.

(22) Noel, A. R.; Attwood, M.; Bowker, K. E.; MacGowan, A. P. Pharmacodynamics of Taniborbactam in Combination with Cefepime Studied in an *in Vitro* Model of Infection. *International Journal of Antimicrobial Agents* **2024**, *64* (4), 107304. https://doi.org/10.1016/j.ijantimicag.2024.107304.

(23) Phee, L. M.; Kloprogge, F.; Morris, R.; Barrett, J.; Wareham, D. W.; Standing, J. F. Pharmacokinetic-Pharmacodynamic Modelling to Investigate in Vitro Synergy between Colistin and Fusidic Acid against MDR Acinetobacter Baumannii. *Journal of Antimicrobial Chemotherapy* **2019**, *74* (4), 961. https://doi.org/10.1093/jac/dky524.

(24) Menezes, B. K.; Alves, I. A.; Staudt, K. J.; Beltrame, B. M.; Venz, L.; Michelin, L.; Araujo, B. V.; Tasso, L. Time-Kill Curves of Daptomycin and Monte Carlo Simulation for the Treatment of Bacteraemia Caused by Enterococcus Faecium. *Brazilian Journal of Microbiology* **2019**, *51* (1), 169. https://doi.org/10.1007/s42770-019-00200-4.

(25) Akrong, G.; Chauzy, A.; Aranzana-Climent, V.; Lacroix, M.; Deroche, L.; Prouvensier, L.; Buyck, J. M.; Couet, W.; Marchand, S. A New Pharmacokinetic-Pharmacodynamic Model To Characterize the Inoculum Effect of Acinetobacter Baumannii on Polymyxin B In Vitro. *Antimicrobial Agents and Chemotherapy* **2022**, *66* (1), e01789. https://doi.org/10.1128/AAC.01789-21.

(26) Li, H.; Zhou, X.; Huang, Y.; Liao, B.; Cheng, L.; Ren, B. Reactive Oxygen Species in Pathogen Clearance: The Killing Mechanisms, the Adaption Response, and the Side Effects. *Front Microbiol* **2020**, *11*, 622534. https://doi.org/10.3389/fmicb.2020.622534.

(27) El-Shaboury, S. R.; Saleh, G. A.; Mohamed, F. A.; Rageh, A. H. Analysis of Cephalosporin Antibiotics. *J Pharm Biomed Anal* **2007**, *45* (1), 1–19. https://doi.org/10.1016/j.jpba.2007.06.002.

(28) Trimble, M. J.; Mlynárčik, P.; Kolář, M.; Hancock, R. E. W. Polymyxin: Alternative Mechanisms of Action and Resistance. *Cold Spring Harb Perspect Med* **2016**, *6* (10), a025288. https://doi.org/10.1101/cshperspect.a025288.

(29) Moukhtari, S. H. E.; Muñoz-Juan, A.; Campo-Montoya, R. D.; Laromaine, A.; Blanco-Prieto, M. J. Biosafety Evaluation of Etoposide Lipid Nanomedicines in C. Elegans. *Drug Delivery and Translational Research* **2024**, *14* (8), 2158. https://doi.org/10.1007/s13346-023-01466-w.

(30) Ewbank, J. J.; Zugasti, O. C. Elegans: Model Host and Tool for Antimicrobial Drug Discovery. *Dis Model Mech* **2011**, *4* (3), 300–304. https://doi.org/10.1242/dmm.006684.

(31) Wu, Y.; Yu, W.; Chu, X.; Zhang, J.; Jia, P.; Liu, X.; Zhu, Y.; Xu, Y.; Yang, Q. Effect of Ceftazidime-Avibactam Combined with Different Antimicrobials against Carbapenem-Resistant Klebsiella Pneumoniae. *Microbiol Spectr 12* (6), e00107-24. https://doi.org/10.1128/spectrum.00107-24.

(32) Huang, Y.-S.; Yang, J.-L.; Wang, J.-T.; Sheng, W.-H.; Yang, C.-J.; Chuang, Y.-C.; Chang, S.-C. Evaluation of the Synergistic Effect of Eravacycline and Tigecycline against Carbapenemase-Producing Carbapenem-Resistant *Klebsiella Pneumoniae*. *Journal of Infection and Public Health* **2024**, *17* (5), 929–937. https://doi.org/10.1016/j.jiph.2024.03.027.

(33) Byun, J. H. Formulation and Validation of an Extended Sigmoid Emax Model in Pharmacodynamics. *Pharm Res* **2024**. https://doi.org/10.1007/s11095-024-03752-9.

(34) Chen, T.; Zheng, Y.; Roskos, L.; Mager, D. E. Comparison of Sequential and Joint Nonlinear Mixed Effects Modeling of Tumor Kinetics and Survival Following Durvalumab Treatment in Patients with Metastatic Urothelial Carcinoma. *J Pharmacokinet Pharmacodyn* **2023**, *50* (4), 251–265. https://doi.org/10.1007/s10928-023-09848-w.

(35) Tiwari, V.; Mishra, N.; Gadani, K.; Solanki, P. S.; Shah, N. A.; Tiwari, M. Mechanism of Anti-Bacterial Activity of Zinc Oxide Nanoparticle Against Carbapenem-Resistant Acinetobacter Baumannii. *Frontiers in Microbiology* **2018**, *9*.

(36) Retore, Y. I.; Lucini, F.; Rial, R. C.; Cardoso, C. A. L.; Souza, P. F. N.; Simionatto, S.; Rossato, L. Antifungal Activity of *Caryocar Brasiliense* Camb. Alone or along with Antifungal Agents against Multidrug-Resistant *Candida Auris*. *Journal of Ethnopharmacology* **2024**, *330*, 118240. https://doi.org/10.1016/j.jep.2024.118240.

(37) Wang, Y.; Zhang, Y.; Shi, Y.; Pan, X.; Lu, Y.; Cao, P. Antibacterial Effects of Cinnamon (*Cinnamomum Zeylanicum*) Bark Essential Oil on *Porphyromonas Gingivalis*. *Microbial Pathogenesis* **2018**, *116*, 26–32. https://doi.org/10.1016/j.micpath.2018.01.009.

(38) Wang, Y.-C.; Huang, S.-W.; Chiang, M.-H.; Lee, I.-M.; Kuo, S.-C.; Yang, Y.-S.; Chiu, C.-H.; Su, Y.-S.; Chen, T.-L.; Wang, F.-D.; Lee, Y.-T. In Vitro and in Vivo Activities of Imipenem Combined with BLI-489 against Class D β-Lactamase-Producing Acinetobacter Baumannii. *Journal of Antimicrobial Chemotherapy* **2021**, *76* (2), 451–459. https://doi.org/10.1093/jac/dkaa421.

(39) Singulani, J. de L.; Scorzoni, L.; Gomes, P. C.; Nazaré, A. C.; Polaquini, C. R.; Regasini, L. O.; Fusco-Almeida, A. M.; Mendes-Giannini, M. J. S. Activity of Gallic Acid and Its Ester Derivatives in Caenorhabditis Elegans and Zebrafish (Danio Rerio) Models. *Future Med Chem* **2017**, *9* (16), 1863–1872. https://doi.org/10.4155/fmc-2017-0096.

(40) Pimentel Arantes, J.; Dillis Faccin, I.; Coutinho, E. J.; Lima Cardoso, C. A.; Lopes Fernandes, S. S.; Rossato, L.; Simionatto, E.; Simionatto, S. An Approach to Combat Multidrug-Resistant K. Pneumoniae Strain Using Synergistic Effects of Ocotea Diospyrifolia Essential Oil in Combination with Amikacin. *Microb Pathog* **2024**, *193*, 106782. https://doi.org/10.1016/j.micpath.2024.106782.

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Antimicrobial activity of ceftibuten/polymyxin B combination against polymyxin/carbapenem-resistant *Klebsiella pneumoniae*

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Objectives: To evaluate the synergistic effect of a ceftibuten and polymyxin B combination and to determine its capacity to overcome polymyxin B resistance in polymyxin/carbapenem-resistant (PC-R) *Klebsiella pneumoniae*.

Methods: To investigate the combination's antibacterial efficacy, antimicrobial susceptibility tests using broth microdilution methods, chequerboard assays and time–kill testing were performed. Antibiofilm activity was also assessed. The treatment's effect on the bacterial cell membrane was examined by quantifying intracellular protein leakage and conducting scanning electron microscopy. Haemocompatibility tests were conducted to evaluate toxicity. Additionally, an infection model was established using Swiss mice to assess *in vivo* antimicrobial activity.

Results: The ceftibuten/polymyxin B combination demonstrated synergistic effects against several PC-R strains of *K. pneumoniae*, as determined by the FIC index (FICI) values, which ranged from 0.15 to 0.37. This combination was efficacious, exhibiting bactericidal activity at twice the MIC. Ceftibuten/polymyxin B also demonstrated antibiofilm activity. Additionally, ceftibuten/polymyxin B neither damaged the bacterial membrane nor exhibited haemolytic activity. Based on these findings, the *in vivo* therapeutic potential was investigated and it was found that ceftibuten/polymyxin B significantly decreased the bacterial load in the peritoneal lavage fluid of mice, revealing its effectiveness in treating infections caused by PC-R *K. pneumoniae*.

Conclusions: The ceftibuten/polymyxin B combination exhibited synergistic effects *in vitro* and *in vivo*, and thus might be a promising therapeutic alternative for treating PC-R *K. pneumoniae* infections. As the combination was efficacious in preclinical models, researchers may further investigate its potential in clinical studies.

Introduction

Microbial resistance to antibiotics is a major global threat, as it contributes to prolonged patient hospitalization, greater healthcare costs and higher mortality rates.[1–3] Many microorganisms are resistant to commonly used classes of antibiotics, such as β-lactams, aminoglycosides and fluoroquinolones.[4] This has led medical practitioners to use older classes of drugs, such as polymyxins (B and E), for treating Gram-negative MDR bacteria (GNB-MDR).[5] However, multiple cases of microorganisms resistant to these antibiotics have been reported from around the world in the last decade.[1,6] The high prevalence of polymyxin-resistant microorganisms is particularly concerning.[7]

In the pharmaceutical industry, the development of new antibiotics faces challenges in keeping pace with the rapid adaptability of GNB such as *Klebsiella pneumoniae*. Probabilistic study has indicated that the production of new antibiotics is delayed by approximately 30 years compared with the rate at which GNB evolve and become antibiotic resistant.[8] Developing a new antibiotic compound requires 10 to 15 years and is an expensive process.[9]

Combining existing drugs is an effective strategy to minimize costs and accelerate the development of new treatments, based

on pre-existing knowledge regarding their composition and safety profiles. Identifying the synergistic interactions between drugs can provide insights into ways to overcome microbial resistance,[8] which can help expand the antibacterial spectrum against various microorganisms,[10] and decrease the required drug dosage, thus lowering the risk of toxicity.[1,7] Considering these factors, in this study, it was aimed to elucidate the synergistic effects of a ceftibuten/polymyxin B combination, *in vitro* and *in vivo*, against polymyxin/carbapenem-resistant (PC-R) *K. pneumoniae*.

Materials and methods

Bacterial strains and chemicals

The six bacterial PC-R *K. pneumoniae* strains used in the experiments were isolated from patients admitted to a tertiary hospital in Dourados, Mato Grosso do Sul, Brazil.[11] MALDI-TOF MS was performed using a microflex LT spectrometer (Bruker Daltonics, Massachusetts, USA) to validate the bacterial species identified using the Phoenix 100® automated system (BD Diagnostic Systems, Sparks, MD, USA). Ceftibuten hydrate (Lot 0000124698) and polymyxin B solution (Lot BCCG2613) were purchased from Sigma (St. Louis, MO, USA) and prepared following the manufacturer's instructions.

Susceptibility test

The broth microdilution test was conducted to determine the MICs of ceftibuten and polymyxin B alone, following the guidelines of the CLSI[12] with a few changes. Due to Mueller–Hinton broth's nutritional simplicity, it was opted to use brain heart infusion (BHI) broth in the microdilution test. Briefly, both antibiotics were placed in a 96-well plate and diluted in BHI broth at concentrations ranging from 0.0625 to 64 mg/L. Next, 100 µL of PC-R *K. pneumoniae* strains, standardized using a 0.5 McFarland scale and diluted to 1:100, with a final concentration of 1.5×10^6 cfu/mL was added to the wells. A positive control was used to assess bacterial cell viability, while a negative control was included to confirm the sterility of the experiment. After incubation for 24 h at 37°C, MICs were determined as the lowest concentration with no visible bacterial growth.[13,14]

Synergism testing

Chequerboard assays were performed to assess the FIC index (FICI) and the synergistic activity of the antibiotics. Briefly, in a 96-well plate, ceftibuten was diluted horizontally in BHI broth, with concentrations ranging from 0.0625 to 64 mg/L. Polymyxin B was diluted vertically, with concentrations ranging from 2 to 64 mg/L. Each well had a different concentration of both medications. PC-R *K. pneumoniae* (1.5×10^6 cfu/mL) strains were added to the wells. The chequerboard plate was incubated for 24 h at 37°C, and the FICI was calculated using the following formula:

$$FICI = \frac{MIC\ PMB.\ C}{MIC\ PMB.\ I} + \frac{MIC\ CTB.\ C}{MIC\ CTB.\ I}$$

where MIC PMB.C indicates the MIC of polymyxin B in combination, MIC PMB.I indicates the MIC of isolated polymyxin B, MIC CTB.C indicates the MIC of ceftibuten in combination, and MIC CTB.I indicates the MIC of isolated ceftibuten. Synergistic activity between the two antibiotics was defined when the FICI was ≤0.5, indifferent interaction was considered when the FICI was >0.5 but ≤4, and antagonistic activity was defined when the FICI was >4.[13,15]

Growth curves were obtained concurrently with antibacterial testing and the chequerboard assay. The absorbance of the experiment's plates was measured, under the wavelength of 595 nm, with an iMark™ Microplate Absorbance Reader, at 0, 2, 4, 6, 8, 12 and 24 h after the inoculation step.[16]

The SynergyFinder web application was utilized to perform dose–response analyses and calculate the zero interaction potency (ZIP) score for the drug combinations. The ZIP score was interpreted as follows: (i) >10 indicated synergy; (ii) between −10 and 10 suggested an additive effect; and (iii) less than −10 indicated antagonism.

Time–kill assay

Initially, a PC-R *K. pneumoniae* K18 inoculum, prepared based on the 0.5 McFarland standard scale, and diluted 1:100, was added to the treatments (combination and isolated antibiotics) in concentrations of 0.5× MIC (2 mg/L ceftibuten + 1 mg/L polymyxin B), 1× MIC (4 mg/L ceftibuten + 2 mg/L polymyxin B) and 2× MIC (8 mg/L ceftibuten + 4 mg/L polymyxin B). At specific timepoints (0, 2, 4, 6, 8 and 24 h), after inoculation, aliquots (1 µL) were collected from each well and plated onto BHI agar plates and incubated at 37°C for 18–24 h. Subsequently, the plates were examined for growth, and bacterial count values were expressed using the logarithmic scale. Negative (culture medium) and positive (culture medium with bacterial suspension) controls were included in the analysis.[17]

Spot assay

A spot assay was conducted to analyse the treatment's influence on the bacterial load. Solutions containing the drugs in 1× MIC (4 mg/L ceftibuten + 2 mg/L polymyxin B) and 2× MIC (8 mg/L ceftibuten + 4 mg/L polymyxin B) and the inoculum of PC-R *K. pneumoniae* K18 (1.5×10^6 cfu/mL) were diluted on a scale of 1:10, 1:100, 1:1000 and 1:10 000 in BHI broth. Polymyxin B (64 mg/L) was used as the negative control; PC-R *K. pneumoniae* with no antibiotics was used as the positive control. The isolated antibiotics were used as a comparison control. Subsequently, 5 µL of each dilution and controls was deposited on a Petri dish containing BHI agar, followed by incubation at 37°C for 24 h.

Biofilm formation inhibition

The ability of the ceftibuten/polymyxin B combination to inhibit biofilm development was evaluated using chequerboard assay plates, which were maintained at 37°C for 24 h to promote bacterial development and biofilm maturation. Subsequently, planktonic cells were removed through thrice serial washings with sterile water, and the remaining biofilms were stained with 0.1% crystal violet, for 30 min, as described.[18,19] Wells were washed again to remove any excess dye, and the surface-bound dye was dissolved in 200 µL of 96% ethanol for 20 min at 4°C to prevent ethanol evaporation. Biofilm biomass was then quantified by measuring the OD at 490 nm. A positive control containing only PC-R *K. pneumoniae* and a negative control (no bacteria) to confirm sterility were also included. All procedures were performed in triplicate. The percentage of inhibition was calculated with the following formula:

$$Biofilm\ inhibition\ (\%) = \frac{OD_{positive\ control} - OD_{treatment}}{OD_{positive\ control}} \times 100$$

Cell membrane integrity assay

To investigate the effect of the ceftibuten/polymyxin B combination on the PC-R *K. pneumoniae* cell at 1× MIC (4 mg/L ceftibuten + 2 mg/L polymyxin B), protein leakage was monitored. In a microplate, the treatment and the bacterial inoculum were added and incubated for 4 h at 37°C. Next, the contents of each microplate well were centrifuged at 2500 rpm for 5 min at 4°C. The quantity of protein released from the cytoplasm was determined in the supernatant using the Pierce™ BCA Protein

Assay kit (Thermo Scientific, MA, USA).[20] Amikacin at a concentration of 64 mg/L was used as a control.

