Heliyon 10 (2024) e27984

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Review article

Unraveling resistance mechanisms in combination therapy: A comprehensive review of recent advances and future directions

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ARTICLE INFO

Keywords:
Combination therapy
Monotherapies
Antimicrobial resistance
Multi-resistant bacteria

ABSTRACT

Antimicrobial resistance is a global health threat. Misuse and overuse of antimicrobials are the main drivers in developing drug-resistant bacteria. The emergence of the rapid global spread of multi-resistant bacteria requires urgent multisectoral action to generate novel treatment alternatives. Combination therapy offers the potential to exploit synergistic effects for enhanced antibacterial efficacy of drugs. Understanding the complex dynamics and kinetics of drug interactions in combination therapy is crucial. Therefore, this review outlines the current advances in antibiotic resistance's evolutionary and genetic dynamics in combination therapies-exposed bacteria. Moreover, we also discussed four pivotal future research areas to comprehend better the development of antibiotic resistance in bacteria treated with combination strategies.

1. Introduction

Since the advent of antibiotics, monotherapies have played a pivotal role in combating infectious diseases [1]. However, with the growing prevalence of antimicrobial resistance (AMR), the scientific community has sought alternative strategies to enhance treatment efficacy. In 2017, the World Health Organization (WHO) acknowledged the escalating threat of antimicrobial resistance (AMR) by publishing a list of microorganisms categorized as high and medium priority. This action underscored the urgent need for novel antibiotic agents to address AMR challenges [2]. Concurrently, the global action plan on AMR was established, focusing on two pivotal objectives: first, to intensify awareness regarding the overuse of antibiotics; and second, to enhance the surveillance and optimization of existing antibiotic treatments [3].

Amid these developments, combination therapy, defined as the concurrent use of multiple antibiotics, has gained prominence as a strategic countermeasure against AMR. This approach holds significant promise; however, its effective translation from preclinical research to clinical application is hampered by a notable disparity in nonclinical diagnostic methodologies. This inconsistency impedes the evaluation of combination therapy, thus obstructing the effective translation of preclinical findings to clinical practices [4]. To address this challenge, it is imperative to harmonize nonclinical diagnostic methods. Such standardization is crucial for two primary reasons: firstly, it enables accurate predictions regarding the efficacy of combination therapies; and secondly, it facilitates the identification of optimal treatment strategies tailored to individual patient needs. Standardization ensures that preclinical data are not only reliable but also applicable in clinical contexts. This will substantially contribute to the development and implementation of more

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https://doi.org/10.1016/j.heliyon.2024.e27984
Received 10 August 2023; Received in revised form 7 March 2024; Accepted 8 March 2024
Available online 11 March 2024

efficacious combination therapy regimens, thereby offering a robust response to the AMR crisis [4–6].

The advancement of more efficacious combination therapies is essential to explore the microbial ecology and genetic and evolutionary dynamics underlying AMR [7][7] to comprehend the distribution and maintenance of AMR in microorganisms. The evolution of resistance caused by spontaneous mutations under controlled conditions varies between antibiotics [8,9]. The discrepancies in evolution rates and diversity of resistance-associated mutational pathways among different drugs warrant further investigation. Advances in genomics and computational biology have enabled researchers to dissect the complex interplay between bacterial genomes and their environment, shedding light on the factors that drive the emergence and spread of resistance [10,11]. These insights have not only facilitated a deeper understanding of the molecular mechanisms underlying AMR but paved the way for innovative approaches in the design and optimization of combination therapies.

Tropack et al. [9] studied the progressive evolution of *Escherichia coli* in response to various drugs, including chloramphenicol, doxycycline, and trimethoprim, over an approximately 20-day period. Their findings revealed that resistance levels significantly increased in parallel populations with similar phenotypic trajectories, in addition to displaying AMR-specific mutations toward individual and multiple drugs [9]. The dose-response effect of antibiotics plays a crucial role in shaping spontaneous mutations throughout the genome, which, in turn, impact the fitness of single cells [8]. Adaptive mutations often occur under antibiotic pressure, with associated costs that other genetic modifications can offset, particularly those related to bacterial fitness when a second drug is administered [12]. Understanding these dynamics, influenced by varying selective pressures due to the irrational use of antibiotics, is critical in reducing the emergence of resistant microorganisms and improving antibiotic treatments for countering infections [13,14]. Despite this, only a limited number of studies have delved into the evolutionary and genetic dynamics leading to the emergence of antibiotic resistance and multi-drug resistance in pathogenic bacteria when exposed to combination therapies. Timely monitoring and identification of resistance development are essential, as they form the cornerstone of combination therapy success, minimizing the potential for resistance development throughout the treatment process [15].

Furthermore, the integration of advanced diagnostic tools and techniques, such as next-generation sequencing and machine learning algorithms, can aid in the early detection of emerging resistance patterns, allowing for more targeted and personalized treatment strategies [10,11,16,17]. Considering the increasing threat of antimicrobial resistance and the potential of combination therapies to address this global health challenge, the scientific community must continue investigating the molecular and evolutionary underpinnings of resistance mechanisms. This knowledge will contribute to developing novel therapeutic approaches, optimizing existing combination therapies, and facilitating evidence-based clinical decision-making. Moreover, a deeper understanding of the factors influencing the emergence and persistence of resistance will inform public health policies and strategies to promote the rational use of antibiotics, thereby mitigating the risk of resistance development and ensuring the continued efficacy of these life-saving drugs.

Therefore, this review outlines the current advances in antibiotic resistance's evolutionary and genetic dynamics in combination therapies-exposed bacteria. Moreover, we also discussed four pivotal future research areas to comprehend better the development of antibiotic resistance in bacteria treated with combination strategies.

2. Combination therapies against clinically relevant microorganisms

Combination therapies have emerged as a practical and alternative treatment approach for diseases caused by global concerning pathogens [1,18,19]. For instance, multidrug-resistant *Mycobacterium tuberculosis* (MTB)strains pose a significant challenge to tuberculosis control due to their increasing prevalence within the last decade [20,21]. Streptomycin was the first antibiotic (in monotherapy regimens) against MTB, leading to resistance development, ultimately sidelining its use [22]. Subsequently, combination therapy with isoniazid, ethambutol, and rifampicin was introduced as a drug combination therapy [23]. Treating MTB strains, both drug-susceptible and drug-resistant, requires a minimum of three to four antibiotics in combination, resulting in intricate drug susceptibility and resistance patterns [24].

MTB drug and multi-drug resistance primarily stem from spontaneous single nucleotide polymorphisms (SNPs) [25]. These SNPs are linked to mutations in various genes associated with resistance (*inhA, rpoB, pncA, embB, rrs, gyrA, gyrB, katG, ahpC, ndh, furA, rpsL)* to specific antibiotics (pyrazinamide, rifampicin, isoniazid, ethambutol, streptomycin, capreomycin, and fluoroquinolone) [26–29]. Although fluctuation tests have been conducted for these antibiotics, the mutations arise spontaneously at rates ranging from 10^{-6} to 10^{-9}, depending on the antibiotic. The probability of developing resistance to two antibiotics is estimated at 10^{-16}. However, as the population of antibiotic-sensitive bacteria decreases, the resistant population increases, paving the way for resistance to a second antibiotic. Consequently, exposure to a second antibiotic causes the selection of doubly resistant bacteria [30]. The genotypic diversity and various mutations exhibited by MTB suggest multiple evolutionary pathways leading to resistance and multi-resistance, warranting further investigation [31].

In 2021, the WHO promulgated revised definitions for extensively drug-resistant tuberculosis [32]. The term pre-extensively drug-resistant tuberculosis (Pre-XDR TB) is now assigned to MTB strains that satisfy the criteria for both multidrug-resistant or rifampicin-resistant (RR) tuberculosis and concomitant resistance to fluoroquinolones. Extensively drug-resistant tuberculosis (XDR-TB) categorizes MTB strains that are both MDR/RR and demonstrate resistance to at least one fluoroquinolone—such as levofloxacin or moxifloxacin—and one additional agent from group A, which includes drugs like bedaquiline and linezolid [33]. It is critical to note that certain genetic mutations in MTB may be "occult" or "disputed" as they elude detection by standard drug susceptibility testing (DST) methodologies. These undetected mutations have the potential to instigate a spectrum of drug resistance, ranging from low to high levels [34–36].