Scanning electron microscopy (SEM)

SEM was used to capture images and assess the morphological changes in PC-R *K. pneumoniae* cells induced by the ceftibuten/polymyxin B combination. The combination of ceftibuten (4 mg/L)/polymyxin B (2 mg/L) was prepared based on the result of the time–kill assay at 24 h. Subsequently, the cultures were centrifuged in phosphate buffer (0.1 M, pH 7.4) at 3000 rpm for 5 min and then fixed with 2.5% glutaraldehyde. The samples were post-fixed with 0.2% osmium tetroxide for 90 min. Subsequently, the samples were dehydrated by a graded series of concentrations of ethanol (30%, 50%, 70% and 100% v/v) for 10 min each.[21,22] Finally, a micropipette was used to add 20 µL of the final product to glass coverslips (0.8×0.8 cm). After drying, the coverslips containing the cells were sputtered with gold and observed under SEM (JSM-6380LV, JEOL, USA).

Haemolysis assay

Haemolysis assays were performed as previously described,[23] with some modifications. Briefly, 100 µL of fresh mouse blood was incubated with 100 µL of antibiotics (4 mg/L ceftibuten and 2 mg/L polymyxin B; in combination or alone) for 4 h. Subsequently, the samples were centrifuged for 5 min at 2500 rpm, after which the supernatant aliquots were removed and quantified by measuring the OD at 595 nm using the iMark™ Microplate Absorbance Reader. Triton (0.1%, v/v) and Dulbecco's PBS (D-PBS) were used as positive and negative controls, respectively. Haemolytic rate (HR) was calculated using the following formula:

$$HR(\%) = (OD_{treatment} - OD_{D-PBS}/OD_{Triton} - OD_{D-PBS}) \times 100$$

Mice in vivo assay

In vivo experiments were conducted using female Swiss mice (*Mus musculus*; 8 weeks old; weight: 18–20 g). The mice were housed in polypropylene cages under controlled humidity (40%–60%), temperature (22°C ± 3°C) and light (12 h/12 h light/dark cycle); all mice received standard commercial feed and water *ad libitum*. All animal experiments followed the recommendations of the National Council for the Control of Animal Experimentation (CONCEA). The study was approved by the Research Ethics Committee on Animal Use of the Universidade Federal da Grande Dourados (no. 23018) and Centro Universitário da Grande Dourados (Unigran; no. 080/18). The institutional animal ethics committee reviewed and approved the study design.

In vivo antibacterial activity

To investigate the therapeutic effect of ceftibuten/polymyxin B *in vivo*, an intraperitoneal infection model induced by PC-R *K. pneumoniae* (strain K18), which exhibited the lowest FICI, was performed.[14,24] Briefly, dexamethasone (20 mg/kg) was administered to mice 24 h before the experiments to induce immunosuppression. Neutropenic mice were randomized into various treatment groups (six mice per group) as follows: polymyxin B [2 mg/kg every 12 h, intraperitoneally (IP)]; ceftibuten [10 mg/kg every 12 h, oral gavage (OG)];[25] ceftibuten/polymyxin B (10 mg/kg ceftibuten every 12 h OG and 2 mg/kg polymyxin B every 12 h IP). The mice in the positive control group received tigecycline (10 mg/kg every 12 h IP), and those in the negative control group received a saline solution (OG). A naive group (without infection) was included to evaluate basal indices. All animals, except those in the naive group, received an IP injection of 0.2 mL of a bacterial suspension at a concentration of 3×10^8 cfu/mL, prepared in 0.9% saline solution; the dose was determined in a pilot study (data not shown). Treatments commenced 1 h after bacterial inoculation. After receiving the treatments for 48 h, the animals were euthanized with a combination of xylazine and ketamine (10 and 60 mg/kg IP, respectively). Peritoneal lavage fluid (PLF) samples were collected to assess bacterial colonization, and the bacteria were subsequently plated on BHI agar to count cfu.

Statistical analyses

The data were expressed as mean ± standard error (SE). To evaluate the variations between groups, one-way analysis of variance (ANOVA) was conducted. The data were analysed, statistical tests were conducted, and graphs were plotted using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). All results were considered to be statistically different at $P<0.05$ (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$).

Results

In this study, analysed bacterial strains were classified as ST 11 (K1, K3, K15, K18, K28) and ST 345 (K4). These strains were identified as carrying the bla_{KPC-2} gene, in addition to ESBL genes such as $bla_{CTX-M-15}$, bla_{TEM-1} and bla_{SHV-11}, which provide resistance to β-lactam antibiotics, including cephalosporins. WGS identified mutations in the *mgrB* gene, a known determinant of polymyxin resistance.[11]

The antibacterial activity of ceftibuten/polymyxin B, either alone or in combination, was evaluated against these MDR strains. The MICs of ceftibuten and polymyxin B were 8–64 mg/L and 32–64 mg/L, respectively, matching the bacterial resistance profiles. A reduction in inhibitory concentrations was observed when evaluating the ceftibuten/polymyxin B combination; the MICs of conjugated ceftibuten ranged from 0.065 to 4 mg/L, and those of conjugated polymyxin B ranged from 2 to 16 mg/L. Additionally, the FICI values across all tested bacteria ranged from 0.15 to 0.37, indicating a synergistic interaction between the drugs for all tested bacterial strains (Table 1).

The growth curves showed significant synergistic interactions between ceftibuten and polymyxin B in all tested PC-R *K. pneumoniae* strains. The combination exhibited antimicrobial effects and effectively suppressed bacterial growth throughout the experiment (24 h), similar to the findings recorded for the negative control. In contrast, when drugs were administered individually, bacterial growth persisted, similar to the positive control for the PC-R *K. pneumoniae* strains K18, K4 and K1 (Figure 1). Polymyxin B inhibited the growth of PC-R *K. pneumoniae* K18 and K1 for 8 h and K4 for 12 h but failed to sustain suppression over 24 h.

The ceftibuten/polymyxin B combination inhibited PC-R *K. pneumoniae* bacterial growth along many concentration variations; synergism was proved with the low FICI values of 0.156 for PC-R *K. pneumoniae* K18, 0.25 for K4 and 0.18 for K1 (Figure 2a–c). Moreover, the ZIP index of the association was 45.745, which confirmed the presence of a synergistic interaction. The optimal inhibition rates (>80%) were observed when ceftibuten (2–8 mg/L) was combined with polymyxin B (16–64 mg/L) (Figure 2d and e), which matched the results of the chequerboard assay and the growth curves.

The results of the time–kill experiments indicated that the combination at 0.5× MIC did not kill bacteria (Figure 3a). Moreover, ceftibuten/polymyxin B only partially suppressed

Table 1. Antibacterial test and chequerboard results for the ceftibuten/polymyxin B combination against multiple PC-R *K. pneumoniae* strains

	MIC (mg/L)					
Bacterial strain/ID	CTB.I	PMB.I	CTB.C	PMB.C	FICI	Interaction
K1/27588809	32	32	4	2	0.18	Synergistic
K3/28030301	64	32	16	2	0.31	Synergistic
K4/28030501	8	64	0.065	16	0.25	Synergistic
K15/32089801	32	64	0.065	16	0.31	Synergistic
K18/34923502	32	64	4	2	0.15	Synergistic
K28/1316	8	64	2	8	0.37	Synergistic

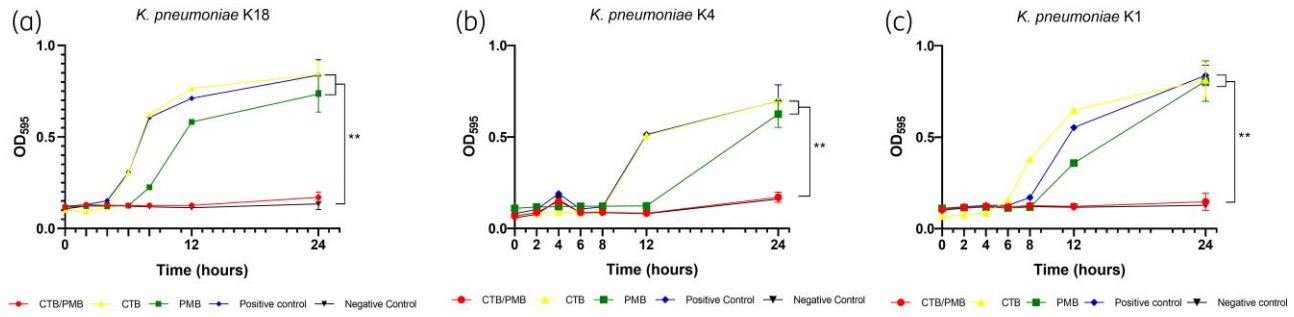

Figure 1. Growth curves obtained in the study of: (a) PC-R *K. pneumoniae* K18 treated with ceftibuten (CTB; 4 mg/L) and polymyxin B (PMB; 2 mg/L), in combination and alone; (b) PC-R *K. pneumoniae* K4 treated with CTB (0.065 mg/L) and PMB (16 mg/L), in combination and alone; (c) PC-R *K. pneumoniae* K1 treated with CTB (4 mg/L) and PMB (2 mg/L), in combination and alone. All curves included a positive control (bacteria only) and a negative control (no bacteria) for comparison. One-way ANOVA was used for the 24 h result; significance was shown with $P < 0.001$ (**). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

bacterial survival at 1× MIC; however, PC-R *K. pneumoniae* K18 cfu decreased considerably (by 4 logs) when treated with the combination, compared with that recorded after administering the drugs individually, suggesting a synergistic effect between the drugs (Figure 3b). The combination at 2× MIC exhibited potent biocidal activity, arresting bacterial growth within 2 h of treatment (Figure 3c). In contrast, individual doses of polymyxin B and ceftibuten at 2× MIC demonstrated a bacteriostatic activity by affecting bacteria for 6 and 8 h, respectively, but failing to sustain this effect over 24 h.

The results of the spot assay showed that bacterial load was inhibited throughout the treatment (Figure 3d). For ceftibuten/polymyxin B (1× MIC and 2× MIC) and the antibiotic control polymyxin B (64 mg/L), no bacterial growth was observed. The isolated drugs could not kill bacteria at any dilution, similar to the negative control.

The effect of the ceftibuten/polymyxin B combination on the integrity of the bacterial cell membrane was assessed through protein leakage as an indicator. The results showed that the PC-R *K. pneumoniae* cells treated with ceftibuten/polymyxin B did not exhibit protein externalization (Figure 4a). The surface of the cells treated with ceftibuten/polymyxin B displayed intact morphology with no evident surface damage, as well as the control group (PC-R *K. pneumoniae* K18) (Figure 4b–e). Further, when PC-R *K. pneumoniae* K18 was treated with a ceftibuten/polymyxin B combination, a significant reduction in bacterial population was observed.

Biofilm inhibition was observed after PC-R *K. pneumoniae* was treated with ceftibuten/polymyxin B. The combination of ceftibuten (8 mg/L)/polymyxin B (16 mg/L) inhibited bacterial biofilm formation more effectively compared with the antibiotics alone. Ceftibuten/polymyxin B inhibited 63% (OD 0.064) of the biofilm mass, whereas isolated ceftibuten and polymyxin B inhibited 23% (OD 0.121) and 1.12% (OD 0.139) of the biofilm mass, respectively. A negative control, without any microbial biofilm, represented 100% (OD 0.054) inhibition, and a positive control with a fully developed biofilm and no treatment with antibiotics represented 0% (OD 0.179) inhibition (Figure 5a).

Haemolysis tests were performed to evaluate the haemocompatibility of ceftibuten/polymyxin B alone and in combination. The results showed no significant difference between ceftibuten/polymyxin B and D-PBS (negative control), indicating that the combination was haemocompatible. The cytotoxicity decreased when the antibiotics were used in combination compared with the cytotoxicity recorded after the antibiotics were administered alone, with ceftibuten and polymyxin B showing haemolytic rates of 17% and 13.3%, respectively. The treatments did not visibly haemolyse blood cells, in contrast to the positive control (Triton 0.1%), and blood coagulated in the same way as that recorded for D-PBS (negative control) (Figure 5b).

The results of *in vivo* experiments indicated that ceftibuten/polymyxin B significantly decreased the bacterial load of PC-R *K. pneumoniae* in the PLF of mice compared with that recorded in the untreated control group. Additionally, the combination

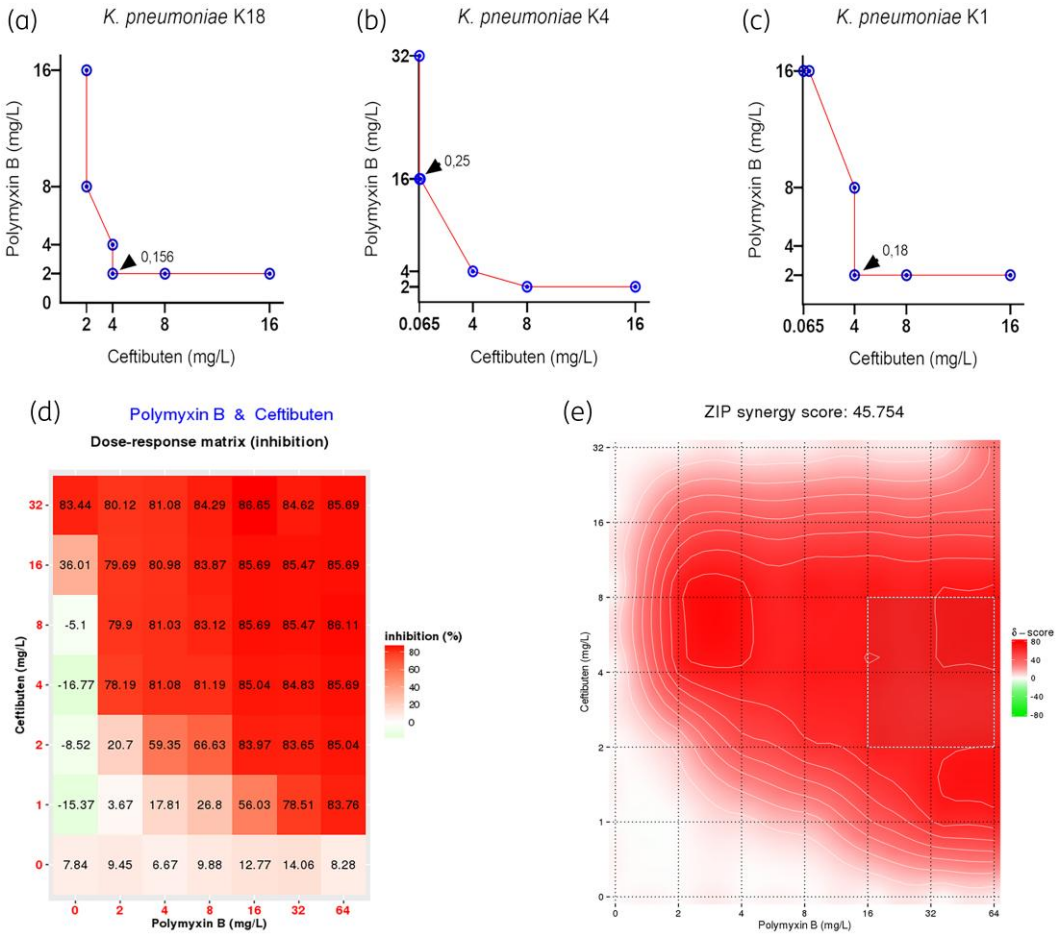

Figure 2. (a) Different combinations of ceftibuten and polymyxin B that inhibited *K. pneumoniae* K18 growth; (b) different combinations of ceftibuten and polymyxin B that inhibited *K. pneumoniae* K4 growth; (c) different combinations of ceftibuten and polymyxin B that inhibited *K. pneumoniae* K1 growth. The best combination doses are identified with the FICI; synergy is defined as a FICI of ≤0.5. (d) Dose–response matrix of ceftibuten/polymyxin B combination. The red (dark) area identifies the dose combinations that inhibited >80% of bacterial growth. (e) ZIP synergy score (45.754) demonstrates that ceftibuten/polymyxin B combination is synergistic. The reddest (darkest) areas indicate better dose combinations related to bacterial growth inhibition. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

treatment showed outcomes similar to those of tigecycline, which is commonly administered to treat PC-R *K. pneumoniae*. Antibiotics tested individually did not show similar results due to the microbial resistance profile of the inoculum used (PC-R *K. pneumoniae* K18) (Figure 6).