H. pylori is another globally prevalent pathogen, infecting over 50% of the world's population, primarily in developing countries [37]. It is implicated in gastric carcinogenesis due to the production of virulence factors that result in peptic ulcers and gastric cancer

[38]. *H. pylori* includes proton pump inhibitors (PPI) in conjunction with different treatment regimens, including triple and quadruple therapies. In triple therapy, first-line antibiotics (amoxicillin and clarithromycin) are usually given with a PPI. Patients with resistance to clarithromycin use the antibiotic metronidazole as an alternative. In some instances where multi-resistance occurs, quadruple therapy containing bismuth is used [39]. Clarithromycin is an antibiotic that is highly relevant for the treatment of *H. pylori*. Clarithromycin resistance is attributed to mutations (A2146G and A2147G) in the 23S rRNA gene [40]. On the other hand, the *hefA* efflux pump gene has already been shown to play an essential role in resistance to multiple antibiotics in *H. pylori* [41]. Multi-resistant strains for both triple and quadruple therapies also present mutations associated with the *23S rRNA, 16S rRNA, gyrA,* and *rpoB* genes; these genes were found exclusively in isolates from patients who had already been unsuccessfully treated on multiple occasions [42].

Understanding the evolution of AMR can inform efforts to combat multi-drug resistance [43]. As already mentioned, triple and quadruple combination therapies have been required to treat these pathogens of global concern, and treating two antibiotics is no longer an option [44]. Despite the statistically low probability of resistance emergence in combination therapies, the significant selective pressure exerted on pathogens may ultimately lead to resistance development, as observed in monotherapies for *H. pylori* and *M. tuberculosis* [45]. Therefore, evolutionary studies are necessary to elucidate the dynamics underlying the emergence of resistance and inform the optimization of combination therapies, which are currently the most effective strategy.

The rise in the prevalence of multi-resistant bacteria has diminished the efficacy of conventional treatments [46]. As a result, This is why alternatives have been sought that improve traditional methods, such as monotherapy and combination therapies with antibiotics [46]. Alternative approaches, such as metallic-based nanoparticles, have been explored to enhance the effectiveness of monotherapies and combination therapies with antibiotics against AMR [47–53]. Although nanoparticle-based treatments have been under investigation for several decades, studies have also reported the development of resistance to these materials. Graves et al. [49] carried out an evolutionary survey demonstrating that bacteria could develop resistance to silver nanoparticles (AgNP), through the presence of simple mutations, especially in the *crl, gatC, glpR, gltP, yjcO* genes in the bacteria *E. coli*. In recent years, it is estimated that the world market for products based on nanotechnology has had an annual increase of 25%. For 2020, the annual production was approximately 58,000 tons globally. This has led to a surge in nanomaterial release into the environment, particularly silver-based products, due to their antimicrobial properties [53,54].

Consequently, microorganisms have evolved strategies to counteract new antimicrobial agents and adapt to the changing landscape that has put them in an evolutionary race [52][52]. Bacterial plasticity, a high capacity for mutations, and the acquisition of resistance genes contribute to this adaptation, driven by the selective pressure exerted since the advent of antibiotics and emerging technologies [52,55,56]. In some cases, bacteria can develop resistance to nanoparticles without requiring significant genetic changes, such as minor phenotypic modifications. A study on *E. coli* and *P. aeruginosa* exposed to silver nanoparticles (NPs-Ag) found that increased production of flagellin protein could confer resistance by causing NPs-Ag aggregation and reducing their antimicrobial effect. It was determined that there were no significant alterations in the flagellar genes (fliC, fLgE, fliD) in the gene that encodes for the master transcriptional regulator gene (FlhDC) and the flagellar regulator gene (FliA) between the strains that presented resistance and those that offered resistance. In control strains, resistance only occurred due to an increase in the production of the flagellin protein [57].