Discussion

Polymyxins are the final option for treating carbapenem-resistant GNB due to the risks of nephrotoxicity and neurotoxicity.[26] Despite these concerns, polymyxins need to be used to combat infections, particularly due to increasing bacterial multidrug resistance.[27] Preventing the development of polymyxin resistance among microorganisms is essential.[28] Therefore, combinations of drugs with polymyxins must be assessed to lower the required dose in clinical treatments, given that toxicity and dosage are directly correlated.[10] The synergy observed between ceftibuten, a third-generation cephalosporin effective against GNB such as *K. pneumoniae*,[25] and polymyxin B is promising; thus, combination treatment is a novel strategy to enhance the antibacterial efficacy of drugs while mitigating resistance.

The ceftibuten/polymyxin B combination exhibited synergism, as determined by the FICI values of ≤0.5 for all tested bacterial strains. Ceftibuten also decreased the *in vitro* dose of polymyxin B 5-fold for the K18 strain of PC-R *K. pneumoniae*. Additionally, the ZIP synergy score highlighted the synergistic effect between the compounds. The combination also achieved a reduction of 4 logs in bacterial growth in the time–kill assay at a concentration of 1× MIC; synergism is achieved when there is a decrease in 2 logs compared with the effects of the drugs alone.[7] These findings confirmed the synergism between ceftibuten and polymyxin B, which indicated that the combination therapy could effectively overcome bacterial resistance,[8] and emphasized the potential of ceftibuten in increasing the antimicrobial efficacy of polymyxin B.

Interestingly, no direct correlation was observed between drug synergy and the resistance genes of PC-R *K. pneumoniae*. This suggests that the observed synergy may not be directly

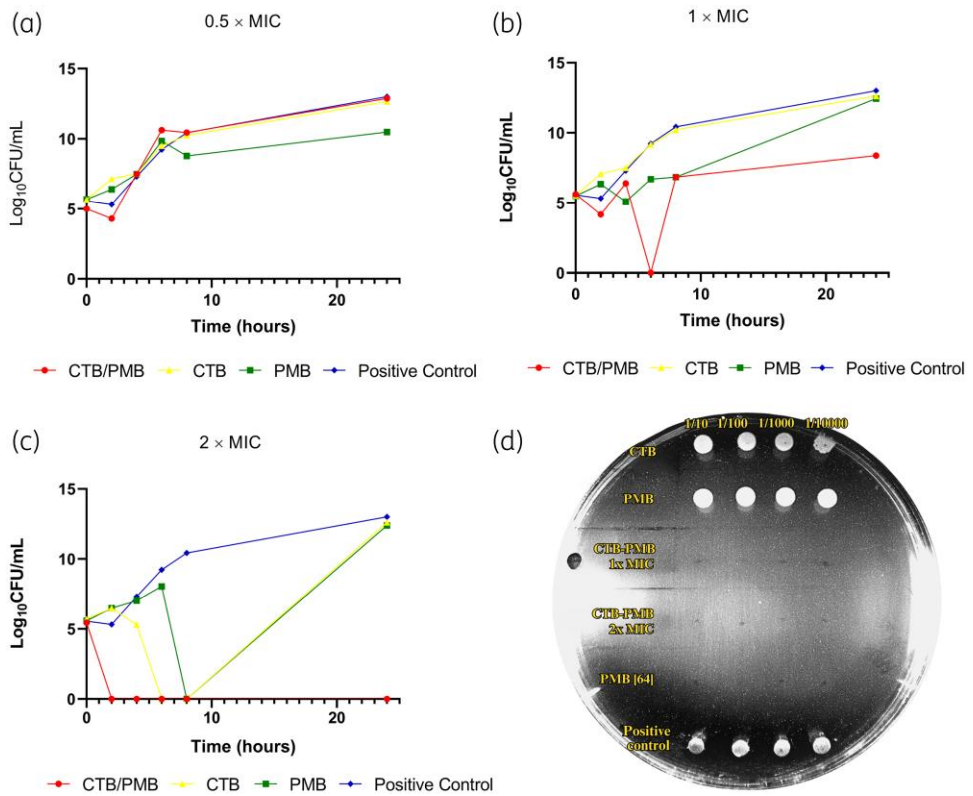

Figure 3. (a) Time–kill results from 0.5× MIC ceftibuten/polymyxin B (CTB/PMB) combination and isolated antibiotics. (b) Time–kill results from 1× MIC CTB/PMB combination and isolated antibiotics. (c) Time–kill results from 2× MIC CTB/PMB combination and isolated antibiotics. (d) Spot assay results. CTB/PMB was able to inhibit bacterial growth between the two concentrations (1× MIC and 2× MIC), as well as the control (polymyxin B 64 mg/L). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

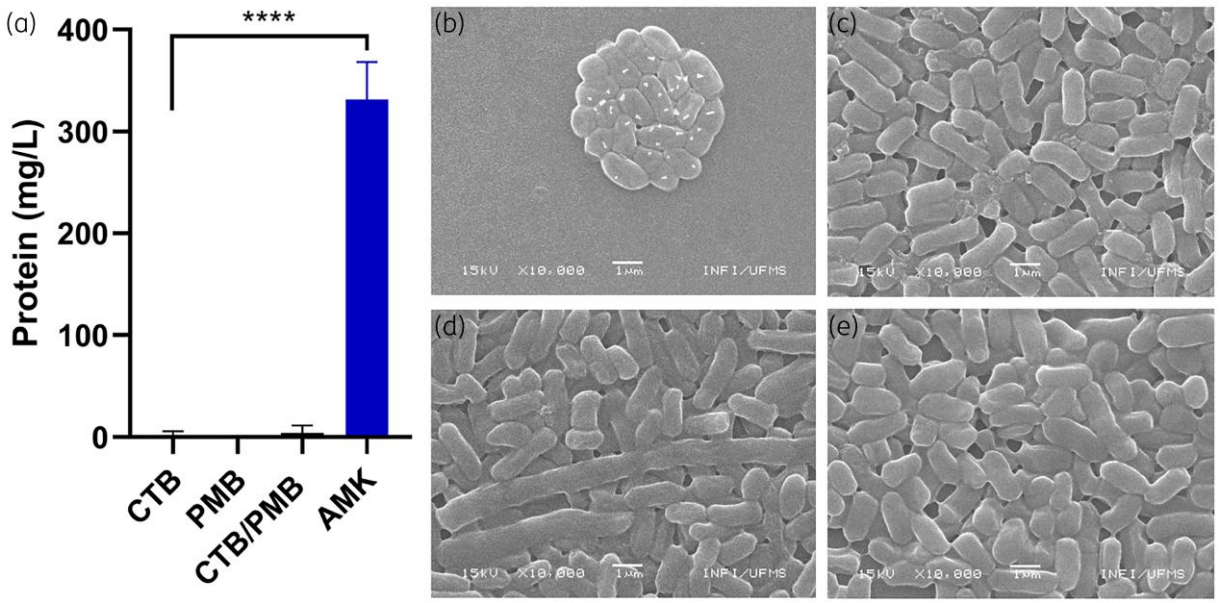

Figure 4. (a) Bacterial protein leakage after 4 h of treatment exposure. Amikacin (AMK; 64 mg/L) was used as positive control. One-way ANOVA was used for data analyses; significance was observed for $P < 0.0001$ (****). SEM images of: *K. pneumoniae* K18 treated with (b) polymyxin B (PMB; 2 mg/L) and ceftibuten (CTB; 4 mg/L) combination; (c) *K. pneumoniae* K18 treated with isolated PMB (2 mg/L); (d) *K. pneumoniae* treated with isolated CBT (4 mg/L); (e) bacterial control, PC-R *K. pneumoniae* K18 images. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

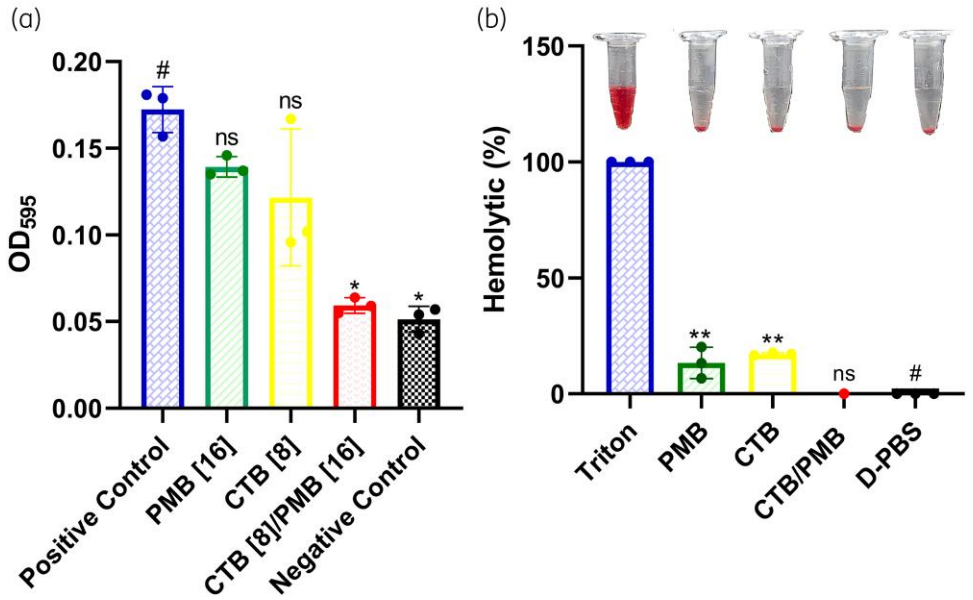

Figure 5. (a) Biofilm inhibition assay results performed with PC-R *K. pneumoniae* K18 for ceftibuten/polymyxin B combination at a concentration of 8 mg/L for ceftibuten and 16 mg/L for polymyxin B. (b) Haemolysis results. Percentage of haemolysed cells on exposure to the treatment combinations, compared with a negative control (D-PBS). Eppendorf tubes with the final haemolysis reaction are shown above the graph columns. Red colour solutions demonstrate a high HR. Coagulated blood demonstrates a low haemolysis rate. One-way ANOVA was applied to the data, and difference was considered significant for $P < 0.05$ (*) and $P < 0.01$ (**). ns, not significant. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

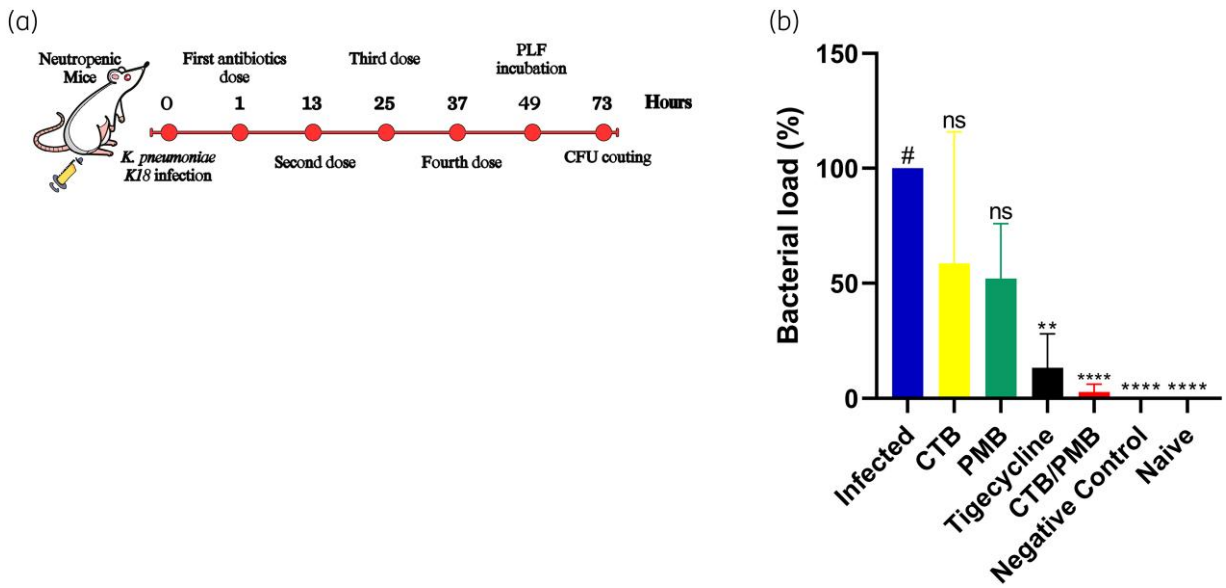

Figure 6. *In vivo* results. (a) Experimental timeline of neutropenic mice peritonitis/sepsis model. Treatment began at 1 h post-infection. In total, four doses were applied. After 2 days, PLFs were incubated for 24 h for colony counting. (b) Percentage of PLF bacterial load in each treatment group. Infected group was considered a positive control, representing 100% bacterial load. One-way ANOVA was used for data analyses; significance was observed for $P < 0.01$ (**) and $P < 0.0001$ (****). ns, not significant. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

driven by the presence of specific resistance genes. Therefore, further experiments are needed to gain a deeper understanding of the complex interactions between these factors.

Generally, polymyxins are compatible with various antibiotics.[29] Recently, scientists reported the synergism between colistin (polymyxin E) and meropenem, and between tigecycline and tazobactam against strains of *Acinetobacter baumannii* resistant to carbapenems.[30] Other studies also confirmed the presence of synergistic interactions among polymyxins and other compounds besides antibiotics.[7,31]

Polymyxins act by binding to LPS on the bacterial outer membrane, consequently causing membrane destabilization and affecting its permeability, which leads to bacterial cell death.[5] The ceftibuten/polymyxin B combination did not induce the release of proteins, suggesting that it did not lead to cell membrane rupture or alteration of membrane permeability. Third-generation cephalosporins, such as ceftibuten, inhibit microbial cell wall synthesis by binding to PBPs, affecting the cell division process.[32] Polymyxin B could contribute to this process by inducing the microbial cell wall to become rigid[33] and by penetrating the cell membrane to directly interfere with the cell division machinery.[34] However, further studies are required to confirm whether polymyxin B enhances the effect of ceftibuten on microbial cell division.

SEM images did not reveal any visible morphological changes in the bacteria treated with a ceftibuten/polymyxin B combination. However, a significant reduction in bacterial population was observed. This indicates that, despite the apparent morphological integrity, treatment with ceftibuten/polymyxin B might have induced other forms of stress or submicroscopic damage that are not detectable by SEM. The decrease in bacterial population could be due to factors such as alterations in cellular functionality, metabolic effects or damage affecting bacterial viability without altering external morphology. Further studies, including viability assays and tests of cellular integrity, may help elucidation of the underlying mechanisms responsible for the observed reduction in bacterial population.

The ceftibuten/polymyxin B combination also inhibited the formation of the PC-R *K. pneumoniae* biofilm when the dose used was higher than the MIC, making it a valuable tool for treating biofilm related ailments.[1,35] Biofilms represent a form of microbial resistance, where microorganisms develop an extracellular polymer matrix to unite and adhere to surfaces.[36] Microorganisms that exhibit resistance in their planktonic forms often show even greater antibiotic resistance in their biofilm-associated forms.[37] Infections associated with biofilms, such as chronic rhinosinusitis and chronic wounds, are particularly challenging to treat, often requiring prolonged therapy that can significantly impact patient wellbeing.[38,39] Consequently, new therapeutic approaches need to be developed to prevent the spread of MDR bacteria biofilm.

The results of haemolytic assays showed that ceftibuten/polymyxin B was haemocompatible, considering that the differences in HR between the combination and D-PBS (the negative control) were not significant. The HR for the combination treatment was 0%, which was within the acceptable maximum limit of 5%.[40] Given the known toxicity of polymyxin B,[26] its combination with ceftibuten offers a promising alternative for safer and more effective management of nosocomial infections caused by PC-R *K. pneumoniae*.

The *in vivo* results showed that ceftibuten/polymyxin B significantly decreased the bacterial load in model mice, and the bacterial load for the combination treatment decreased by 95% (on average), compared with the bacterial load recorded in the infected control group. Plus, ceftibuten/polymyxin B results were similar to tigecycline, a control antibiotic, which postulates the combination as a treatment alternative. Wang *et al.*[16] found similar results by administering oxacillin with nisin; their association significantly reduced the bacterial load on skin wound infections caused by MRSA.

In conclusion, the ceftibuten/polymyxin B combination could overcome polymyxin resistance *in vitro* and *in vivo* in a PC-R *K. pneumoniae* infection model, plus it holds the potential of bacterial biofilm formation inhibition *in vitro*. These findings provide new insights into the effectiveness of ceftibuten/polymyxin B treatment. Additionally, the safety profiles suggest that the combination is suitable for clinical applications. These results confirm that a ceftibuten/polymyxin B combination is a promising therapeutic strategy and may be further investigated in clinical trials for the development of new methods for treating PC-R *K. pneumoniae*.

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Transparency declarations

No conflicts of interest to declare.