The efficacy of combination therapy has been corroborated by checkerboard assay utilizing fractional inhibitory concentration indices (FICI). Specifically, the efficacy of three distinct antibiotic pairings—roxithromycin with doxycycline, vancomycin with ofloxacin, and vancomycin with fosfomycin—was investigated against methicillin-resistant *Staphylococcus aureus* (MRSA). *In vitro* results revealed that these combinations, at minimal inhibitory concentrations, achieved up to 99% inhibition in bacterial colonies, indicating the potential of this synergistic combination to prevent the development of resistance [58]. Further, within the family *Enterobacteriaceae*, notorious for carbapenemase production, efficacious antibiotic combinations were identified. An *in vitro* study evaluated multiple antibiotic combinations against other members of the family *Enterobacteriaceae*, including colistin with meropenem, tigecycline, rifampicin, and erythromycin, as well as meropenem with tigecycline and rifampicin, and a triad of meropenem-tigecycline-colistin. These combinations were tested against strains harboring bla genes, responsible for various carbapenemase enzymes (bla_{KPC}, bla_{NDM}, and bla_{IMP}) and *mcr* −1/8/9 linked to colistin resistance. The checkerboard assay demonstrated that the colistin - rifampicin combination had the most significant impact with a synergistic effect of up to 76%, while the colistin - erythromycin exhibited a synergistic effect of up to 60%. Remarkably, the triple antibiotic combination of meropenem-tigecycline-colistin exhibited synergistic effects reaching 100%. These *in vitro* studies suggest that the colistin-rifampicin combination could significantly enhance treatment outcomes for patients afflicted with infections caused by carbapenemase-producing *Enterobacteriaceae* strains [59].

Acinetobacter baumannii is classified as a pathogenic microorganism of significant clinical concern due to its extensive resistance profile against a broad spectrum of available antimicrobial agents [60]. Investigative efforts have been directed towards evaluating the synergistic potential of colistin and ampicillin/sulbactam in combination with cephalosporins, fluoroquinolones, and aminoglycosides against multidrug-resistant (MDR) strains of *A. baumannii*. The *in vitro* synergy observed when colistin and ampicillin/sulbactam are combined with doxycycline, cotrimoxazole, azithromycin, and amikacin, particularly with the ampicillin/sulbactam-amikacin combination, suggests an enhanced efficacy against MDR *A. baumannii* [60]. Furthermore, the presence of carbapenem-resistant *A. baumannii* (CRAB) in intensive care units poses a formidable challenge [61]. Research led by Kragh et al. [62] focused on the comparative effectiveness and safety of colistin monotherapy versus its combination with meropenem in critically ill patients with CRAB infections. A cohort study spanning from 2015 to 2017 at Chiang Mai University Hospital provided evidence that combination therapy did not result in increased nephrotoxicity compared to monotherapy. Notably, the combined regimen was associated with a decreased mortality rate and improved outcomes from both clinical and microbiological perspectives [61].

Antibiotic combinations that employ distinct mechanisms of action are pivotal in enhancing treatment efficacy through synergistic interactions [62,63]. The quest for improved therapeutic strategies has led to the implementation of innovative techniques such as

microcalorimetry to ascertain the synergistic potential of antibiotic regimens against multidrug-resistant organisms, with validation efforts conducted in murine infection models [62]. Microcalorimetry serves as a critical tool for metabolic phenotyping, offering a viable method for determining the MIC [63]. This technique was utilized to assess the combined effects of meropenem with colistin, rifampin, and amikacin against pathogens including *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *A. baumannii*. The isothermal microcalorimeter, which quantifies metabolic activity, provided insights into the *in vivo* efficacy of these combinations in a mouse model of peritonitis and sepsis [62]. Out of various assessments, thirteen showcased a notable synergistic impact, with significant correlation observed between microcalorimetric readings and the logarithmic reduction of the peritoneal fluid in mice [62]. The application of microcalorimetry not only facilitates the rapid identification of synergistic antibiotic combinations but also holds promise for expediting the development of effective treatment protocols against resistant bacterial infections [62].

The combination of antimicrobial peptides (AMPs) with established antibiotics has emerged as a compelling strategy in the fight against antibiotic resistance, capturing the interest of the scientific community due to the observed synergistic effects [64]. Research involving the peptide Pt5-1c, a derivative of phosvitin, has indicated potent antimicrobial activity against *E. coli*, *S. aureus*, and *K. pneumoniae*. Notably, Pt5-1c has demonstrated synergistic interactions with conventional antibiotics such as oxacillin, vancomycin, streptomycin, and azithromycin. These combinations have proven effective against the biofilm-forming capabilities of these bacteria both *in vitro* and *in vivo*, positioning AMPs as a potent adjunct in combination therapies targeting clinically significant bacterial pathogens [64].