Author contributions

Conceptualization, investigation: M.C.S., G.H.A.S., N.S.D.; formal analysis: N.M.L.F., M.G.S.A., K.L.C.M.; animal experimentation: A.A.M., T.L.F.; writing—original draft preparation; M.C.S., O.N.S., T.M.A., L.R.; methodology: S.B. and O.A.D.: writing—review & editing, supervision, project administration, funding acquisition: S.S.

Data availability

All data are available from the corresponding authors upon reasonable request.

References

1 Li Z, Cao Y, Yi L *et al.* Emergent polymyxin resistance: end of an era? *Open Forum Infect Dis* 2019; **6**: ofz368. https://doi.org/10.1093/ofid/ofz368

2 Roca I, Akova M, Baquero F *et al.* The global threat of antimicrobial resistance: science for intervention. *New Microbes New Infect* 2015; **6**: 22–9. https://doi.org/10.1016/j.nmni.2015.02.007

3 Tanwar J, Das S, Fatima Z *et al.* Multidrug resistance: an emerging crisis. *Interdiscip Perspect Infect Dis* 2014; **2014**: 541340. https://doi.org/10.1155/2014/541340

4 Bialvaei AZ, Samadi Kafil H. Colistin, mechanisms and prevalence of resistance. *Curr Med Res Opin* 2015; **31**: 707–21. https://doi.org/10.1185/03007995.2015.1018989

5 Olaitan AO, Morand S, Rolain J-M. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014; **5**: 643. https://doi.org/10.3389/fmicb.2014.00643

6 Giamarellou H. Epidemiology of infections caused by polymyxin-resistant pathogens. *Int J Antimicrob Agents* 2016; **48**: 614–21. https://doi.org/10.1016/j.ijantimicag.2016.09.025

7 Miró-Canturri A, Ayerbe-Algaba R, Villodres ÁR *et al*. Repositioning rafoxanide to treat Gram-negative bacilli infections. *J Antimicrob Chemother* 2020; **75**: 1895–905. https://doi.org/10.1093/jac/dkaa103

8 Coates ARM, Hu Y, Holt J *et al*. Antibiotic combination therapy against resistant bacterial infections: synergy, rejuvenation and resistance reduction. *Expert Rev Anti Infect Ther* 2020; **18**: 5–15. https://doi.org/10.1080/14787210.2020.1705155

9 Freire-Moran L, Aronsson B, Manz C *et al*. Critical shortage of new antibiotics in development against multidrug-resistant bacteria—time to react is now. *Drug Resist Updat* 2011; **14**: 118–24. https://doi.org/10.1016/j.drup.2011.02.003

10 Hanci H, Igan H. Antimicrobial synergistic effects of apigenin, (-)-epigallocatechin-3-gallate, myricetin and luteolin in combination with some antibiotics. *Ann Agric Environ Med* 2023; **30**: 61–4. https://doi.org/10.26444/aaem/161220

11 da Silva KE, Thi Nguyen TN, Boinett CJ *et al*. Molecular and epidemiological surveillance of polymyxin-resistant *Klebsiella pneumoniae* strains isolated from Brazil with multiple *mgrB* gene mutations. *Int J Med Microbiol* 2020; **310**: 151448. https://doi.org/10.1016/j.ijmm.2020.151448

12 Humphries RM, Ambler J, Mitchell SL *et al*. CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. *J Clin Microbiol* 2018; **56**: e01934-17. https://doi.org/10.1128/JCM.01934-17

13 Zhang W, Guo Y, Li J *et al*. In vitro and in vivo bactericidal activity of ceftazidime-avibactam against carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Resist Infect Control* 2018; **7**: 142. https://doi.org/10.1186/s13756-018-0435-9

14 Song M, Liu Y, Huang X *et al*. A broad-spectrum antibiotic adjuvant reverses multidrug-resistant Gram-negative pathogens. *Nat Microbiol* 2020; **5**: 1040–50. https://doi.org/10.1038/s41564-020-0723-z

15 Chaiben V, Yamada CH, Telles JP *et al*. A carbapenem-resistant *Acinetobacter baumannii* outbreak associated with a polymyxin shortage during the COVID pandemic: an *in vitro* and biofilm analysis of synergy between meropenem, gentamicin and sulbactam. *J Antimicrob Chemother* 2022; **77**: 1676–84. https://doi.org/10.1093/jac/dkac102

16 Wang J, Ma X, Li J *et al*. The synergistic antimicrobial effect and mechanism of nisin and oxacillin against methicillin-resistant *Staphylococcus aureus*. *Int J Mol Sci* 2023; **24**: 6697. https://doi.org/10.3390/ijms24076697

17 Wu Y, Yu W, Chu X *et al*. Effect of ceftazidime-avibactam combined with different antimicrobials against carbapenem-resistant *Klebsiella pneumoniae*. *Microbiol Spectr* 2024; **12**: e0010724. https://doi.org/10.1128/spectrum.00107-24

18 Ribeiro SM, de la Fuente-Núñez C, Baquir B *et al*. Antibiofilm peptides increase the susceptibility of carbapenemase-producing *Klebsiella pneumoniae* clinical isolates to β-lactam antibiotics. *Antimicrob Agents Chemother* 2015; **59**: 3906–12. https://doi.org/10.1128/AAC.00092-15

19 Rossato L, Arantes JP, Ribeiro SM *et al*. Antibacterial activity of gallium nitrate against polymyxin-resistant *Klebsiella pneumoniae* strains. *Diagn Microbiol Infect Dis* 2022; **102**: 115569. https://doi.org/10.1016/j.diagmicrobio.2021.115569

20 Vasconcelos NG, Queiroz JHFS, da Silva KE *et al*. Synergistic effects of *Cinnamomum cassia* L. essential oil in combination with polymyxin B against carbapenemase-producing *Klebsiella pneumoniae* and *Serratia marcescens*. *PLoS One* 2020; **15**: e0236505. https://doi.org/10.1371/journal.pone.0236505

21 Dias LP, Souza PFN, Oliveira JTA *et al*. RcAlb-PepII, a synthetic small peptide bioinspired in the 2S albumin from the seed cake of *Ricinus communis*, is a potent antimicrobial agent against *Klebsiella pneumoniae* and *Candida parapsilosis*. *Biochim Biophys Acta Biomembr* 2020; **1862**: 183092. https://doi.org/10.1016/j.bbamem.2019.183092

22 Yang Z, Song M, Li X *et al*. Synergy of outer membrane disruptor SLAP-S25 with hydrophobic antibiotics against Gram-negative pathogens. *J Antimicrob Chemother* 2023; **78**: 263–71. https://doi.org/10.1093/jac/dkac387

23 Sæbø IP, Bjørås M, Franzyk H *et al*. Optimization of the hemolysis assay for the assessment of cytotoxicity. *Int J Mol Sci* 2023; **24**: 2914. https://doi.org/10.3390/ijms24032914

24 de Almeida de Souza GH, Dos Santos Radai JA, Mattos Vaz MS *et al*. *In vitro* and *in vivo* antibacterial activity assays of carvacrol: a candidate for development of innovative treatments against KPC-producing *Klebsiella pneumoniae*. *PLoS One* 2021; **16**: e0246003. https://doi.org/10.1371/journal.pone.0246003

25 Lasko MJ, Asempa TE, Nicolau DP. Pharmacodynamics of ceftibuten: an assessment of an oral cephalosporin against Enterobacterales in a neutropenic murine thigh model. *Antibiotics (Basel)* 2021; **10**: 201. https://doi.org/10.3390/antibiotics10020201

26 Zhang X, Guo F, Shao H *et al*. Clinical translation of polymyxin-based combination therapy: facts, challenges and future opportunities. *J Infect* 2017; **74**: 118–30. https://doi.org/10.1016/j.jinf.2016.11.015

27 de Souza GH, Vaz MS, Dos Santos Radai JA *et al*. Synergistic interaction of polymyxin B with carvacrol: antimicrobial strategy against polymyxin-resistant *Klebsiella pneumoniae*. *Future Microbiol* 2024; **19**: 181–93. https://doi.org/10.2217/fmb-2023-0070

28 da Costa Júnior SD, de Almeida Campos LA, Cavalcanti IDL *et al*. Dissemination and prevalence of polymyxin-resistant bacteria of clinical and environmental origin. *Res Soc Dev* 2022; **11**: e94111334996. https://doi.org/10.33448/rsd-v11i13.34996

29 Scudeller L, Righi E, Chiamenti M *et al*. Systematic review and meta-analysis of in vitro efficacy of antibiotic combination therapy against carbapenem-resistant Gram-negative bacilli. *Int J Antimicrob Agents* 2021; **57**: 106344. https://doi.org/10.1016/j.ijantimicag.2021.106344

30 Ju YG, Lee HJ, Yim HS *et al*. In vitro synergistic antimicrobial activity of a combination of meropenem, colistin, tigecycline, rifampin, and ceftolozane/tazobactam against carbapenem-resistant *Acinetobacter baumannii*. *Sci Rep* 2022; **12**: 7541. https://doi.org/10.1038/s41598-022-11464-6

31 Amaral SC, Pruski BB, de Freitas SB *et al*. *Origanum vulgare* essential oil: antibacterial activities and synergistic effect with polymyxin B against multidrug-resistant *Acinetobacter baumannii*. *Mol Biol Rep* 2020; **47**: 9615–25. https://doi.org/10.1007/s11033-020-05989-0

32 El-Shaboury SR, Saleh GA, Mohamed FA *et al*. Analysis of cephalosporin antibiotics. *J Pharm Biomed Anal* 2007; **45**: 1–19. https://doi.org/10.1016/j.jpba.2007.06.002

33 Mortensen NP, Fowlkes JD, Sullivan CJ *et al*. Effects of colistin on surface ultrastructure and nanomechanics of *Pseudomonas aeruginosa* cells. *Langmuir* 2009; **25**: 3728–33. https://doi.org/10.1021/la803898g

34 Trimble MJ, Mlynárčik P, Kolář M *et al*. Polymyxin: alternative mechanisms of action and resistance. *Cold Spring Harb Perspect Med* 2016; **6**: a025288. https://doi.org/10.1101/cshperspect.a025288

35 Ramya S, Shanmugasundaram T, Balagurunathan R. Biomedical potential of actinobacterially synthesized selenium nanoparticles with special reference to anti-biofilm, anti-oxidant, wound healing, cytotoxic and anti-viral activities. *J Trace Elem Med Biol* 2015; **32**: 30–9. https://doi.org/10.1016/j.jtemb.2015.05.005

36 Sauer K, Stoodley P, Goeres DM *et al*. The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nat Rev Microbiol* 2022; **20**: 608–20. https://doi.org/10.1038/s41579-022-00767-0

37 Borowski RGV, Barros MP, da Silva DB *et al*. Red pepper peptide coatings control *Staphylococcus epidermidis* adhesion and biofilm formation. *Int J Pharm* 2020; **574**: 118872. https://doi.org/10.1016/j.ijpharm.2019.118872

38 Huang Y, Qin F, Li S *et al*. The mechanisms of biofilm antibiotic resistance in chronic rhinosinusitis: a review. *Medicine (Baltimore)* 2022; **101**: e32168. https://doi.org/10.1097/MD.0000000000032168

39 Lu R, Luo Z, Zhang Y *et al*. A multifunctional tissue-engineering hydrogel aimed to regulate bacterial ferroptosis-like death and overcoming infection toward bone remodeling. *Adv Sci (Weinh)* 2024; **11**: e2309820. https://doi.org/10.1002/advs.202309820

40 Gao W, Han X, Sun D *et al*. Antibacterial properties of antimicrobial peptide HHC36 modified polyetheretherketone. *Front Microbiol* 2023; **14**: 1103956. https://doi.org/10.3389/fmicb.2023.1103956

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Synergistic antimicrobial combination of third-generation cephalosporins and polymyxin B against carbapenem-polymyxin-resistant *Klebsiella pneumoniae:* an *in vitro* and *in vivo* analysis

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ABSTRACT Antibiotic combination therapy is a promising approach to address the urgent need for novel treatment options for infections caused by carbapenem-polymyxin-resistant *Klebsiella pneumoniae* (CPR-Kp). The present study aimed to investigate the synergistic potential of four cephalosporins in combination with polymyxin B (PMB). A checkerboard assay was performed to evaluate the synergistic effects of cephalexin (CLX), cefixime, cefotaxime (CTX), and cefmenoxime (CMX) in combination with PMB. Subsequently, experiments evaluating the use of CTX or CMX in combination with PMB (CTX-PMB or CMX-PMB, respectively), including growth curve and SynergyFinder analysis, antibiofilm activity assays, cell membrane integrity assays, and scanning electron microscopy, were performed. Safety assessments were also conducted, including hemolysis and toxicity evaluations, using *Caenorhabditis elegans*. Furthermore, an *in vivo* model in *C. elegans* was adopted to assess the treatment efficacy against CPR-Kp infections. CTX-PMB and CMX-PMB exhibited low fractional inhibitory concentration indexes ranging from 0.19 to 0.50 and from 0.25 to 1.5, respectively, and zero interaction potency scores of 37.484 and 15.076, respectively. The two combinations significantly reduced growth and biofilm formation in CPR-Kp. Neither CTX-PMB nor CMX-PMB compromised bacterial cell integrity. Safety assessments revealed a low hemolysis percentage and high survival rates in the *C. elegans* toxicity evaluations. The *in vivo* model revealed that the CTX-PMB and CMX-PMB treatments improved the survival rates of *C. elegans*. The synergistic effects of the CTX-PMB and CMX-PMB combinations, both *in vitro* and *in vivo*, indicate that these antibiotic pairings could represent effective therapeutic options for infections caused by CPR-Kp.

KEYWORDS cephalosporins, combination therapy, antimicrobial resistance, *Klebsiella pneumoniae*, *Caenorhabditis elegans*

Multidrug-resistant (MDR) strains of *Klebsiella pneumoniae* seriously threaten public health because they are associated with high morbidity and mortality rates (1, 2). This pathogen is particularly challenging due to its ability to develop resistance to multiple classes of antibiotics, including third-generation cephalosporins. The resistance of *K. pneumoniae* to cephalosporins is mediated by several mechanisms, such as the production of extended-spectrum β-lactamases (ESBLs) and carbapenemases, which complicate treatment regimens (3).

With the increasing failure rates of traditional antibiotics, polymyxin B (PMB) has re-emerged as a therapeutic option. Originally sidelined due to its nephrotoxic and

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neurotoxic side effects, polymyxin B is now widely used as a last-resort antibiotic in Brazil for the treatment of infections caused by MDR bacteria (4, 5). However, resistance to polymyxin B is also increasing, and several mechanisms are reportedly involved, such as modifications in the lipopolysaccharide (LPS) layer, mutations in the *mgr*B gene, and the presence of *mcr*-1 (6).

The rise of carbapenem-polymyxin-resistant strains of *K. pneumoniae* (CPR-Kp) is particularly concerning. The therapeutic options available for infections caused by these strains are limited, which leads to increased treatment failure rates and prolonged hospitalization. Moreover, the economic cost of treating these infections is substantially higher due to the need for more aggressive and prolonged therapies as well as the occurrence of complications related to antimicrobial resistance (7).

Therefore, the development of novel treatment strategies is imperative to effectively combat infections caused by MDR bacteria. In this regard, the use of a combination of existing antibiotics has emerged as a promising approach. Such combinations are considered valuable due to their ability to overcome microbial resistance, broaden the antibacterial spectrum of the treatment, and reduce the required drug dosages (8, 9). In this context, the present study aimed to evaluate the synergistic interactions of different cephalosporins with polymyxin B and the ability of each combination to overcome resistance via CPR-Kp.

MATERIALS AND METHODS

Chemicals

Cefmenoxime (CMX; lote T1190) was purchased from Start Bioscience. Cephalexin (CLX; Lot LRAC0286), cefixime (CFX; Lot SPBB4723), cefotaxime (CTX; Lot 0000146939), and polymyxin B solution (Lot BCCG2613) were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals used in the experiments were prepared as instructed by the respective manufacturers.

Bacterial strains and antibacterial test

The six bacterial CPR-Kp strains were isolated from patients admitted to a tertiary hospital in Dourados, Mato Grosso do Sul, Midwest, Brazil, as previously described (10). Species identification was performed using the automated Vitek2 system (bioMerieux, Hazelwood, MO), followed by validation of the identified species through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a Microflex LT spectrometer (Bruker Daltonics, Massachusetts, USA) (10). The minimum inhibitory concentrations (MICs) for the combinations of cephalexin, cefixime, cefotaxime, and cefmenoxime with polymyxin B were determined using the broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (11).