In response to the escalating threat posed by multidrug-resistant bacteria, the scientific endeavor has increasingly pivoted towards developing therapeutic strategies that exhibit resilience to resistance development, aiming to forestall or interrupt the emergence of resistance in pathogenic bacteria [65]. It is imperative to acknowledge, however, that research into the resistance patterns elicited by these novel antibiotic combinations remains limited. The evolution of drug resistance is an intricate phenomenon influenced by a multitude of factors, including the specific drug and dosage, the patient's genetic constitution, and additional bacterial mutations or genetic variations [66]. Despite these complexities, the scientific community remains steadfast in its commitment to developing innovative approaches to counteract drug resistance. Ongoing research efforts are dedicated to enhancing the effectiveness of antibacterial treatments, with the ultimate objective of delivering more robust therapeutic solutions [45,67].

Given the emergence of AMR in novel therapeutic alternatives [52,55,56], it is imperative to conduct research focused on understanding the contribution of bacterial systems (e.g., signal transduction, regulators, virulence, or membrane permeabilization) and evolutionary biology. Nevertheless, despite the promise of combination therapy in combating bacterial infections, there is a notable discrepancy between the supportive *in vitro* evidence and the clinical validation in humans [68]. *In vitro* assays are invaluable for discerning the synergistic potential of antibiotic combinations, yet they often do not fully encapsulate the complex dynamics and intricate behavior of bacterial infections within a human host [69]. The translation of these effects into a clinical context is profoundly affected by a myriad of factors, including pharmacokinetics, pharmacodynamics, and the individual's immune response, all of which play a decisive role in the real-world efficacy of combination treatments [70]. The lack of robust *in vivo* data constitutes a significant barrier to translating *in vitro* research into viable clinical applications. This underscores the imperative for a concerted effort to bridge the *in vitro-in vivo* gap, a critical step in the advancement of combination therapeutic strategies against infections caused by bacterial pathogens [71].

Animal models are indispensable in translational research since they bridge the gap between *in vitro* studies and clinical trials for combination antibacterial therapies [72]. They mimic human pathophysiological processes and disease progression, offering a nuanced platform for evaluating the efficacy and safety of diverse antibiotic combinations [73]. These models are instrumental in elucidating the pharmacokinetics and pharmacodynamics of antibiotics within a living organism, thus enabling the exploration of drug interactions and synergistic effects [74]. Moreover, Moreover, they provide an observational window into how combination therapies modulate host immune responses and influence the development of microbial resistance [4,75]. Through methodical investigations of antibiotic combinations in animal models, researchers can discern and advance the most promising therapeutic candidates toward clinical evaluation, thereby enhancing the probability of success in formulating potent combination therapies to counteract bacterial infections [75].

3. Resistance in combination therapies: Reality or Myth?

In the current landscape marked by an escalation of antibiotic-resistant infections, the strategic application of empiric antimicrobial therapy, employing multiple agents, is increasingly recognized as a means to enhance coverage against suspected pathogens [69,76,77]. The timely initiation of an effective antimicrobial regimen is essential for the management of seriously ill patients where bacterial infections are implicated [69]. The rationale for utilizing empiric combination therapy is reinforced by the growing prevalence of infections attributable to multidrug-resistant organisms. Contemporary evidence underscores that the primary advantage of such combination therapy is the augmented probability of administering an effective antimicrobial agent during the initial, empiric phase of treatment. This approach may be more critical than leveraging *in vitro* synergy or circumventing resistance during subsequent, targeted therapy [78].

Combination therapy is widely employed in the management of bacterial infections, offering substantial advantages such as an enhanced spectrum of activity and the mitigation of resistance emergence. However, this approach is not without its risks [79]. A significant concern associated with the concurrent use of multiple antibiotics is the potential for increased adverse effects [80]. As each antibiotic possesses a unique profile of side effects, their combination can exacerbate the risk of toxicity, a factor that demands careful consideration, especially in patients with impaired organ function or polypharmacy or those taking other medications [81]. The strategy of employing multiple pharmacological mechanisms to outmaneuver bacterial resistance [82] also paradoxically amplifies the

selection pressure for the development of resistant strains [65]. This can lead to the emergence of MDR bacteria, which are difficult to treat with existing antibiotics. A pertinent illustration of this is the use of ampicillin and ceftriaxone in treating serious *Enterococcus faecalis* infections, like endocarditis, which has been implicated in subsequent colonization by vancomycin-resistant enterococci [83]. Therefore, the deployment of combination therapy necessitates a judicious analysis of the benefits in light of the potential for adverse outcomes.

Vestergaard et al. [54] explored the evolution of *P. aeruginosa* resistance exposed to ceftazidime, ciprofloxacin, and combined therapy. Resistant mutants were exposed to antibiotics and reconstructed through allelic substitution and *in vitro* bacterial fitness assays. In contrast to single-antibiotic treatments, combination therapy selected mutants with enhanced fitness expressing broad-spectrum resistance mechanisms. These mutant strains' resistance was associated with the inactivation of the repressor mexR gene, which regulates the multi-drug efflux operon mexAB-oprM and decreases susceptibility to combination therapy, including the antibiotics meropenem and tobramycin.

Berríos-Caro et al. [38] employed mathematical models to compare monotherapy and combination therapy growth methods (exponential, logarithmic, and resource competition-free). They found that resource scarcity and bacterial competition impacted single resistance expression, while double resistance emerged in resource competition-free growth. However, double resistance stemmed from microorganisms with simple (single antibiotic) resistance. This study underscores the potential for double resistance under specific conditions influenced by intrinsic and extrinsic factors of the host organism, pathogen, and antibiotics [38]. The pursuit of synergistic effects between antibiotics and their enhanced activity in clinically relevant microorganisms has prompted investigations into combination therapy characteristics [1,84,85].

Synergy in AMR may yield contradictory effects, accelerating pathogen elimination and reducing the time for multi-resistant mutation development while promoting single antibiotic resistance [86]. Under certain circumstances, this phenomenon may lead to double resistance, rendering combination therapies ineffective [44].

Munck et al. [39][m] demonstrated that collateral sensitivity alters AMR development, as antibiotic interactions change when pathogenic microorganisms develop AMR, resulting in reduced long-term microbial adaptation. The study focused on the adaptive evolution of *E. coli* to five different antibiotics and ten pairwise combinations (ciprofloxacin, tetracycline, amikacin, chloramphenicol, and piperacillin). The data revealed that a specific combination of antibiotics could reduce the evolution of AMR. Furthermore, collateral sensitivity between antibiotics influences the development of AMR in combined therapies, as the sensitivity and resistance that arise when mutations confer resistance to one antibiotic either increase or decrease sensitivity to another antibiotic [45].

Lazar et al. [37] conducted evolutionary and genetic studies on *E. coli* with twelve antibiotics and their combinations. They found that genetic evolution predominates after initial exposure to different treatments, and cross-resistance between two interacting antibiotics is independent of their synergy. However, multi-drug resistance evolution was common under single antibiotic pressure. The authors noted that various mechanisms govern evolutionary interactions, influencing combination treatments and multi-drug resistance, which must be considered when devising new antimicrobial strategies.

Understanding the complexities of antibiotic resistance in the context of combination therapies is crucial for developing effective treatment strategies. The potential emergence of double resistance, collateral sensitivity, and the role of tolerance in these therapies must be carefully considered [44,45,87,88]. While combination therapies have shown promise in controlling the development of resistance, their effectiveness depends on various factors, including the specific drug interactions, the presence of intrinsic and extrinsic factors in both the host and the pathogen, and the genetic makeup of the microorganisms [1,44].

To assess the potential evolution of resistance in these therapies, we must examine the mechanisms of action of antibiotics and microorganisms' intrinsic and extrinsic responses upon drug exposure. Angst et al. [18] highlighted the benefits of combination therapies *in vitro* through epidemiological and bioinformatic studies and supported their superiority over monotherapies if double resistance does not develop in bacteria. The likelihood of a double mutation is reportedly low since resistance-conferring mutations occur independently after a single antibiotic treatment or the subsequent administration of a combination treatment. Understanding drug interactions' complex dynamics and kinetics in combination therapy is crucial [18,87,88].

Resistance-associated genes and mutations impose bacterial fitness costs [87]. However, microbial competition studies reveal that resistance costs for one antibiotic depend on alleles conferring resistance to other antibiotics [89,90]. Trindade et al. [53] found that positive epistasis in combined therapies could induce double resistance in microorganisms, specifically via point mutations. Epistasis often arises when the expression of a phenotype at one locus is contingent on mutations at other loci, which is particularly relevant in evolution and the emergence of AMR [87]. Some allelic combinations rapidly compensate, explaining the prevalence of multi-resistant bacteria [87], potentially linked to combination therapy implementation.