Antimicrobial synergy testing

The synergism between cephalosporins (CLX, CFX, CTX, CMX) with PMB was evaluated using checkerboard assay (12). Briefly, the antibiotic combination was cross-diluted in the wells of a 96-well microplate using brain heart infusion (BHI) broth. PMB was diluted vertically, with concentrations ranging from 1 to 64 mg/L. On the other hand, cephalosporins were diluted horizontally, with concentrations ranging from 0.065 to 64 mg/L. The CPR-Kp inoculum (1.5×10^6 CFU/mL) was then added to the wells, after which the plates were incubated at 37°C for 24 h. The results were interpreted according to the fractional inhibitory concentration index (FICI), as stated below:

$$\mathrm{FICI} = (\mathrm{FIC}\ A + \mathrm{FIC}\ B),$$

where:

$$\text{FIC } A = \frac{\text{MIC of drug } A \text{ in combination}}{\text{MIC of isolated drug } A},$$
$$\text{FIC } B = \frac{\text{MIC of drug } B \text{ in combination}}{\text{MIC of isolated drug } B}.$$

Results are interpreted as follows: synergistic interaction (FICI ≤0.5), no interaction (0.5 < FICI ≤ 4), and antagonistic interaction (FICI >4) (13). The checkerboard results were analyzed using the zero interaction potency (ZIP) model for synergy by employing free and open-source *synergyfinder* software (https://synergyfinder.fimm.fi). The ZIP scores of the antibiotic combinations were interpreted as follows: synergism (>10), additive (<10 and >−10), and antagonistic (<−10) (14).

Simultaneously, growth curves were generated for all combinations using spectrophotometry. The checkerboard plates were analyzed at 0, 2, 4, 6, 8, 12, and 24 h after inoculation with the CPR-Kp strains. The optical density (OD) was measured at a wavelength of 595 nm using an iMark Microplate Absorbance Reader (15).

Biofilm formation inhibition

The ability of each cephalosporin and polymyxin combination to prevent biofilm formation was quantified using crystal violet staining (16). Checkerboard plates were maintained under stable conditions at 37°C for 24 h to allow for bacterial development and biofilm maturation. Subsequently, the planktonic cells were removed, and the biofilms formed were stained with 0.1% crystal violet. A positive control comprising CPR-Kp and a negative control comprising BHI broth were also included in the experiments to confirm sterility. Biofilm biomass was quantified by measuring the OD at 595 nm.

Cell membrane integrity

Modulation of cell membrane permeability was assessed 14). Each combination at a concentration of 1 × MIC and the isolated antibiotics were inserted into the wells of a microplate, followed by the addition of the CPR-Kp strain. The microplate was then incubated at 37°C for 4 h. Next, the contents of each well in the microplate were centrifuged at 2,500 rpm for 5 min at 4°C. The resulting supernatant was then evaluated for the quantity of protein released from the cytoplasm using the Pierce BCA Protein Assay Kit (Thermo Scientific, MA, USA), followed by OD measurement at 595 nm using the iMark Microplate Absorbance Reader (Bio-Rad, São Paulo, Brazil).

Scanning electron microscopy

The influence of each treatment on the cellular structure of CPR-Kp was determined through scanning electron microscopy (SEM) (17). CPR-Kp cells were first treated with 8 mg/L CTX and 4 mg/L PMB (isolated or associated) or 8 mg/L CMX and 8 mg/L PMB (isolated or associated), followed by fixation in a 2.5% glutaraldehyde solution. Each sample was then dehydrated in a gradient of ethanol [30%, 50%, 70%, and 100% (vol/vol)] for 10 min at each concentration. Finally, 20 µL of the final product was placed on a glass coverslip (0.8 × 0.8 cm) using a micropipette. After drying, the coverslips containing the cells were sputtered with gold and examined under an SEM (JSM-6380LV, JEOL, USA).

Hemolysis assay

Hemolysis assays were conducted as described in a previous study (18), with slight adaptations. In brief, 100 µL of freshly collected mouse blood [the study was approved by the Research Ethics Committee on Animal Use of the Universidade Federal da Grande Dourados (no. 23018)] was mixed with 100 µL of the antibiotic (8 mg/L CTX in combination with 4 mg/L PMB, 8 mg/L CMX in combination with 8 mg/L PMB, 64 mg/L CTX, 64 mg/L CMX, and 64 mg/L PMB), followed by 4 h of incubation. Subsequently, the samples were centrifuged at 2,500 rpm for 5 min, followed by extracting aliquots from

the resulting supernatants and subjecting them to $OD_{595\ nm}$ measurements utilizing the iMark Microplate Absorbance Reader. Triton (0.1%, vol/vol) and Dulbecco's phosphate-buffered saline (D-PBS) were used as positive and negative controls, respectively. The hemolytic rate (HR) was calculated using the formula provided below:

$$\mathrm{HR}(\%) = (OD_{\mathrm{treatment}} - OD_{D-PBS}/OD_{\mathrm{Triton}} - OD_{D-PBS}) \times 100.$$

Safety in *C. elegans* model

Next, the wild-type *C. elegans* nematode (N2) was exposed to the antibiotic combinations to evaluate their safety. The *C. elegans* strain was propagated using a nematode growth medium (NGM) supplemented with *Escherichia coli* OP50 as the food source. The worms were then age synchronized through bleaching using alkaline hypochlorite and sodium hydroxide. The released embryos were then placed on NGM plates at 16°C until the young adult stage (L4 phase) was reached. At this point, 15–30 worms were transferred to 24-well plates containing M9 liquid medium along with CTX 8 mg/L, PMB 4 mg/L (isolated or combined) or CMX 8 mg/L, PMB 8 mg/L (isolated or combined). Tigecycline 16 mg/L was used as the reference antibiotic for comparison. The numbers of viable and dead nematodes were determined every 24 h during the 6 days of incubation at 16°C. The worms were classified as dead when no spontaneous movement or response upon stimulation with a platinum loop was detected (19).

C. elegans in vivo assay

An *in vivo* infection model was established in the *C. elegans* AU37 (glp-4; sek-1) strain, which was selected for its susceptibility to pathogens due to the sek-1 mutation, which rendered the worms immunocompromised for infection, as described in previous studies (20, 21). The *C. elegans* strain was propagated and age synchronized to the young adult stage (L4 phase), followed by exposing the nematodes to the CPR-Kp K1 (the strain with greater virulence) strain (1.5×10^8 CFU/mL) for 3 h. After washing in M9 buffer, 15–30 worms were transferred to 24-well plates containing M9 liquid medium along with a cephalosporin (CTX or CMX), PMB, or their combinations. The MIC concentrations used in the checkerboard assay were also used in this study. Uninfected nematodes and infected and untreated nematodes were used as negative and positive controls, respectively. Tigecycline at 16 mg/L was used as the reference antibiotic for comparison. The numbers of viable and dead nematodes were determined every 24 h during the 7 days of incubation at 16°C. The worms were classified as dead when no spontaneous movement or response upon stimulation with a platinum loop was detected.

Statistical analyses

Each experiment was conducted in triplicate. One-way ANOVA was performed for the statistical analysis of the resulting data. Differences were considered significant at $P < 0.5$. The survival of *C. elegans* was determined based on Kaplan-Meier survival curves and a log-rank test. All analyses and graph plotting were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

The bacterial strains used in the present study exhibited sequence types (ST) 11 and 345, belonged to clonal complex 258 (CC258), the most important CC associated with KPC production. All strains presented $bla_{\mathrm{KPC-2}}$ gene (responsible for generating KPC-2 enzyme, which provides bacterial resistance to carbapenems) and ESBL genes, such as $bla_{\mathrm{CTX-M-15}}$, $bla_{\mathrm{TEM-1}}$, and $bla_{\mathrm{SHV-11}}$, providing resistance to β-lactams such as cephalosporins. Whole-genome sequencing revealed that isolates presented alteration of *mgrB* gene, which is associated with polymyxin resistance (Table 1). The strains showed sensitivity only to tigecycline and amikacin (10).

TABLE 1 Carbapenem-polymyxin-resistant *Klebsiella pneumoniae* strain features

Strain/strain ID	ST	Carbapenemase	*mgrB* status
K1/27588809	11	KPC-2	*mgrB* repeated sequence at nt 89 + 2 frameshift
K3/28030301	11	KPC-2	Insertional inactivation, IS*Ecp1* at nt 124 (FW)
K4/28030501	345	KPC-2	Insertional inactivation, IS*Kpn13* at nt 125 (FW)
K15/32089801	11	KPC-2	Insertional inactivation, IS*5*-like element at nt 89 (RW)
K18/34923502	11	KPC-2	*mgrB* repeated sequence at nt 89 + 2 frameshift
K28/1316	11	KPC-2	Insertional inactivation, IS903 at nt 89 (FW)

[a]Abbreviations: FW: Forward; RW: Reverse.

To identify the most potential antibiotic combinations and evaluate their synergistic effects, four cephalosporins were initially screened for their effects when used in combination with PMB. In this screening step, CLF and CFX exhibited no synergistic interactions with polymyxin B against CPR-Kp and were therefore not investigated further. CTX and CMX, on the other hand, exhibited synergistic effects with PMB, as evidenced by a decrease in the MICs by more than fourfold for almost all the evaluated strains (Table 2).

The MICs of isolated CTX ranged from 1 to 64 mg/L, while the MICs of isolated PMB ranged from 32 to 64 mg/L. In combination, CTX presented MICs ranging from 0.065 to 32 mg/L, with a notable concentration reduction (e.g., an eightfold reduction was observed against the K18 strain). In combination, PMB presented MICs ranging from 2 to 16 mg/L, accounting for a 32-fold decrease. The FICI of CTX-PMB ranged from 0.19 to 0.50, indicating synergism.

Subsequently, the synergistic potential of the CMX-PMB combination was assessed. The MICs of isolated CMX ranged from 0.25 to 64 mg/L, while the MICs of isolated PMB ranged from 32 to 64 mg/L in these assessments, consistent with previous observations. In combination, CMX presented MICs ranging from 0.065 to 32 µg/mL, accounting for a significant decrease of eightfold. Similarly, PMB in combination presented MICs in the range of 0.065 to 64 µg/mL, accounting for a decrease of 16-fold. The FICI for the CMX-PMB combinations ranged from 0.19 to 1.5. The above values, while being greater than those observed for the CTX-PMB combination, indicated synergism for most of the evaluated strains.

Microbial growth curves corroborated the synergistic effects of the evaluated combinations of cephalosporins with PMB. All combinations generated a growth curve for all strains, including the negative control, over a 24-h period. Notably, while the drugs

TABLE 2 The results of antimicrobial and checkerboard experiments conducted to evaluate the effects of different combinations of cephalosporins and polymyxin B on CPR-Kp strains

		MIC (mg/L)					
		Alone		Associated			
Cephalosporin	Bacterial strain	Cph	PMB	Cph	PMB	FICI	Interaction
Cephalexin	K1	64	32	64	32	2	Indifferent
Cefixime	K1	64	32	64	32	2	Indifferent
Cefotaxime	K1	64	32	16	4	0.37	**Synergic**
	K3	64	32	16	4	0.37	**Synergic**
	K4	1	64	0.065	16	0.31	**Synergic**
	K15	64	64	32	2	0.5	**Synergic**
	K18	64	64	8	4	0.19	**Synergic**
	K28	2	64	1	2	0.53	Indifferent
Cefmenoxime	K1	64	32	16	0.065	0.5	**Synergic**
	K3	64	32	16	0.065	0.5	**Synergic**
	K4	0.25	64	0.065	16	0.5	**Synergic**
	K15	64	64	32	64	1.5	Indifferent
	K18	64	64	8	8	0.25	**Synergic**
	K28	8	32	1	2	0.19	**Synergic**

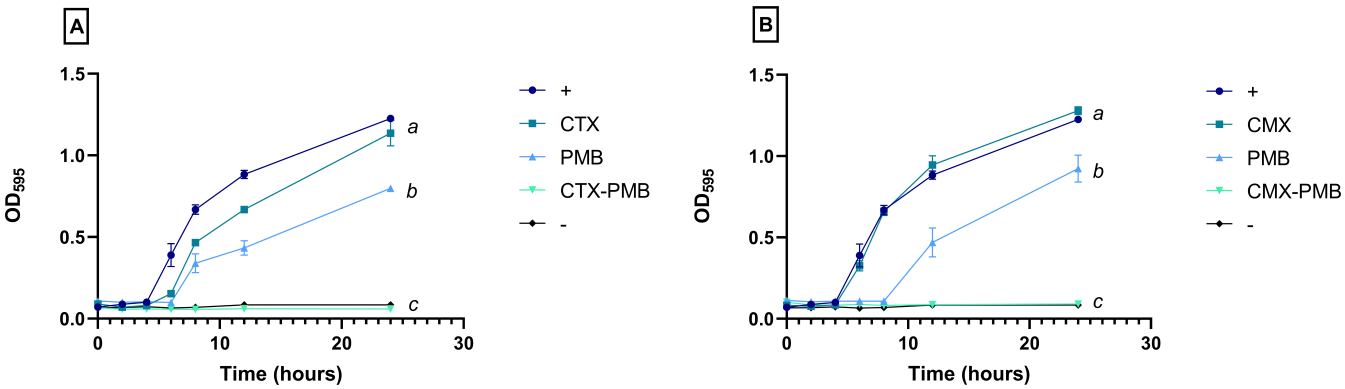

FIG 1 Growth curves generated for CPR-Kp K18 treated with (A) the CTX-PMB combination and the isolated antibiotics at a 1 × MIC; (B) the CMX-PMB combinations and the isolated antibiotics at a 1 × MIC. Positive (+) and negative (–) controls were also included in the experiments. One-way ANOVA was performed, and the differences were considered significant at $P < 0.5$. Significant differences are indicated using different letters (*a-b; b-c; a-c*), while nonsignificant differences are indicated using the same letter (*a-a; b-b; c-c*).

(CTX, CMX, and PMB) failed to inhibit microbial growth effectively when used in isolation, the administration of the same drugs in combination resulted in significant inhibition (Fig. 1).

At lower concentrations (0, 0.25, and 0.5 × MIC), the antibiotic combinations did not significantly reduce the growth of CPR-Kp. However, at concentrations above 1 × MIC, the same combinations led to high growth inhibition (Fig. 2A and B). In contrast, the CTX-PMB and CMX-PMB combinations exhibited potent activity against CPR-Kp. In addition, CTX-PMB inhibited microbial growth at multiple concentrations of the combination, yielding an FICI of 0.19 (Fig. 2C). The CMX-PMB combination also inhibited microbial growth, although at higher concentrations, with an FICI of 0.25.

To further validate the above findings, SynergyFinder analyses were conducted. The CTX-PMB combination achieved a ZIP score of 37.484, indicating synergism. The combination of CTX (4–16 mg/L) and PMB (4–16 mg/L) resulted in inhibition rates exceeding 80% (Fig. 3A and B). Moreover, the CMX-PMB combination led to a lower ZIP score of 15.076, although this could also be classified as synergistic. The combinations of CMX (8–32 mg/L) with PMB (8–32 mg/L) led to high inhibition rates (Fig. 3C and 3D).

The study of the antibiofilm potential of the evaluated antibiotic combinations revealed that the combinations were ineffective for biofilm prevention/inhibition at lower concentrations, while significant activity was noted at higher concentrations. Specifically, the combination of 16 mg/L CTX and 16 mg/L PMB led to significantly decreased biofilm formation, with an OD of 0.05, corresponding to 60.39% inhibition. The combination of 0.065 mg/L CMX with 64 mg/L PMB led to an OD of 0.042, indicating 59.42% inhibition. In contrast, the isolated drugs could not combat biofilm formation, with 16 mg/L CTX resulting in an OD of only 0.102, indicating only 32% inhibition, while 0.065 mg/L CMX led to an OD of 0.106, accounting for 19% inhibition of biofilm development. PMB at concentrations of 16 and 64 mg/L presented similar results, with corresponding OD values of 0.129 and 0.126, respectively, indicating quite low inhibition rates (<1%) (Fig. 4).