Future research should focus on a deeper understanding of the mechanisms driving resistance in combination therapies, including exploring novel antibiotic combinations that minimize the risk of resistance development [88]. Additionally, the identification of synergistic effects between antibiotics, as well as the enhancement of their activity against clinically relevant microorganisms, will prove valuable for more efficient treatment strategies [1]. To optimize the efficacy of combination antibiotic therapies, it is vital to design studies that align treatment strategies with the biological behavior of the pathogen. These studies should focus on selecting antibiotics that not only exhibit synergistic interactions but also avoid antagonistic effects. Further investigation is required to ascertain whether definitive therapy can be augmented by 'rescuing' an antibiotic with a companion drug to which the pathogen remains sensitive, a concept supported by various research findings [1,49,62]. This approach, grounded in a pathogen-centric framework for antibiotic selection and combination therapy, has the potential to markedly enhance patient outcomes by maximizing inter-drug efficacy and reducing the emergence of resistance. Further, we propose that subsequent research should investigate how such strategies may affect definitive clinical endpoints, including the resolution of infection, patient morbidity and mortality, duration of hospitalization, and the long-term impact on antimicrobial resistance patterns.

Studies investigating the dynamics of bacterial competition and the fitness costs associated with resistance development will provide insights into the evolutionary pressures that shape the emergence of antibiotic resistance [18,87].

4. Conclusion

The escalating antibiotic resistance crisis presents a significant global public health challenge, necessitating a reevaluation of monotherapy as the sole therapeutic approach. Combination therapy offers the potential to revitalize previously disused drugs due to resistance development, exploit synergistic effects for enhanced antibacterial efficacy, and reduce resistance emergence. Combination treatments are currently employed in various healthcare settings, particularly for addressing infections caused by multidrug-resistant microorganisms. However, implementing such treatments should be grounded in evolutionary, genetic, and tolerance studies to develop well-rounded antimicrobial strategies that prioritize therapeutic success while minimizing resistance development.

It is crucial to recognize that although existing studies on resistance emergence in combination therapies are limited, the findings indicate the possibility of such occurrences. As a result, adopting combination treatments requires comprehensive laboratory and clinical evaluations that account for the potential consequences of antibiotic resistance emergence in the context of these therapies. By considering the evolutionary, clinical, and epidemiological implications, researchers and healthcare professionals can work collaboratively to develop more effective and sustainable treatment strategies to combat the global challenge of antibiotic resistance.

Assessing the correlation between *in vitro* and *in vivo* results and the efficacy observed in randomized controlled trials is critical to validating combination therapy protocols. Essential questions that must be addressed by future research include a) How well do *in vitro* and *in vivo* models of combination therapy predict clinical outcomes in humans? And b) How can we improve and refine the design of *in vitro* and *in vivo* studies of combination therapy? Addressing these inquiries is a necessary step in the progression of combination therapy evaluation. Through this process, it is anticipated that more effective strategies will be developed to enhance the discovery and optimization of combination therapies, ultimately leading to improved patient outcomes.

5. Future perspectives

Evidence strongly supports the efficacy of combination therapies as a powerful alternative for combating AMR caused by pathogenic bacteria [1]. Nevertheless, it is crucial to consider the short- and long-term implications of using such therapies, as bacteria

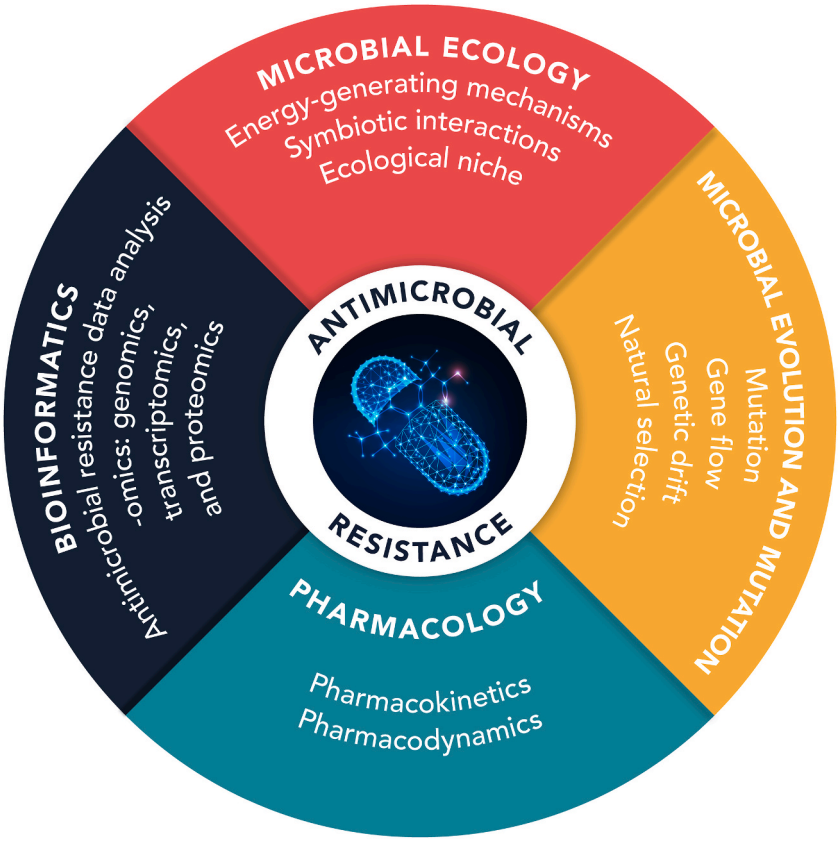

Fig. 1. Four pivotal future research areas (Microbial Ecology, Microbial Evolution, Pharmacology, and Bioinformatics) to better comprehend the development of antibiotic resistance in bacteria treated with combination strategies.

continuously adapt to novel therapeutic landscapes. Investigating the development of AMR mechanisms encompassing evolutionary forces and intrinsic and extrinsic factors will enable a more comprehensive understanding of this pressing global public health issue. Accordingly, we have identified four pivotal future research areas to comprehend better the development of antibiotic resistance in bacteria treated with combination strategies (Fig. 1):

Ecology and microbial evolution: Traditional research on AMR has primarily focused on genetic changes and developing new alternatives to counteract resistance. However, this approach overlooks essential aspects of microbial ecology and evolution [91]. It is known that the genetic and ecological part of microorganisms should not be studied separately since the study of symbiotic associations, the formation of biofilms, and genetic exchange, among others, contribute to the evolutionary trajectory towards dissemination and maintenance of AMR [92–95]. Thus, the study of microbial interactions in different environments and diversity and abundance will contribute to understanding the ecological pressures exerted on microorganisms, which are considered vital in preventing, treating, and stopping the spread of AMR [96,97].

Pharmacology: Carrying out studies focused on selecting and discovering antimicrobials that present an optimal effect for treating infections. Likewise, minimizing side effects and the development of AMR is the main objective of pharmacology and aspects related to pharmacokinetics, pharmacodynamics, and toxicology to achieve this [1,98]. In combination therapies, the interactions between antibiotics play an essential role in treating resistant antibiotics since they can present synergistic, additive, or antagonistic effects that, when determined, can propose treatment strategies that use a combination of more effective drugs [99,100].

Bioinformatics: It is characterized by using structural and sequence analysis techniques that contain large biological databases, which determine new predictions or new contributions in the area of biologics such as molecular biology, genomics, transcriptomic, and proteomics, in addition to providing information on the epidemiology of pathogenic microorganisms, so its role in AMR has become highly relevant [10,16,101,102]. Another valuable contribution is the possibility of creating databases to detect existing genes and new antibiotic-resistance genes in bacterial genomes [106,107]. It allows the creation of genome profiles and metagenomes of microorganisms of clinical interest to study them in an epidemiological and evolutionary context to understand their origin, the form of infection and transmission, as well as the synthesis of new antimicrobial agents [19,103].

Host-pathogen interactions and microbial ecology: Investigating the impact of host-pathogen interactions and the broader microbial ecology on AMR development will offer a more comprehensive understanding of the factors influencing resistance in bacteria treated with combination strategies. This knowledge will inform the development of targeted therapies that consider the complex interplay between bacteria, the host, and their environment [91,104,105].

Addressing these disciplines in developing AMR in combination therapies is necessary to avoid repeating what happened in the golden age with the discovery of antibiotics and the application of monotherapy. Elucidating the molecular basis, genetic trajectory, and rate of evolution of resistance to combination therapies will go a long way in expanding our knowledge thus far about the novel and alternative therapy regimens that could transform the diagnosis, treatment, and predictability of AMR.

Data availability statement

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Nami Morales-Durán: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Angel León-Buitimea:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **José R. Morones-Ramírez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We want to acknowledge Universidad Autónoma de Nuevo León and the Centro de Investigación en Biotecnología y Nanotecnología (CIByN) for the facilities provided to perform the research.

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