Furthermore, the experiments conducted to determine the impact of the antibiotic combinations on bacterial cell integrity revealed that the evaluated combinations did not damage the cell membrane of *K. pneumoniae* nor did the combinations promote intracellular protein release. Scanning electron microscopy revealed that the CPR-Kp cells treated with the CTX-PMB and CMX-PMB combinations at 1 × MIC maintained intact structures. In addition, the SEM images revealed a decrease in bacterial load for the combination treatments compared to that noted for the isolated antibiotics, highlighting the effectiveness of using antibiotic combinations in reducing bacterial presence. The

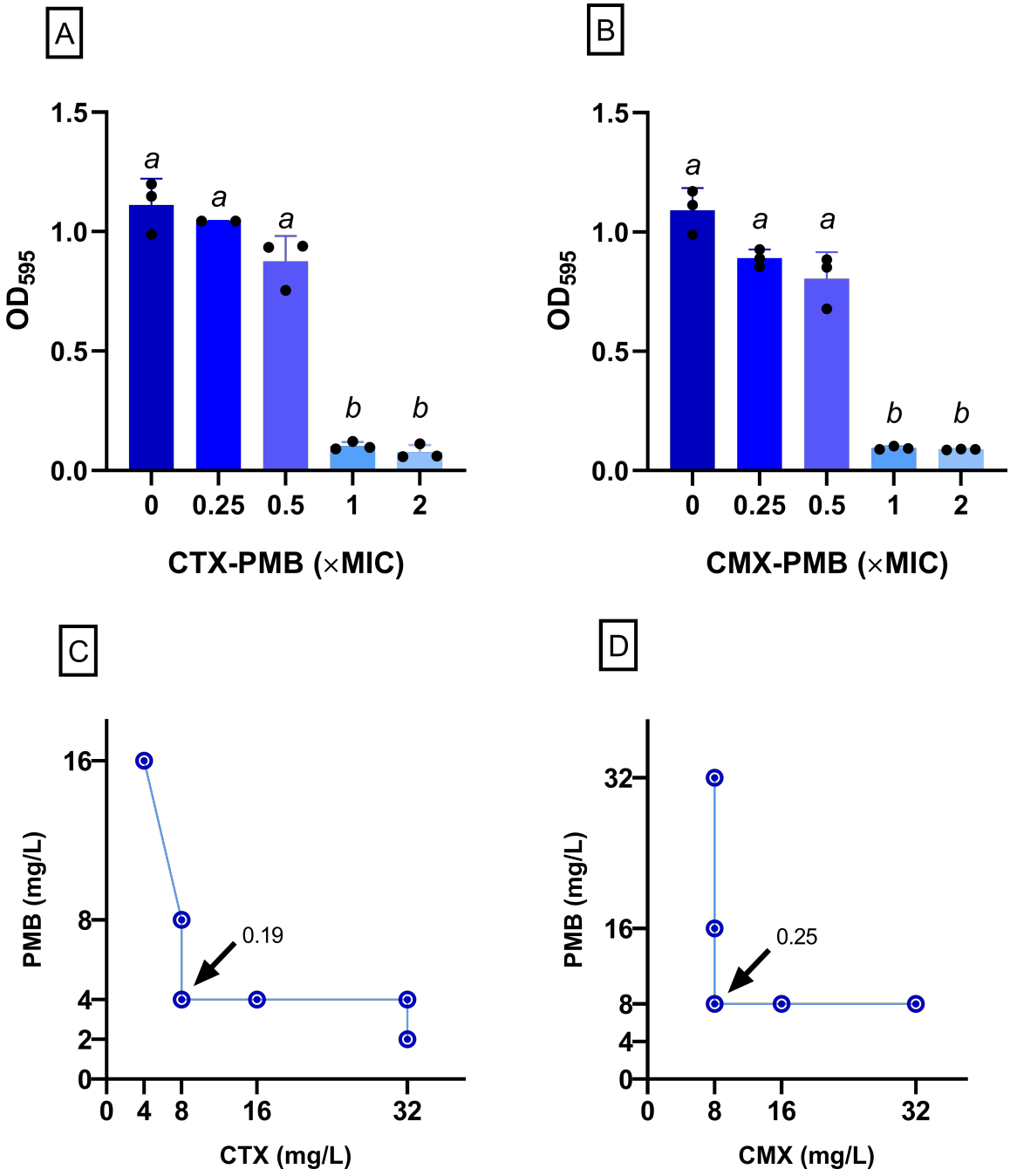

FIG 2 (A) OD of the CTX-PMB combination at 0, 0.25, 0.5, 1, and 2 × MIC against CPR-Kp K18 and (B) OD of the CMX-PMB combination at 0, 0.25, 0.5, 1, and 2 × MIC CPR-Kp K18. The OD is proportional to microbial growth. One-way ANOVA was performed, and the differences were considered significant at $P <$ 0.5. Significant differences are indicated using different letters (*a-b; b-c; a-c*), while nonsignificant differences are indicated using the same letter (*a-a; b-b; c-c*). (C) Multiple concentration combinations of CTX-PMB that inhibited CPR-Kp K18 growth, with the lowest concentration indicated using FICI (0.19). (D) Multiple concentration combinations of CMX-PMB that inhibited CPR-Kp K18 growth, with the lowest concentration indicated using FICI (0.25).

control images confirmed these findings, displaying similar levels of bacterial growth (Fig. 5).

Next, the safety of the antibiotic combinations was assessed using a hemolysis assay. No statistically significant differences in hemolytic activity were noted between each of the combinations and the negative control, indicating that the combinations were relatively safe. The CTX-PMB combination exhibited a hemolytic rate of 3.78%, which was markedly lower than that observed for the isolated CTX (HR 10.9%) used at a

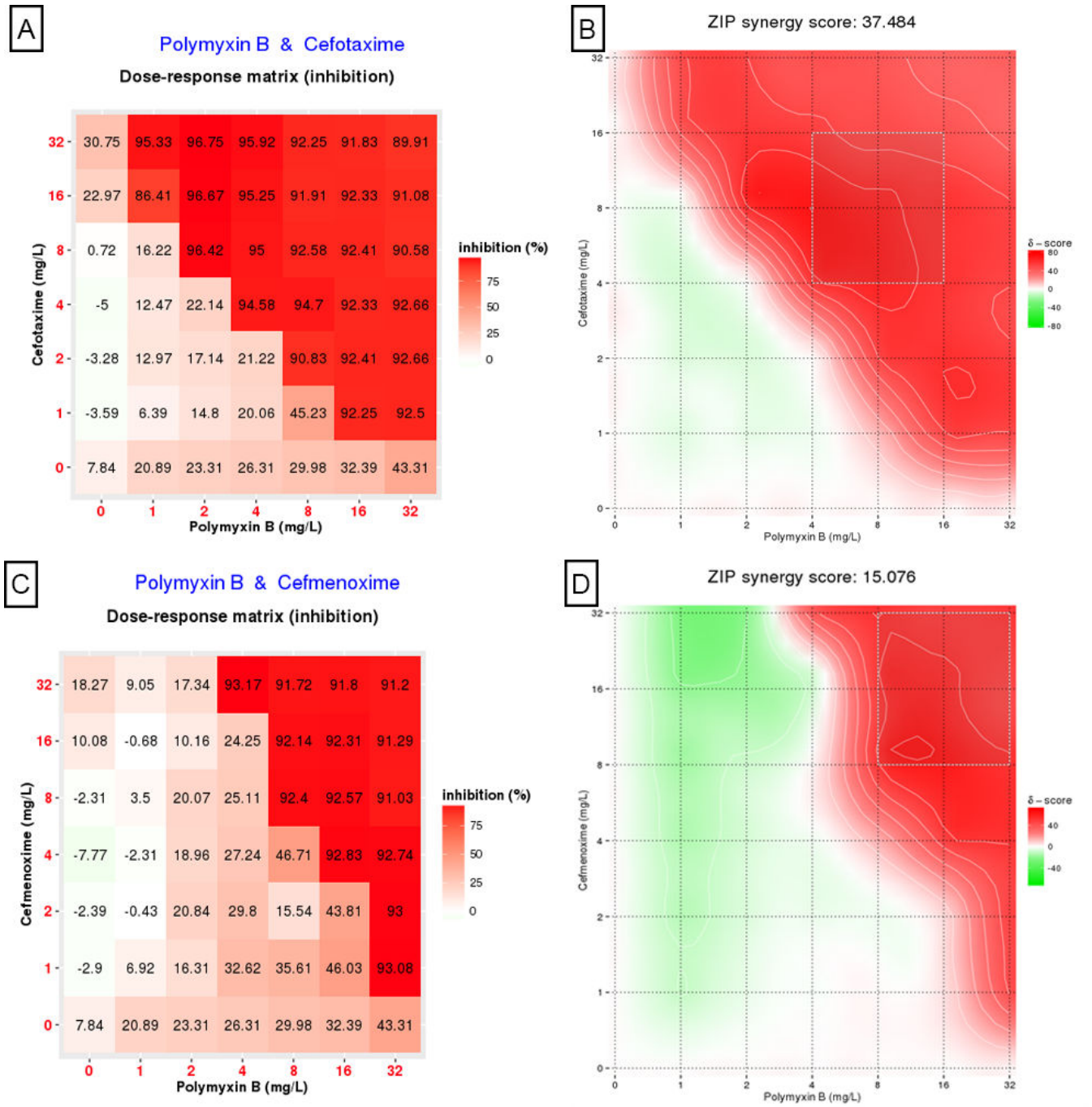

FIG 3 SynergyFinder analysis. (A) Dose-response matrix for the CTX-PMB combination. (B) Dose-response matrix for the CMX-PMB combination. (C) The ZIP synergy score (37.484) demonstrated synergism for the CTX-PMB combination. (D) The ZIP synergy score (15.076) demonstrated synergism for the CMX-PMB combination. The regions depicted in the deepest red indicate better dose combinations related to bacterial growth inhibition (inhibition >80%).

concentration of 64 mg/L. CMX-PMB exhibited an HR of 4.72%, isolated CMX led to an HR of 2.73%, and isolated PMB demonstrated the lowest HR value of 0.2%. Notably, the hemolytic activity was substantially greater in the positive control, i.e., Triton X-100 (0.1% solution) (Fig. 6)

In addition, the CTX-PMB and CMX-PMB combinations, along with the isolated antibiotics, demonstrated safety when administered to the *C. elegans* wild-type strain. The survival rates observed were 60% for CTX-PMB and 69.4% for CMX-PMB, which

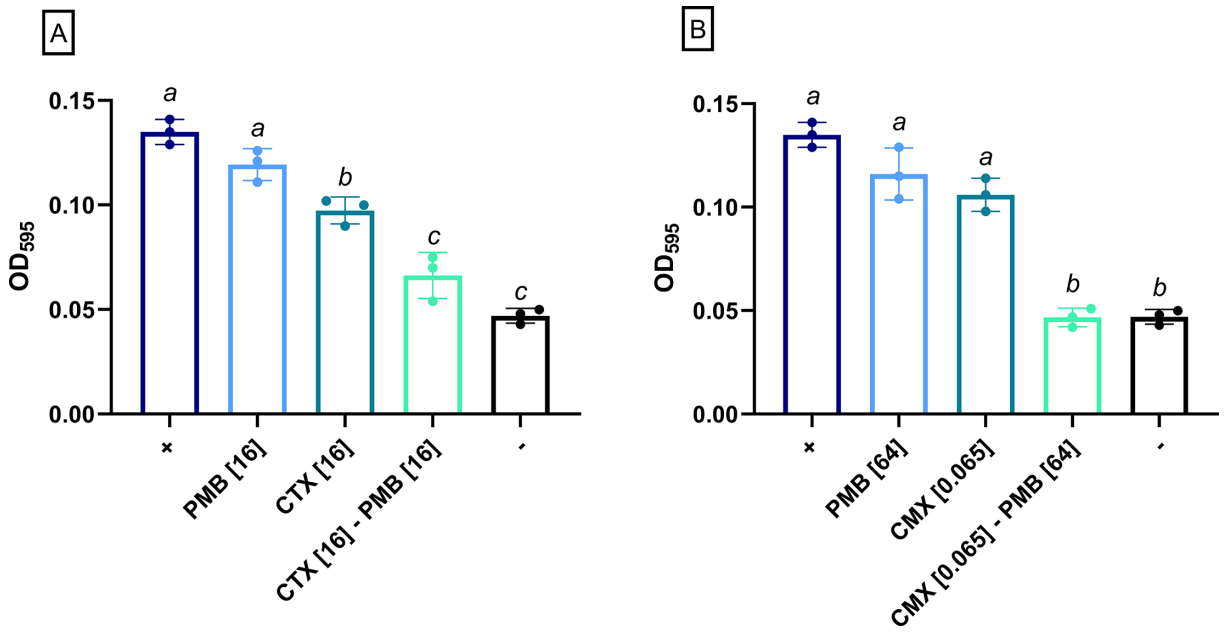

FIG 4 Antibiofilm activity of (A) the CTX-PMB combination and the isolated antibiotics at a concentration of 16 mg/L for each; (B) the CTX-PMB combination and the isolated antibiotics at a concentration of 0.065 mg/L for CMX and 64 mg/L for PMB. Positive (+) and negative (–) controls were also included in the experiments. One-way ANOVA was performed, and the differences were considered significant at $P < 0.5$. Significant differences are indicated using different letters (*a-b; b-c; a-c*), while nonsignificant differences are indicated using the same letter (*a-a; b-b; c-c*). CTX [16], cefotaxime 16 mg/L; CTX [16]-PMB [16], cefotaxime 16 mg/L associated with polymyxin B 16 mg/L; PMB [16], polymyxin B 16 mg/L; CMX [0.065], cefmenoxime 0.065 mg/L; CMX [0.065]-PMB [64], cefmenoxime 0.065 mg/L associated with polymyxin B 64 mg/L; PMB [64], polymyxin B 64 mg/L.

indicated that these treatments were well tolerated by the nematodes. None of the treatments were significantly different from the experimental control (Fig. 6B).

The *in vivo* infection model in *C. elegans* revealed the synergistic potential of the CTX-PMB and CMX-PMB combinations against CPR-Kp. CTX-PMB treatment led to a survival rate of 70% for the nematode model. The isolated antibiotics, on the other hand, could not maintain *C. elegans* survival. The CTX group had a survival rate of 41.6%, while the PMB group had a survival rate of 26.6% (Fig. 7A). CMX-PMB treatment also maintained *C. elegans* survival at a rate of 85.7%. The survival rate of the isolated CMX group was lower, 53.3% (Fig. 7B). Tigecycline, which was used as the reference antibiotic, led to a survival rate of 80%, which closely matched the survival rates noted for the evaluated antibiotic combinations. Furthermore, the antibiotic combinations effectively preserved the reproductive capabilities of the nematodes, resulting in substantial egg deposition (Fig. 7C). Conversely, the isolated antibiotic groups led to an inability to produce eggs, coupled with intestinal damage due to CPR-Kp infection, ultimately resulting in mortality (Fig. 7D).

DISCUSSION

The present study explored the synergistic interplay of four cephalosporins with PMB against multiple strains of CPR-Kp strains with alterations in the *mgr*B gene responsible for polymyxin resistance in bacteria (10). Few studies to date have addressed this topic, which underscores the significance of unraveling the synergistic potential of the evaluated combinations to identify alternative therapies against CPR-Kp pathogens.

CTX and CMX are third-generation cephalosporin antibiotics used for the treatment of GNB; for example, *K. pneumoniae*. Due to cephalosporins' low associated toxicity, they were chosen for the present study (22). Therefore, these antibiotics exhibited enhanced synergetic interaction with PMB. The two studied combinations led to low FICI values of ≤0.5 for almost all evaluated strains, indicating synergism. This finding is important, particularly considering the need for novel and effective antimicrobial therapies. In

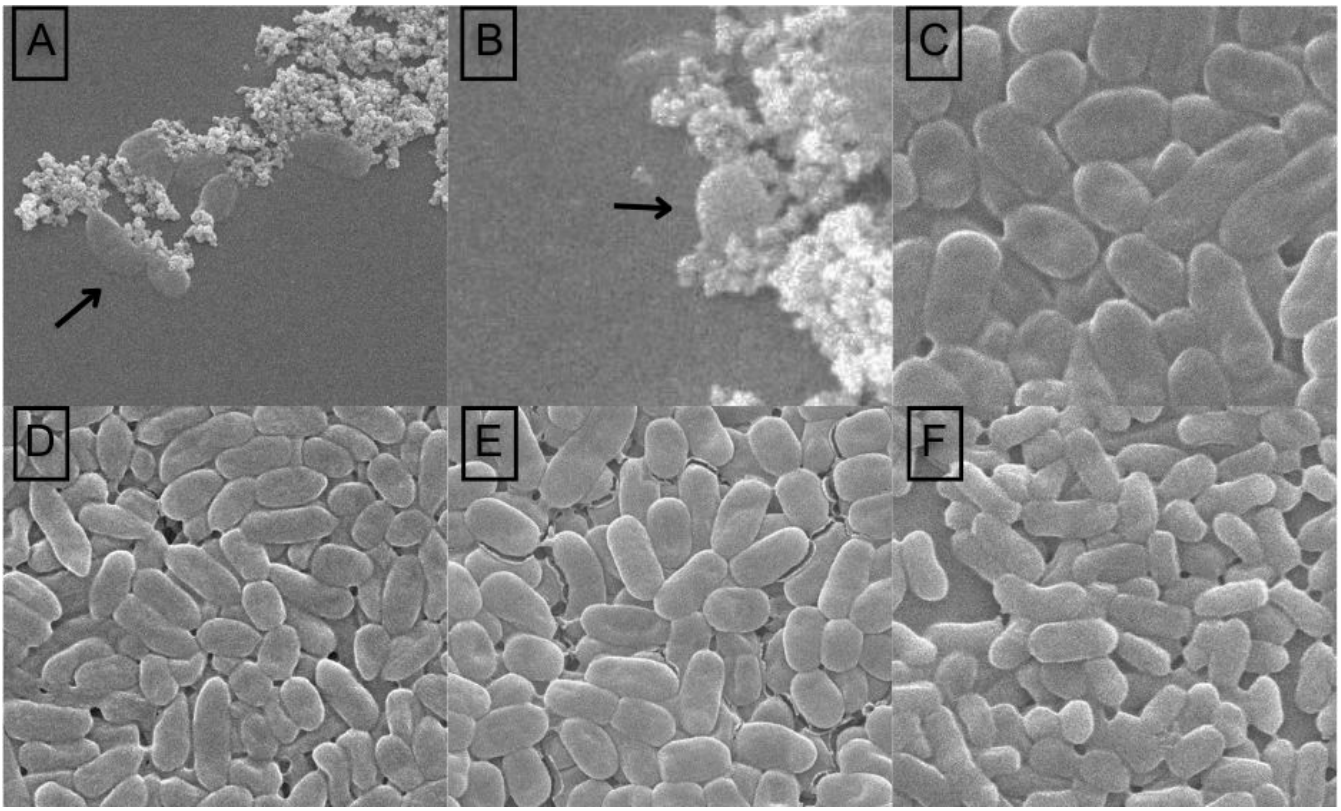

FIG 5 SEM images at 5,000× magnification for the CPR-Kp K18 strain treated with (A) CTX at 8 mg/L in combination with PMB at 4 mg/L; the microbial cell membrane is indicated using an arrow; (B) CMX at 8 mg/L in combination with PMB at 8 mg/L; the microbial cell membrane is indicated using an arrow; (C) PMB at 8 mg/L; (D) CTX at 8 mg/L; (E) CMX at 8 mg/L; (F) BHI broth only, used as the bacterial growth control.

addition, these combinations led to decreased antibiotic dosages necessary for the inhibited CPR-Kp growth by more than fourfold, which again confirmed their synergistic activity. This dose reduction is particularly important, considering the direct correlation between antibiotic toxicity and antibiotic concentration levels (23). Interestingly, Elemam et al. (24) reported no evidence of synergism for the combination of ceftriaxone and PMB against CRP-Kp, which suggests that our findings may not be applicable to all third-generation cephalosporins. Therefore, further investigation into combinations of PMB with other third-generation cephalosporins is warranted.

PMB is used as a last-resource antibiotic in the treatment of GNB because it presents nephrotoxicity, and its microbial resistance is currently considered a significant health threat, leading to critical treatment limitations (25, 26). Concomitantly, antimicrobial resistance contributes to high mortality rates in nosocomial infections, prolonged hospitalization, and increased healthcare costs (27, 28). Consequently, it is imperative to identify novel therapeutic alternatives, such as antibiotic combinations (8). PMB is known for being a good adjuvant for antibiotics; therefore, it was chosen for this study (29).

The antibiotic combinations evaluated in the present study exhibited antibiofilm activity, specifically at higher doses; therefore, these combinations are considered promising for addressing concerns about CPR-Kp biofilm formation. Bacterial biofilm formation is a significant concern in the context of microbial resistance and represents a considerable challenge within hospital environments. Planktonic-resistant microorganisms tend to exhibit even greater antibiotic resistance when they are present in biofilms (30). Nosocomial infections, frequently associated with *K. pneumoniae*, occur in 60%–70% of cases involving biofilm on medical devices (31). Discovering new candidates to combat bacterial biofilm is crucial. Our combinations showed antibiofilm activity, at higher doses, making them promising for tackling CPR-Kp biofilm. PMB sensitizes the

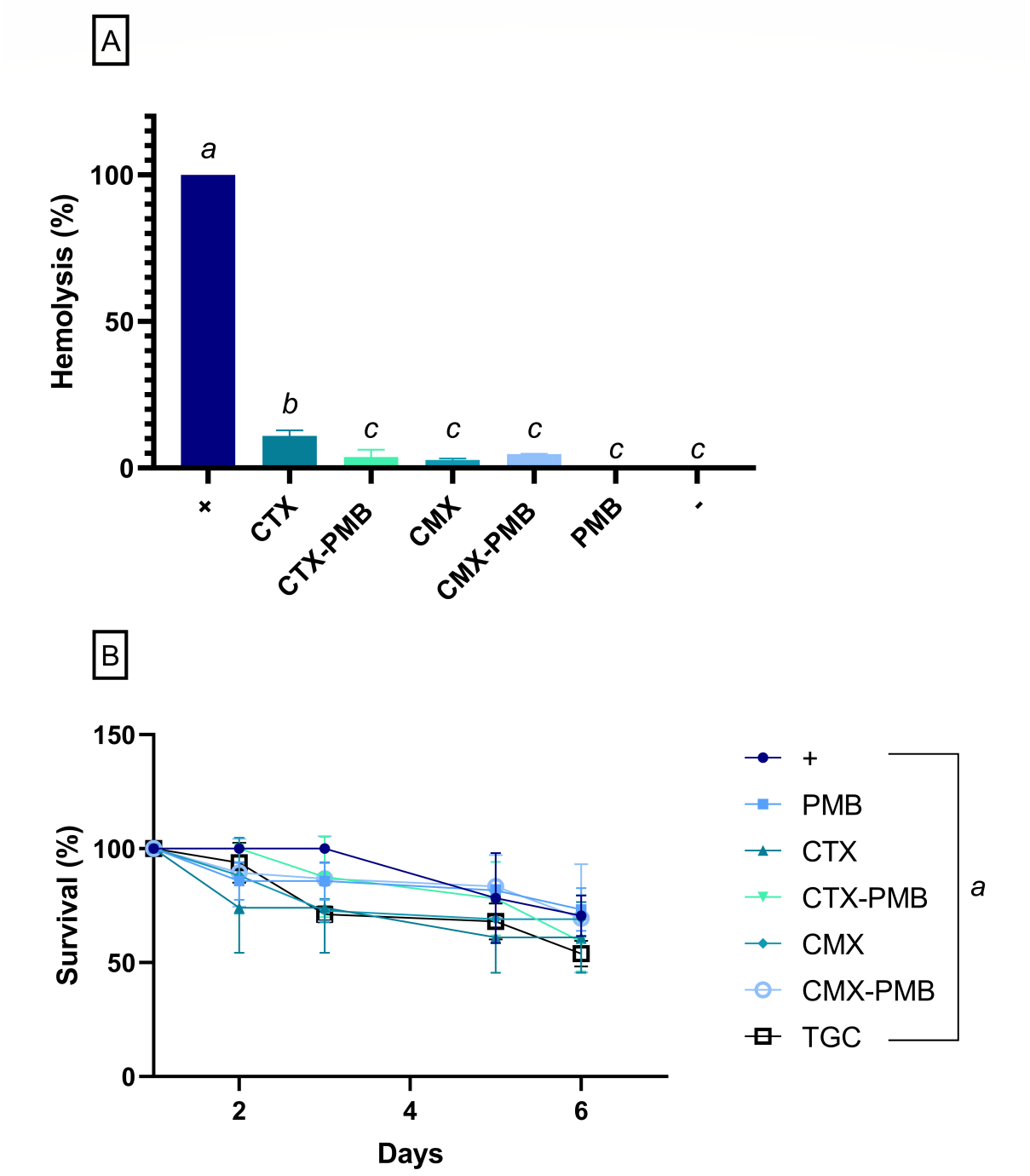

FIG 6 Safety experiment results. (A) Hemolysis rates achieved by the CTX-PMB and CMX-PMX combinations at a concentration of 1 × MIC for each, and for comparison, the hemolysis rates achieved by the isolated antibiotics CTX, CMX, and PMB at a concentration of 64 mg/L for each. Triton was used as the positive control (+), and D-PBS was used as the negative control (–). (B) *C. elegans* (N2) survival rate when exposed to the CTX-PMB and CMX-PMB combinations and isolated antibiotics at a concentration of 1 × MIC. M9 buffer was used as the positive control (+), and 16 mg/L tigecycline was used as the reference antibiotic. One-way ANOVA was performed, and the differences were considered significant at $P < 0.5$. Significant differences are indicated using different letters (*a-b; b-c; a-c*), while nonsignificant differences are indicated using the same letter (*a-a; b-b; c-c*). TGC, tigecycline.

bacterial biofilms of GNB and eradicates them when used in combination with other antibiotics. The synergetic effects of PMB with ceftazidime, meropenem, and levofloxacin on *Acinetobacter baumannii* biofilms confirmed that antibiotics exhibited better activity when used in combination than when used in monotherapy (32). The effect of PMB/

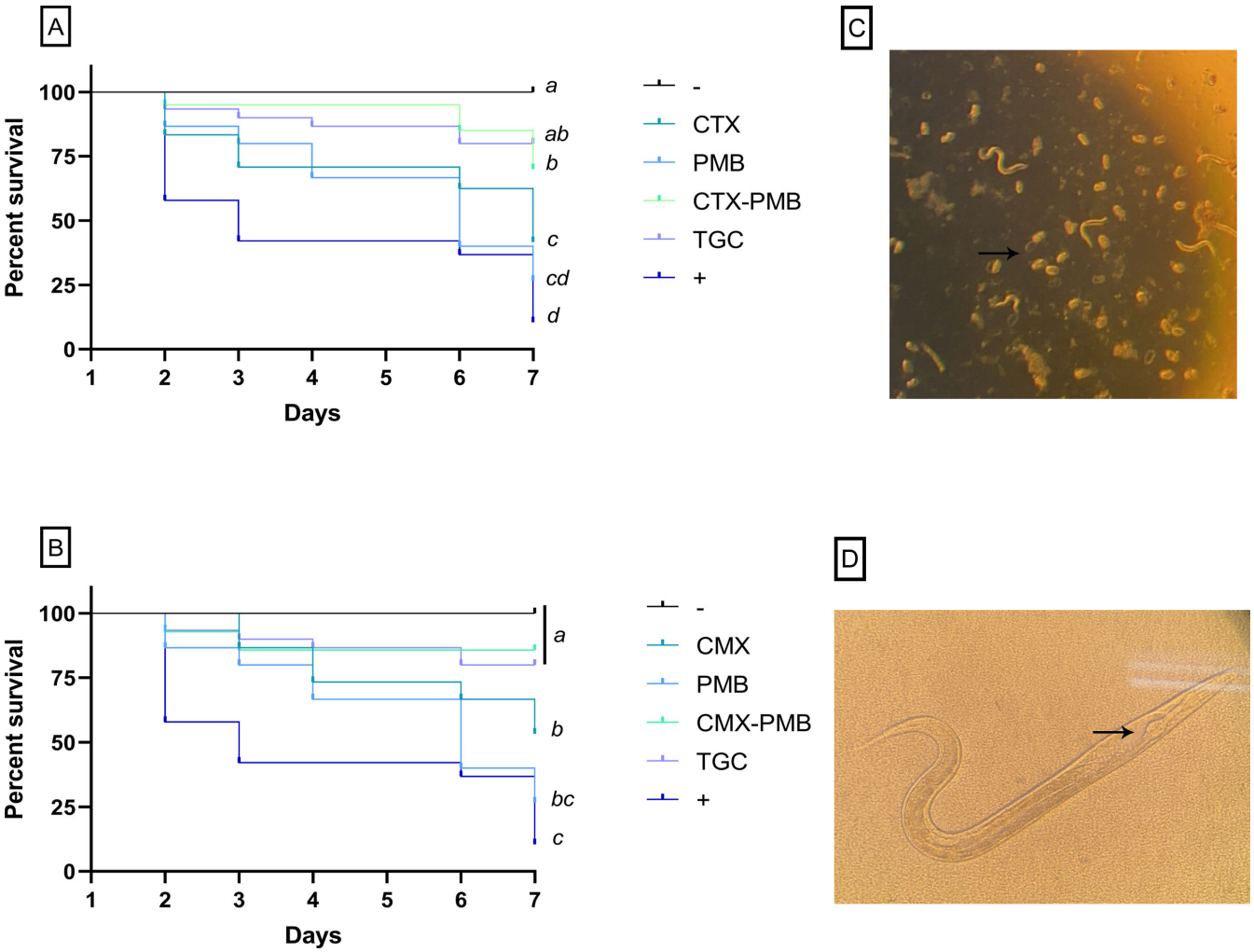

FIG 7 (A) Kaplan-Meier survival curves for *C. elegans* (Au37) infected with the CPR-Kp K1 strain and treated with the CTX-PMB combination and isolated antibiotics. (B) Kaplan-Meier survival curves for the CMX-PMB combination and isolated antibiotics for the infection of *C. elegans* (Au37) with the CPR-Kp K1 strain. Log-rank was made for both curves, and they were considered statistically different ($P < 0.5$). (C) Microscopy image of the CTX-PMB-treated C. elegans (Au37) group, highlighting numerous eggs, indicated by an arrow, demonstrating the treatment's success. (D) Microscopy image of isolated CMX-treated *C. elegans* (Au37), with an arrow indicating intestinal obstruction due to CPR-Kp K1 infection ($P < 0.5$). TGC, tigecycline.

meropenem on *Pseudomonas aeruginosa* biofilms has also been reported (33). Still, PMB effectively inhibited GNB biofilm formation at higher doses than the combinations of MIC, which renders intravenous administration of PMB impractical, considering its toxicity. However, novel administration routes, such as PMB inhalation, could be preventing and safer alternatives, delivering the drug directly to the lungs (34, 35).

Polymyxins function by binding to the LPS present on the outer membrane of the bacterium, causing destabilization and increased permeability, ultimately leading to bacterial cell death (36). In the present study, the CTX-PMB and CMX-PMB combinations did not induce protein release, indicating preserved membrane integrity. The SEM images corroborated this finding, as no membrane damage was observed in the treated bacteria, suggesting additional mechanisms of bacterial death. Third-generation cephalosporins, such as CTX and CMX, disrupt microbial cell wall synthesis by binding to penicillin-binding proteins, thereby hindering cell division (37). Polymyxins may also impact cell division by inducing rigidity (38) in the microbial cell wall and directly interfering with the cell division machinery through cell membrane penetration (39). However, further research is warranted to validate whether PMB enhances the effect of cephalosporins on microbial cell division.

Next, the toxicity of the CTX-PMB and CMX-PMX combinations was evaluated using a hemolysis assay and *C. elegans* model experiment. The combinations led to low hemolysis rates (<5%), indicating their hemocompatibility (40). Interestingly, CTX used alone at a dose capable of inhibiting CPR-Kp (64 mg/L) led to a greater hemolysis rate than the CTX-PMB combination. This observation was attributed to the reduced antibiotic dose in the associated treatment. CMX and PMB exhibited negligible hemolysis, consistent with the existing literature (41). In the *C. elegans* model, the antibiotic combinations were safety, as exposure did not affect the nematode's lifespan (42). However, further studies are needed to validate these findings in mammalian models and clinical setting.

The CTX-PMB and CMX-PMB combinations also exhibited antimicrobial efficacy against CPR-Kp *in vivo*, improving the survival of immunosuppressed *C. elegans* (Au37) nematodes. Notably, the nematodes treated with these combinations maintained their reproductive cycle, which highlights the efficacy of these treatments (21). Conversely, nematodes exposed to isolated antibiotics exhibited visible damage due to CPR-Kp infection, with compromised intestinal epithelial cell integrity and a diminished lifespan of *C. elegans* (43). Considering the role of *C. elegans* as a robust tool for antimicrobial agent screening (44), the above findings offer promising prospects for the development of novel therapeutics against CPR-Kp.

In conclusion, both the CTX-PMB and CMX-PMB combinations effectively surmounted bacterial resistance to polymyxin, reactivating PMB effectively and exhibiting antimicrobial activity both *in vitro* and *in vivo* in a CPR-Kp infection model in *C. elegans*. The combinations also exhibited potential in terms of inhibiting CPR-Kp biofilm formation, which is considered a potent form of bacterial resistance, *in vitro*. Moreover, the combinations had favorable safety profiles, which indicated their suitability for clinical applications. Overall, these findings underscore the promising potential of CTX-PMB and CMX-PMB as compelling therapeutic strategies, meriting further investigation in preclinical and clinical trials, considering the known commercial profile of antibiotics, for novel and rapid approaches to treating CPR-Kp infections.

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DATA AVAILABILITY STATEMENT

All data are available from the corresponding authors upon reasonable request.

REFERENCES

1. Wang G, Zhao G, Chao X, Xie L, Wang H. 2020. The characteristic of virulence, biofilm and antibiotic resistance of *Klebsiella pneumoniae*. Int J Environ Res Public Health 17:6278. https://doi.org/10.3390/-ijerph17176278
2. Gonzalez-Ferrer S, Peñaloza HF, Budnick JA, Bain WG, Nordstrom HR, Lee JS, Van Tyne D. 2021. Finding order in the Chaos: outstanding questions in *Klebsiella pneumoniae* pathogenesis. Infect Immun 89:e00693-20. https://doi.org/10.1128/IAI.00693-20
3. Stewart AG, Bauer MJ, Butkiewicz D, Hinton A, Henderson A, Harris PNA, Paterson DL. 2023. *In-vitro* activity of oral third-generation cephalosporins plus clavulanate against ESBL-producing Enterobacterales isolates from the MERINO trial. Int J Antimicrob Agents 62:106858. https://doi.org/10.1016/j.ijantimicag.2023.106858
4. da Silva KE, Baker S, Croda J, Nguyen TNT, Boinett CJ, Barbosa LS, Tetila A, Simionatto S. 2020. Risk factors for polymyxin-resistant carbapenemase-producing Enterobacteriaceae in critically ill patients: an epidemiological and clinical study. Int J Antimicrob Agents 55:105882. https://doi.org/10.1016/j.ijantimicag.2020.105882
5. Mattos KPH, Gouvêa IR, Quintanilha JCF, Cursino MA, Vasconcelos PENS, Moriel P. 2019. Polymyxin B clinical outcomes: a prospective study of patients undergoing intravenous treatment. J Clin Pharm Ther 44:415–419. https://doi.org/10.1111/jcpt.12801
6. de Souza GH, Vaz MS, Dos Santos Radai JA, Fraga TL, Rossato L, Simionatto S. 2024. Synergistic interaction of polymyxin B with carvacrol: antimicrobial strategy against polymyxin-resistant *Klebsiella pneumoniae*. Future Microbiol 19:181–193. https://doi.org/10.2217/fmb-2023-0070
7. Gomes MZR, de Lima EM, Martins Aires CA, Pereira PS, Yim J, Silva FH, Rodrigues CAS, Oliveira TRTE, da Silva PP, Eller CM, de Souza CMR, Rybak MJ, Albano RM, de Miranda AB, Machado E, Catanho MNucleus of Hospital Research (NPH) study collaborators2023. Outbreak report of polymyxin-carbapenem-resistant *Klebsiella pneumoniae* causing untreatable infections evidenced by synergy tests and bacterial genomes. Sci Rep 13:6238. https://doi.org/10.1038/s41598-023-31901-4
8. Coates ARM, Hu Y, Holt J, Yeh P. 2020. Antibiotic combination therapy against resistant bacterial infections: synergy, rejuvenation and resistance reduction. Expert Rev Anti Infect Ther 18:5–15. https://doi.org/10.1080/14787210.2020.1705155
9. Hanci H, Igan H. 2023. Antimicrobial synergistic effects of apigenin, (-)-epigallocatechin-3-gallate, myricetin and luteolin in combination with some antibiotics. Ann Agric Environ Med 30:61–64. https://doi.org/10.26444/aaem/161220
10. da Silva KE, Thi Nguyen TN, Boinett CJ, Baker S, Simionatto S. 2020. Molecular and epidemiological surveillance of polymyxin-resistant *Klebsiella pneumoniae* strains isolated from Brazil with multiple *mgrB* gene mutations. Int J Med Microbiol 310:151448. https://doi.org/10.1016/j.ijmm.2020.151448
11. Humphries RM, Ambler J, Mitchell SL, Castanheira M, Dingle T, Hindler JA, Koeth L, Sei K, CLSI Methods Development and Standardization Working Group of the Subcommittee on Antimicrobial Susceptibility Testing. 2018. CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. J Clin Microbiol 56:e01934-17. https://doi.org/10.1128/JCM.01934-17
12. Bellio P, Fagnani L, Nazzicone L, Celenza G. 2021. New and simplified method for drug combination studies by checkerboard assay. MethodsX 8:101543. https://doi.org/10.1016/j.mex.2021.101543
13. Chaiben V, Yamada CH, Telles JP, de Andrade AP, Arend L, Ribeiro VST, Dantas LR, Suss PH, Tuon FF. 2022. A carbapenem-resistant *Acinetobacter*

baumannii outbreak associated with a polymyxin shortage during the COVID pandemic: an *in vitro* and biofilm analysis of synergy between meropenem, gentamicin and sulbactam. J Antimicrob Chemother 77:1676–1684. https://doi.org/10.1093/jac/dkac102
14. Vasconcelos NG, Queiroz J de S, Silva K da, Vasconcelos P de P, Croda J, Simionatto S. 2020. Synergistic effects of *Cinnamomum cassia* L. essential oil in combination with polymyxin B against carbapenemase-producing *Klebsiella pneumoniae* and *Serratia marcescens*. PLoS One 15:e0236505. https://doi.org/10.1371/journal.pone.0236505
15. Tiwari V, Mishra N, Gadani K, Solanki PS, Shah NA, Tiwari M. 2018. Mechanism of anti-bacterial activity of zinc oxide nanoparticle against carbapenem-resistant *Acinetobacter baumannii*. Front Microbiol 9:1218. https://doi.org/10.3389/fmicb.2018.01218
16. Ribeiro SM, de la Fuente-Núñez C, Baquir B, Faria-Junior C, Franco OL, Hancock REW. 2015. Antibiofilm peptides increase the susceptibility of carbapenemase-producing *Klebsiella pneumoniae* clinical isolates to β-lactam antibiotics. Antimicrob Agents Chemother 59:3906–3912. https://doi.org/10.1128/AAC.00092-15
17. Yang Z, Song M, Li X, Zhang Q, Shen J, Zhu K. 2023. Synergy of outer membrane disruptor SLAP-S25 with hydrophobic antibiotics against Gram-negative pathogens. J Antimicrob Chemother 78:263–271. https://doi.org/10.1093/jac/dkac387
18. Sæbø IP, Bjørås M, Franzyk H, Helgesen E, Booth JA. 2023. Optimization of the hemolysis assay for the assessment of cytotoxicity. Int J Mol Sci 24:2914. https://doi.org/10.3390/ijms24032914
19. Wang Y-C, Huang S-W, Chiang M-H, Lee I-M, Kuo S-C, Yang Y-S, Chiu C-H, Su Y-S, Chen T-L, Wang F-D, Lee Y-T. 2021. *In vitro* and *in vivo* activities of imipenem combined with BLI-489 against class D β-lactamase-producing *Acinetobacter baumannii*. J Antimicrob Chemother 76:451–459. https://doi.org/10.1093/jac/dkaa421
20. Eduvirgem J, Rossato L, Melo AL, Valiente AC, Plaça LF, Wender H, Vaz MS, Ribeiro SM, Simionatto S. 2023. Antimicrobial and antibiofilm activities of desloratadine against multidrug-resistant *Acinetobacter baumannii*. Future Microbiol 18:15–25. https://doi.org/10.2217/fmb-2022-0085
21. Kamaladevi A, Balamurugan K. 2017. Global proteomics revealed *Klebsiella pneumoniae* induced autophagy and oxidative stress in *Caenorhabditis elegans* by inhibiting PI3K/AKT/mTOR pathway during infection. Front Cell Infect Microbiol 7:393. https://doi.org/10.3389/fcimb.2017.00393
22. Kobayashi K, Hata A, Imoto W, Kakuno S, Shibata W, Yamada K, Kawaguchi H, Sakurai N, Nakaie K, Nakatsuka Y, Ito T, Uenoyama K, Takahashi T, Ueda S, Katayama T, Onoue M, Kakeya H. 2023. The clinical evaluation of third-generation cephalosporins as definitive therapy for *Enterobacter* spp. and *Klebsiella aerogenes* bacteremia. Intern Med 62:1921–1929. https://doi.org/10.2169/internalmedicine.0612-22
23. Zhang W, Guo Y, Li J, Zhang Y, Yang Y, Dong D, Zhu D, He P, Hu F. 2018. *In vitro* and *in vivo* bactericidal activity of ceftazidime-avibactam against carbapenemase-producing *Klebsiella pneumoniae*. Antimicrob Resist Infect Control 7:142. https://doi.org/10.1186/s13756-018-0435-9
24. Elemam A, Rahimian J, Doymaz M. 2010. *In vitro* evaluation of antibiotic synergy for polymyxin B-resistant carbapenemase-producing *Klebsiella pneumoniae*. J Clin Microbiol 48:3558–3562. https://doi.org/10.1128/JCM.01106-10
25. Liang Z, Shi Y-L, Peng Y, Xu C, Zhang C, Chen Y, Luo X-Q, Li Q-M, Zhao C-L, Lei J, Yuan Z-Q, Peng Y-Z, Song B-Q, Gong Y-L. 2023. BL02, a phage against carbapenem- and polymyxin-B resistant *Klebsiella pneumoniae*, isolated from sewage: a preclinical study. Virus Res 331:199126. https://doi.org/10.1016/j.virusres.2023.199126
26. Yu Z, Liu X, Du X, Chen H, Zhao F, Zhou Z, Wang Y, Zheng Y, Bergen PJ, Li X, Sun R, Fang L, Li W, Fan Y, Wu H, Guo B, Li J, Yu Y, Zhang J. 2022. Pharmacokinetics/pharmacodynamics of polymyxin B in patients with bloodstream infection caused by carbapenem-resistant *Klebsiella pneumoniae*. Front Pharmacol 13:975066. https://doi.org/10.3389/fphar.2022.975066
27. Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, Cohen J, Findlay D, Gyssens I, Heuer OE, Kahlmeter G, Kruse H, Laxminarayan R, Liébana E, López-Cerero L, MacGowan A, Martins M, Rodríguez-Baño J, Rolain J-M, Segovia C, Sigauque B, Tacconelli E, Wellington E, Vila J. 2015. The global threat of antimicrobial resistance: science for intervention. New Microbes New Infect 6:22–29. https://doi.org/10.1016/j.nmni.2015.02.007
28. Tanwar J, Das S, Fatima Z, Hameed S. 2014. Multidrug resistance: an emerging crisis. Interdiscip Perspect Infect Dis 2014:541340. https://doi.org/10.1155/2014/541340
29. Hou S-Y, Wu D, Feng X-H. 2020. Polymyxin monotherapy versus polymyxin-based combination therapy against carbapenem-resistant *Klebsiella pneumoniae*: a systematic review and meta-analysis. J Glob Antimicrob Resist 23:197–202. https://doi.org/10.1016/j.jgar.2020.08.024
30. Mahamuni-Badiger PP, Patil PM, Badiger MV, Patel PR, Thorat- Gadgil BS, Pandit A, Bohara RA. 2020. Biofilm formation to inhibition: role of zinc oxide-based nanoparticles. Mater Sci Eng C Mater Biol Appl 108:110319. https://doi.org/10.1016/j.msec.2019.110319
31. Borowski RGV, Barros MP, da Silva DB, Lopes NP, Zimmer KR, Staats CC, de Oliveira CB, Giudice E, Gillet R, Macedo AJ, Gnoatto SCB, Zimmer AR. 2020. Red pepper peptide coatings control *Staphylococcus epidermidis* adhesion and biofilm formation. Int J Pharm 574:118872. https://doi.org/10.1016/j.ijpharm.2019.118872
32. Li Y, Wang B, Lu F, Ahn J, Zhang W, Cai L, Xu J, Yin Y, Cao Q, Ren Z, He X. 2022. Synergistic inhibitory effect of polymyxin B in combination with ceftazidime against robust biofilm formed by *Acinetobacter baumannii* with genetic deficiency in AbaI/AbaR quorum sensing. Microbiol Spectr 10:e0176821. https://doi.org/10.1128/spectrum.01768-21
33. Wickremasinghe H, Yu HH, Azad MAK, Zhao J, Bergen PJ, Velkov T, Zhou QT, Zhu Y, Li J. 2021. Clinically relevant concentrations of polymyxin B and meropenem synergistically kill multidrug-resistant *Pseudomonas aeruginosa* and minimize biofilm formation. Antibiotics (Basel) 10:405. https://doi.org/10.3390/antibiotics10040405
34. Tang T, Li Y, Xu P, Zhong Y, Yang M, Ma W, Xiang D, Zhang B, Zhou Y. 2023. Optimization of polymyxin B regimens for the treatment of carbapenem-resistant organism nosocomial pneumonia: a real-world prospective study. Crit Care 27:164. https://doi.org/10.1186/s13054-023-04448-z
35. Liu J, Shao M, Xu Q, Liu F, Pan X, Wu J, Xiong L, Wu Y, Tian M, Yao J, Huang S, Zhang L, Chen Y, Zhang S, Wen Z, Du H, Liu Y, Li W, Xu Y, Teboul J-L, Chen D, TaoWang. 2022. Low-dose intravenous plus inhaled versus intravenous polymyxin B for the treatment of extensive drug-resistant Gram-negative ventilator-associated pneumonia in the critical illnesses: a multi-center matched case-control study. Ann Intensive Care 12:72. https://doi.org/10.1186/s13613-022-01033-5
36. Olaitan AO, Morand S, Rolain J-M. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5:643. https://doi.org/10.3389/fmicb.2014.00643
37. El-Shaboury SR, Saleh GA, Mohamed FA, Rageh AH. 2007. Analysis of cephalosporin antibiotics. J Pharm Biomed Anal 45:1–19. https://doi.org/10.1016/j.jpba.2007.06.002
38. Mortensen NP, Fowlkes JD, Sullivan CJ, Allison DP, Larsen NB, Molin S, Doktycz MJ. 2009. Effects of colistin on surface ultrastructure and nanomechanics of *Pseudomonas aeruginosa* cells. Langmuir 25:3728–3733. https://doi.org/10.1021/la803898g
39. Trimble MJ, Mlynárčik P, Kolář M, Hancock REW. 2016. Polymyxin: alternative mechanisms of action and resistance. Cold Spring Harb Perspect Med 6:a025288. https://doi.org/10.1101/cshperspect.a025288
40. Gao W, Han X, Sun D, Li Y, Liu X, Yang S, Zhou Z, Qi Y, Jiao J, Zhao J. 2023. Antibacterial properties of antimicrobial peptide HHC36 modified polyetheretherketone. Front Microbiol 14:1103956. https://doi.org/10.3389/fmicb.2023.1103956
41. Wu Y, Wu P, Wu R, Li H, Duan Y, Cai C, Liu Z, She P, Zhang D. 2023. Simeprevir restores the anti-*Staphylococcus* activity of polymyxins. AMB Express 13:122. https://doi.org/10.1186/s13568-023-01634-8
42. Huang S-T, Lu J-H, Jualo SM, Tayo LL, Mansor W-N-W, Lai Y-C, Wang C-L, Chao H-R. 2023. Titanium dioxide (TiO_2) nanoparticle toxicity in a *Caenorhabditis elegans* model. Toxics 11:989. https://doi.org/10.3390/toxics11120989
43. Yang W-H, Chen P-H, Chang H-H, Kwok HL, Stern A, Soo P-C, Chen J-H, Yang H-C. 2023. Impaired immune response and barrier function in GSPD-1-deficient *C. elegans* infected with *Klebsiella pneumoniae*. Curr Res Microb Sci 4:100181. https://doi.org/10.1016/j.crmicr.2023.100181
44. Ewbank JJ, Zugasti O. 2011. *C. elegans*: model host and tool for antimicrobial drug discovery. Dis Model Mech 4:300–304. https://doi.org/10.1242/dmm.006684

6. CONCLUSION AND FUTURE PERSPECTIVES

The results of this study raise several important questions that merit further attention. Firstly, our extensive patent review provides valuable insights into the innovative potential of cephalosporin-based combination therapies targeting ESKAPE pathogens. However, it also highlights key gaps that must be addressed to advance this approach. These include the need for more robust *in vivo* assays, a deeper understanding of the mechanisms driving the synergistic effects of these combinations, and the initiation of clinical trials to enable the translation of this promising technology into effective healthcare solutions.

Considering these findings and the scarcity of new antimicrobials, we conducted targeted experiments to explore a promising therapeutic alternative for infections caused by carbapenem- and polymyxin-resistant *K. pneumoniae*. This approach focused on combinations of cefotaxime, cefmenoxime, and ceftibuten with polymyxin B. These combinations demonstrated synergistic antibacterial activity in both *in vitro* and *in vivo* assays, along with notable antibiofilm potential—an important factor in combating bacterial resistance. While the combinations effectively reduced the survival of multidrug-resistant *K. pneumoniae*, interestingly, they did not alter bacterial cell morphology, suggesting a possible mechanism involving disruption of bacterial cell division that needs to be elucidated. Additionally, no hemolytic activity or signs of toxicity were observed in the *C. elegans* model.

This study has the potential to become a real-world applicable therapy, similar to established combinations of cephalosporins and β-lactamase inhibitors, such as ceftazidime-avibactam and ceftolozane-tazobactam, whose have proven effectivity in overcoming resistance mechanisms. However, to advance our combinations toward clinical application, further research is essential. This includes comprehensive toxicity assessments to ensure safety, detailed studies to elucidate mechanisms of action, and progression to advanced-phase clinical trials to validate efficacy and safety in real-world healthcare settings.

7. ANNEXES

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA

Dourados-MS, 18 de novembro 2023.

CERTIFICADO

Certificamos que o projeto intitulado " **Abordagens de baixo custo baseadas em triagem virtual e planejamentos de novos hits para controlar a disseminação de bactérias multirresistentes**", protocolo 23018, sob a responsabilidade de **Simone Simionatto**, - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei no. 11.794, de 8 de outubro de 2008, do Decreto no 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), tendo sido aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Federal da Grande Dourados (UFGD).

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